ADVANCES IN
EXPERIMENTAL MEDICINE AND BIOLOGY

VOLUME 86B

PROTEIN CROSSLINKING

Nutritional and Medical Consequences

ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Recent Volumes in this Series

Volume 81
PHOSPHATE METABOLISM
Edited by Shaul G. Massry and Eberhard Ritz • 1977

Volume 82
ATHEROSCLEROSIS: Metabolic, Morphologic, and Clinical Aspects
Edited by George W. Manning and M. Daria Haust • 1977

Volume 83
FUNCTION AND BIOSYNTHESIS OF LIPIDS
Edited by Nicolás G. Bazán, Rodolfo R. Brenner, and Norma M. Giusto • 1977

Volume 84
MEMBRANE TOXICITY
Edited by Morton W. Miller, Adil E. Shamoo, and John S. Brand • 1977

Volume 85A
ALCOHOL INTOXICATION AND WITHDRAWAL – IIIa: Biological Effects of Alcohol
Edited by Milton M. Gross • 1977

Volume 85B
ALCOHOL INTOXICATION AND WITHDRAWAL – IIIb: Studies in Alcohol Dependence
Edited by Milton M. Gross • 1977

Volume 86A
PROTEIN CROSSLINKING: Biochemical and Molecular Aspects
Edited by Mendel Friedman • 1977

Volume 86B
PROTEIN CROSSLINKING: Nutritional and Medical Consequences
Edited by Mendel Friedman • 1977

Volume 87
HYPOTHALAMIC PEPTIDE HORMONES AND PITUITARY REGULATION
Edited by John C. Porter • 1977

PROTEIN CROSSLINKING

Nutritional and Medical Consequences

Edited by

Mendel Friedman

Western Regional Research Laboratory
Agricultural Research Service
U.S. Department of Agriculture
Berkeley, California

SPRINGER SCIENCE+BUSINESS MEDIA, LLC

Library of Congress Cataloging in Publication Data

Symposium on Protein Crosslinking, San Francisco, 1976.
Protein crosslinking.

(Advances in experimental medicine and biology; v. 86A-86B)
"Proceedings of the [1st and 2d halves] of a Symposium on Protein Crosslinking held in San Francisco, California, August 30-September 3, 1976, with additional invited contributions."
Includes index.
CONTENTS: v. 1. Biochemical and molecular aspects. – v. 2. Nutritional and medical consequences.
1. Proteins – Crosslinking – Congresses. I. Friedman, Mendel. II. Title. III. Series. [DNLM: 1. Proteins – Congresses. W1 AD559 v. 86 pt. A etc. / QU55 S98644p 1976]
QP551.S939 1976 574.1'9245 77-23448
ISBN 978-1-4757-9115-0 ISBN 978-1-4757-9113-6 (eBook)
DOI 10.1007/978-1-4757-9113-6

Proceedings of the second half of a Symposium on Protein Crosslinking held in San Francisco, California, August 30-September 3, 1976, with additional invited contributions

Originally published by Plenum Press, New York in 1977
Softcover reprint of the hardcover 1st edition 1977

PREFACE

The word crosslinking implies durable combination of usually large, distinct elements at specific places to create a new entity that has different properties as a result of the union. In the case of proteins, such crosslinking often results in important changes in chemical, physical, functional, nutritional, and biomedical properties, besides physical properties simply related to molecular size and shape. (Nucleic acids, carbohydrates, glycoproteins, and other biopolymers are correspondingly affected.) Since proteins are ubiquitous, the consequences of their crosslinking are widespread and often profound. Scientists from many disciplines including organic chemistry, biochemistry, protein chemistry, food science, nutrition, radiation biology, pharmacology, physiology, medicine, and dentistry are, therefore, very much interested in protein crosslinking reactions and their implications.

Because protein crosslinking encompasses so many disciplines, in organizing the Symposium on Nutritional and Biochemical Consequences of Protein Crosslinking sponsored by the Protein Subdivision of the Division of Agricultural and Food Chemistry of the American Chemical Society, I sought participants with the broadest possible range of interests, yet with a common concern for theoretical and practical aspects of protein crosslinking.

An important function of a symposium is to catalyze progress by bringing together ideas and experiences needed for interaction among different, yet related disciplines. To my pleasant surprize, nearly everone invited came to San Francisco to participate. Furthermore, those that could not come usually agreed to contribute a paper for the Proceedings. Many participants told me privately that they had made a special effort to come to San Francisco to help celebrate the combined Centennial of the American Chemical Society and Bicentennial of the United States. I am grateful for this friendly gesture. To supplement the verbal presentations further, the Proceedings include several closely related, invited contributions. The distinguished international participation from at least nine countries increases the authority and usefulness of the Proceedings.

These papers are being published in two volumes in the series Advances in Experimental Medicine and Biology under the following titles: PROTEIN CROSSLINKING: BIOCHEMICAL AND MOLECULAR ASPECTS (Part A) and PROTEIN CROSSLINKING: NUTRITIONAL AND MEDICAL CONSEQUENCES (Part B). The two volumes are intended to be complementary, but their interests necessarily overlap.

Part A, the first volume, encompasses detailed discussions of natural crosslinks such as disulfide and peptide bonds, various artificial crosslinks formed by means of bifunctional reagents, radiation-induced crosslinks, and techniques to determine crosslinks.

Ultraviolet and gamma radiations are widely used to increase vitamin D content of foods, to sterilize food and drug products, and to treat diseases such as psoriasis and cancer. However, our knowledge about the molecular and nutritional consequences of irradiating food products and other proteins and biopolymers is still imperfect. Such consequences include crosslink formation. Several contributions report recent results in these areas. The results directly concern those interested in radiation biology and cancer therapy as well as food scientists and food technologists responsible for balancing good and bad effects of radiation.

Part B, the second volume, includes detailed discussions of crosslink formation in food proteins through lysinoalanine, isopeptide bonds, and products derived from protein-carbohydrate reactions. Such crosslinks not only lower the nutritional quality and digestibility of food products but sometimes introduce toxicity. This volume discusses nutritional and biological consequences of crosslink formation in food proteins, various factors that govern crosslink formation, effects of crosslinks on protein structure, reactivity, and digestibility, and ways to minimize crosslinking.

Part B also discusses structural and tissue proteins, such as collagen and elastin, which contain many natural crosslinks derived from lysine. Several papers report evidence that these crosslinks are important in aging and connective tissue disease. The chemistry and biochemistry of such natural crosslinks are thus important to anyone concerned with the relation of nutrition, health, and aging.

I want to emphasize considerations supporting the diversity of the subject matter presented in these volumes and of contributors backgrounds and interests. The widest possible interaction of viewpoints and ideas is needed to transcend present limitations in our knowledge as expeditiously as possible and to catalyze progress in the field of crosslinking. Scientists from related disciplines need one another's results; results with different biopolymers need to be compared; scientists and physicians

responsible for practical applications need to share experiences and problems with basic researchers. These volumes bring together many elements needed for such interactions. The range of material includes a great variety of specific and general topics. This scope should interest at least a similar range of readers, but it challenges all of us to think seriously about subjects beyond our primary interests. It is my hope, therefore, that the reader will look not only to those articles of primary interest to him but to others as well and so profit by a broad overview.

I am particularly grateful to all contributors and participants for excellent cooperation, to Dr. Wilfred H. Ward for constructive contributions to several manuscripts, to my son Alan David Friedman for his help with the preparation of the subject index, to Dawn M. Thorne for final typing of several manuscripts, to Roy Oliver of Pierce Chemical Company and Dr. Rao Makineni of Bachem Fine Chemicals for financial assistance, and to the Protein Subdivision of the Division of Agricultural and Food Chemistry of the American Chemical Society for sponsoring the symposium. I hope that PROTEIN CROSSLINKING will be a valuable record and resource for further progress in this very active interdisciplinary field.

Finally, I dedicate this work to the late Professor S. Morris Kupchan, with whom I had the privilege of spending a post-doctoral year at the University of Wisconsin. His untimely death deprives us of a very great scientific benefactor whose twenty-year global search for natural anti-tumour protein (enzyme) alkylating compounds is just now beginning to bear fruit.

Mendel Friedman
Moraga, California
March, 1977

GENESIS 44:30...because the father's (Jacob's) life is crosslinked to his son's (Benjamin's)...

SAMUEL I 18:1...Jonathan's soul was crosslinked to David's...

CONTENTS OF PART B

CONTENTS OF PART A

CROSSLINKING AMINO ACIDS -- STEREOCHEMISTRY AND NOMENCLATURE

Mendel Friedman

Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California 94710

ABSTRACT

The stereochemical factors that would be expected to operate during crosslinking of amino acids residues in proteins are analyzed on the basis of mechanistic and theoretical considerations. Names are assigned both to the known amino acids and to the unknown, but theoretically possible, amino acids that result from the crosslinking reactions.

INTRODUCTION

Crosslinked amino acids have been found in hydrolysates of both alkali and heat-treated proteins. These derivatives are thought to be derived from nucleophilic addition of active-hydrogen-bearing protein function groups to the double bonds of dehydroalanine and 3-methyldehydroalanine residues. Dehydroalanine residues could be derived from cystine, cysteine, and serine side chains; 3-methyldehydroalanine groups from threonine residues (Friedman, 1973a,1977; Gross, 1977; Asquith and Otterburn, 1977; Whitaker and Feeney, 1977; Snow et al., 1976). In this paper I (a) examine some of the stereochemical and mechanistic factors that would be expected to operate during crosslinking of amino acids, (b) outline a nomenclature for both known and unknown, but theoretically possible, crosslinked amino acids that may result from the cited interactions, and (c) speculate about possible pharmacological and biochemical consequences of formation of these crosslinks at the molecular level.

STEREOCHEMICAL AND MECHANISTIC CONSIDERATIONS

The stereochemistry of amino acids and related compounds and methods for assigning absolute configurational designations have been detailed (Cahn, 1964; Hanson, 1966; Bentley, 1969,1970; Alworth, 1972; Klyne and Buckingham, 1974; Meloche and Monti, 1975; Brooks, 1976; Spencer et al., 1976; Prelog, 1976, Hansen, 1976).

Nucleophilic elimination-addition reactions of amino acid residues to form crosslinked proteins that yield, on hydrolysis, crosslinked amino acids are illustrated in Figures 1-7. Note that the addition reaction may restore or reverse the configuration of the original asymmetric center in the alanine part of 3-N^{δ}-lysinoalanine and that the derivatives of 3-methyldehydroalanine would have one or more asymmetric carbon atom than those of dehydroalanine (Figure 7). In general, the chiral α-center derived from L-lysine, L-ornithine, L-cysteine, L-serine, L-threonin, L-histidine, and L-tyrosine would remain S as in the original amino acids. Similarly, the β-center of the threonine adduct should remain R as in the natural L_S-threonine. However, the chirality of the restored assymmetric center derived from proton

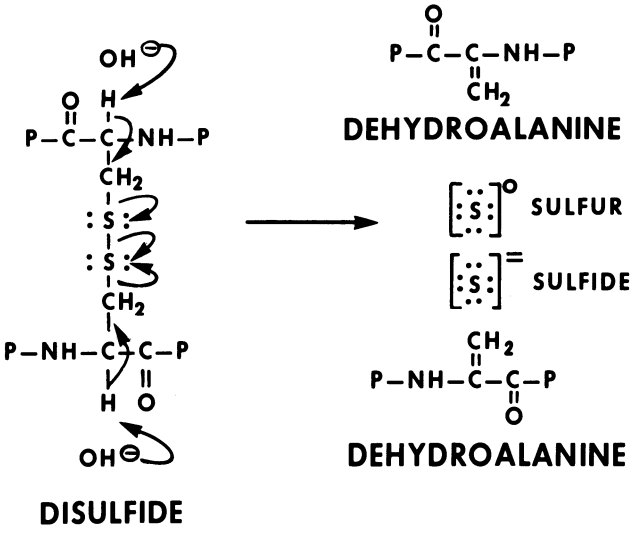


Fig. 1. Base-catalyzed transformation of a protein disulfide bond to two dehydroalanine side chains, a sulfide ion, and elemental sulfur.

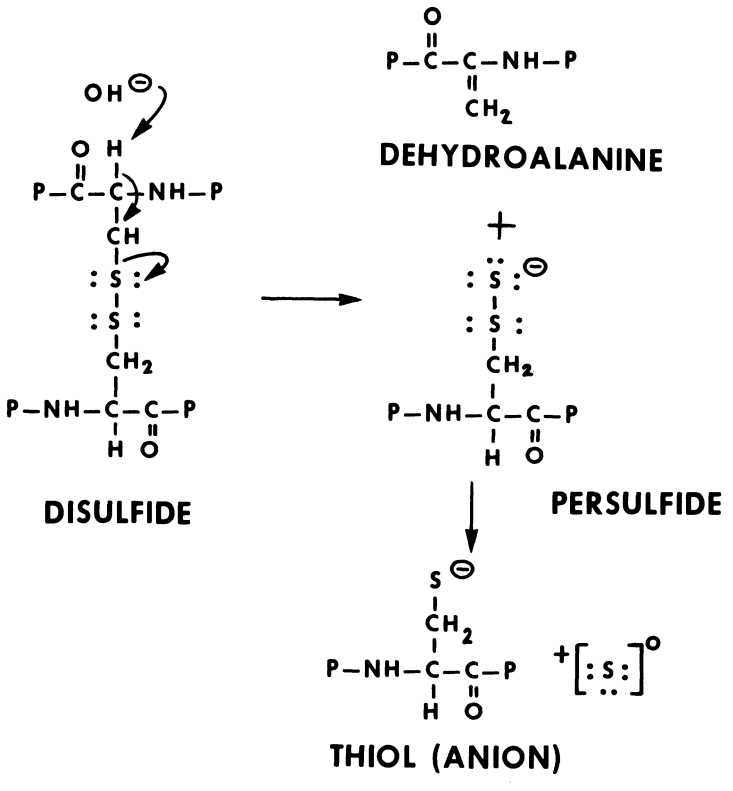


Fig. 2. Base-catalyzed formation of one dehydroalanine and one persulfide anion from a disulfide bond. The persulfide probably decomposes to a cysteine residue and elemental sulfur. It can also combine with a dehydroalanine residue to reform a (different) disulfide bond.

addition to the α-carbon of the dehydroalanine or methyldehydroalanine moieties, would depend upon the stereochemistry of the coupling reaction and the priorities of the ligand added to the β-carbon, as illustrated in Figure 8. Si face protonation would yield and R center; re face protonation would yield an S chirality for both dehydro moieties, except for lanthionine where a sulfur- and oxygen-containing ligands (S > O > N) supercedes the relative priority of carboxyl group in determining the chirality designa-

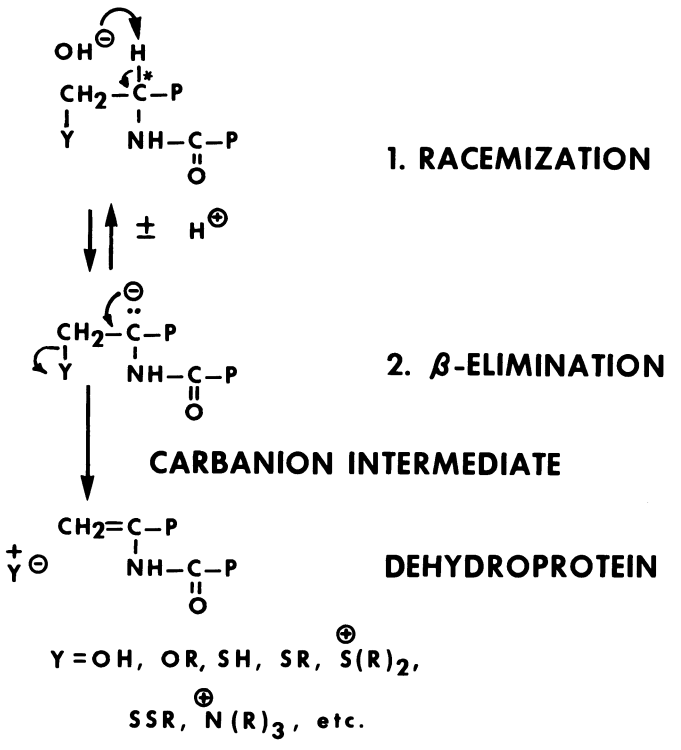


Fig. 3. Racemization and elimination pathways via a common carbanion intermediate.

tions. Note that the L-configuration of the sulfur-containing amino acids (cysteine, etc.) is (R), while the L-configuration of the oxygen-containing amino acids (serine, etc.) is (S).

The chirality of the center produced by nucleophilic attack at the β-carbon of 3-methyldehydroalanine will depend upon the stereospecificity of addition at the β-center, as illustrated in Figure 9. Note, however, that since the added ligands (O, S, N) in all the derivatives will have higher priority than C or H, the chirality designation (configuration) of all the derivatives, whether threoninyl, histidino, or tyrosinyl, will be the same if the stereospecificity of addition is the same.

Friedman and Wall (1964) previously noted than an additional

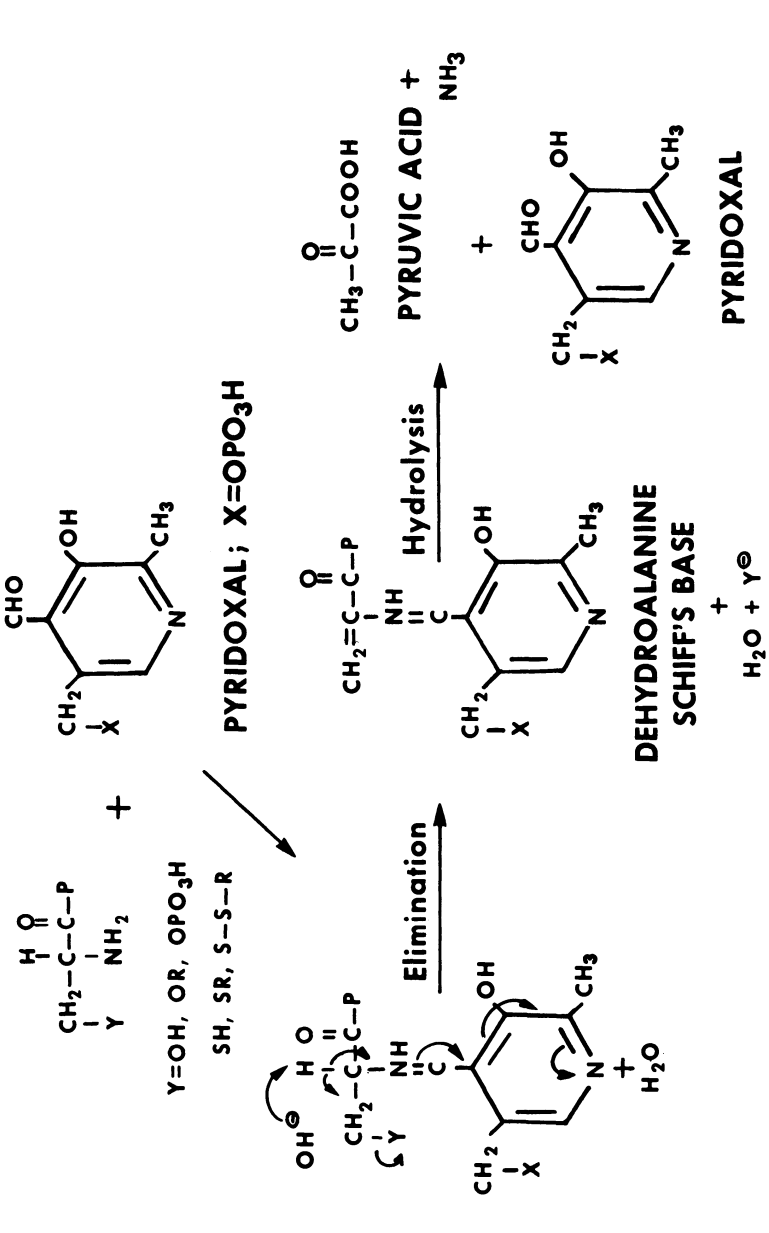


Fig. 4. Pyridoxal-catalyzed formation of a dehydroalanine intermediate via a resonance-stabilized Schiff's base. Such elimination reactions have been postulated to take place at physiological pH (Morino and Snell, 1967; Friedman, 1973).

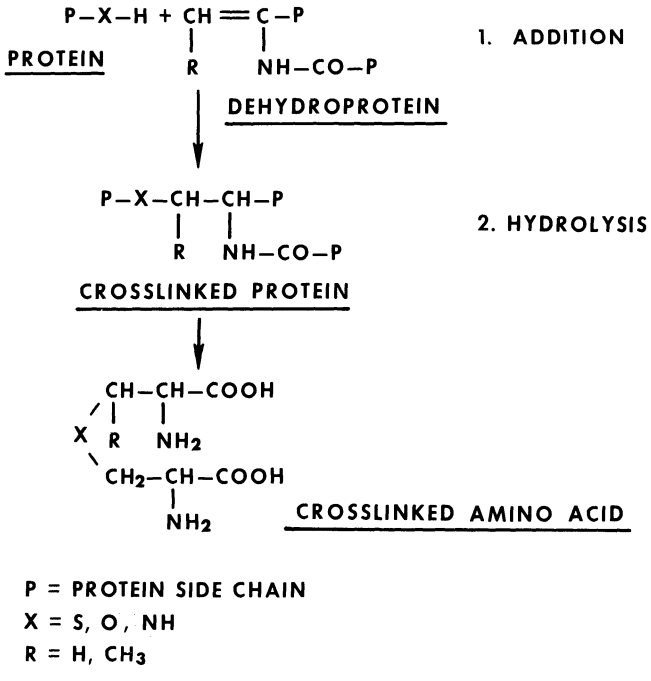
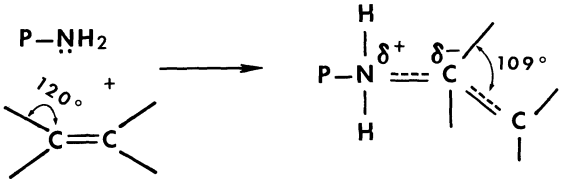


Fig. 5. Formation of crosslinked amino acids via addition of protein functional groups to a dehydroprotein.

element that causes increased steric strain in the activation process during addition of an amino or other functional group to an α,β-unsaturated system such as dehydroalanine is the change in hybridization of the sp^2-carbon atoms in the unsaturated compound to sp^3 in the activated complex, as illustrated:



GROUND STATE TRANSITION STATE

The steric effect of this angle compression will depend on the size of the reacting components. The rate of sp^3 hybridization, therefore, and, hence, the chirality of the α- and β-carbons could be affected by the bulkiness of the attacking protein side chains.

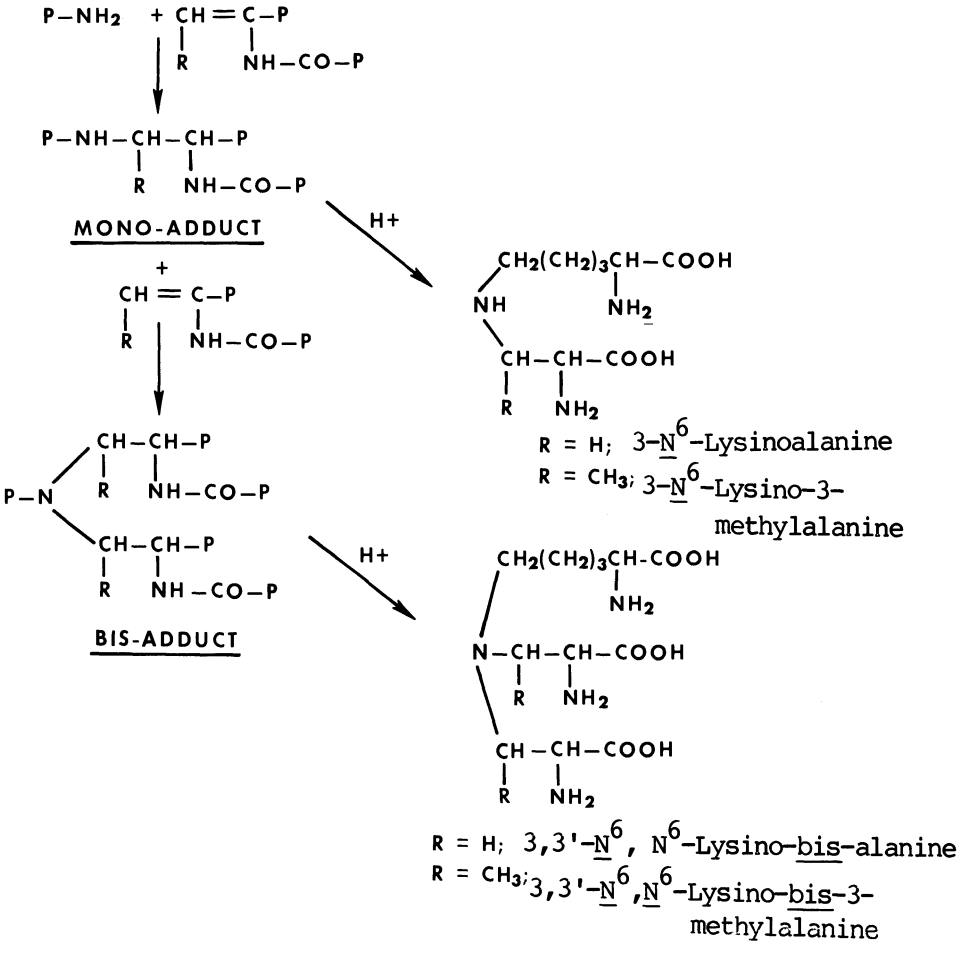


Fig. 6. Mono- and bis-alkylation of the ε-amino group in a lysine residue by a dehydroalanine residue.

Two points previously mentioned (Friedman and Wall, 1964; Friedman, 1972,1973) are also relevant. Model studies of transition states show that although both amino and mercaptide groups have to approach the double bond of an α,β-unsaturated compound, such as acrylonitrile or dehydroalanine, almost at right angles to the plane of the molecule, the amino group has to assume a more restricted orientation than the sulfur anion to form the transition state. The mechanisms by which both groups form the respective transition states differ in several features, and energetically the reaction of the mercaptide ion is more favored than that of the amino group. As the electron pair on nitrogen approaches, double bond breakage requires energetically unfavorable distribution of electrons. The antibonding empty orbitals of the double

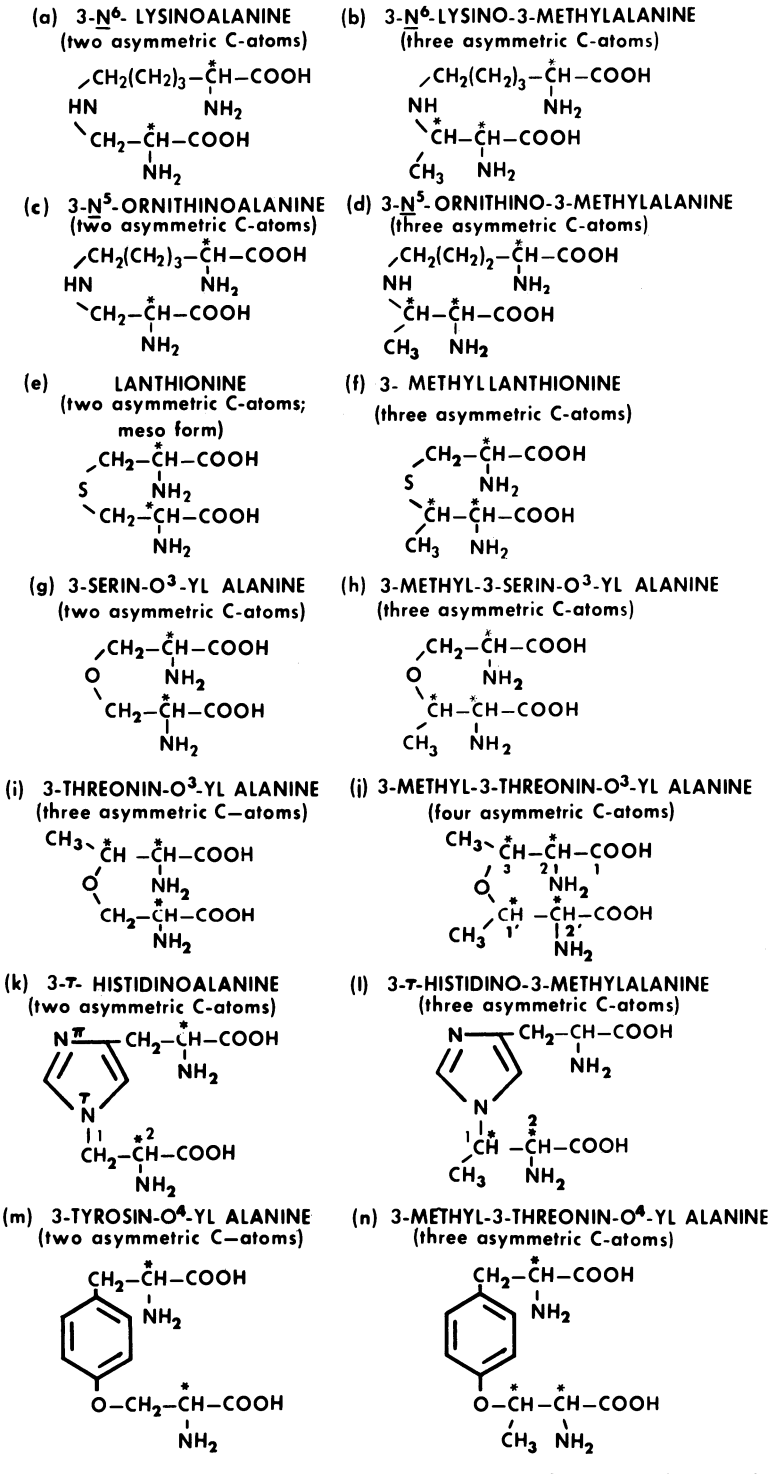


Fig. 7. Postulated structures of crosslinked amino acids.

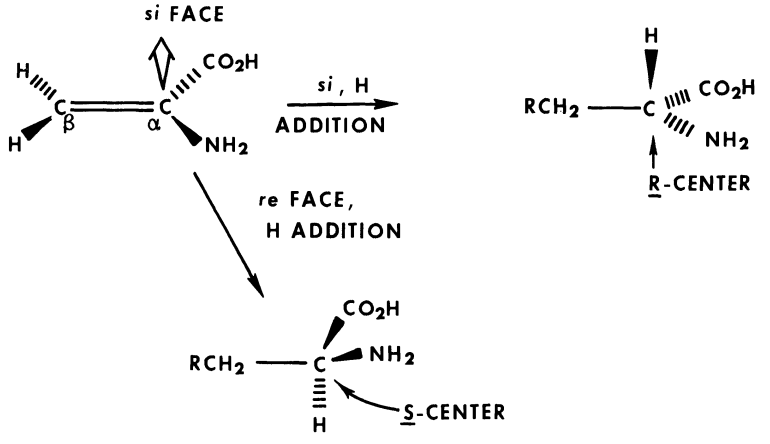


Fig. 8. Stereochemical course of proton addition of the α-carbon of dehydroalanine. Note, however, that when R is a sulfur atom, the change in priorities (S > O) reverses the configuration above.

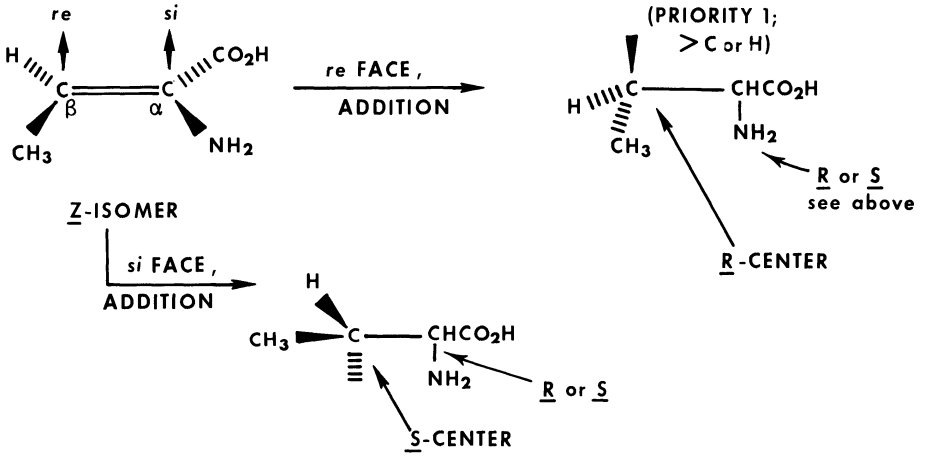


Fig. 9. Stereochemical course of addition of the β-carbon of 3-methyldehydroalanine, where X = ligand (S, O, N). The stereochemistry at the β-center will be the same for the E-isomer; i.e. re addition of X at β-carbon gives R center at β-carbon; si addition of X gives S center at β-carbon. However, re(β-C)-si(α-C) addition of X and H is a syn addition with Z-isomer; whereas re(β-C-si(α-C) addition of X and H is an anti process with E-isomer.

bond, which are in the process of assuming tetrahedral geometry, can overlap the nitrogen orbitals. On the other hand, the sulfur anion has two lone pairs of electrons left after bonding is initiated, and the sulfur atom has empty 3d-orbitals. If the energetics are favorable, these 3d-orbitals may stabilize the high-energy electrons of the double bond displaced towards the center α-carbon atom of dehydroalanine during formation of the transition state. This concept is akin to "orbital steering" discussed by Koshland (1973).

Transition states involving mercaptide and amino groups differ in still another aspect. When the sulfur anion goes from the negatively charged ground state to its transition state, charge dissipation takes place. In the case of the amino group, however, charge separation occurs. This difference in charge rearrangement again favors reaction (the transition state) of the sulfur anion, as illustrated:

$$^{-}OOC\text{-}R\text{-}S\text{---}CH_2\text{---}\overset{\delta^-}{C}H\text{-}R \qquad \text{vs.}$$

$$^{-}OOC\text{-}R\text{-}\overset{\delta^+}{N}H_2\text{---}\overset{\delta^-}{C}H_2\text{---}CH\text{-}R$$

Oxygen anions of serine, threonine, and tyrosine are negatively charged but have no 3d-orbitals.

None of these factors would operate during formation of crosslinks by substitution rather than elimination-addition, as discussed below.

ELIMINATION-ADDITION VS. SUBSTITUTION

In principle, formation of crosslinks between, for example, an ε-amino group of lysine and a serine residue can take place in two ways, by β-elimination followed by addition (Figs. 1-9) or by substitution (Fig. 10). In β-elimination, a base abstracts a proton from the serine carbon atom that has nitrogen attached to it (α-center), initiating a β-elimination to form a dehydroalanine derivative that can combine with an amino group to give a crosslinked 3-N^6-lysinoalanine side chain. Since the serine asymmetric center is first destroyed and then regenerated, the stereochemistry of the restored asymmetric center is uncertain, as discussed. The crosslink formed *via* substitution does not affect an asymmetric center, so the original configuration is retained, unless racemization occurs during exposure of the protein to strong base.

It would be worthwhile to establish conditions that select one pathway over the other proteins (*Cf.* Touloupis and Vassiliadis, 1977). Ginsburg and Wilson (1964) attempted this for several cysteine and serine derivatives. These authors studied factors that control relative yields of oxazoline or thiazoline (substitution pathway) and dehydroalanine (elimination pathway) derivatives. They concluded that the nature of the leaving group and the basicity and polarity of the solvent medium are decisive in determin-

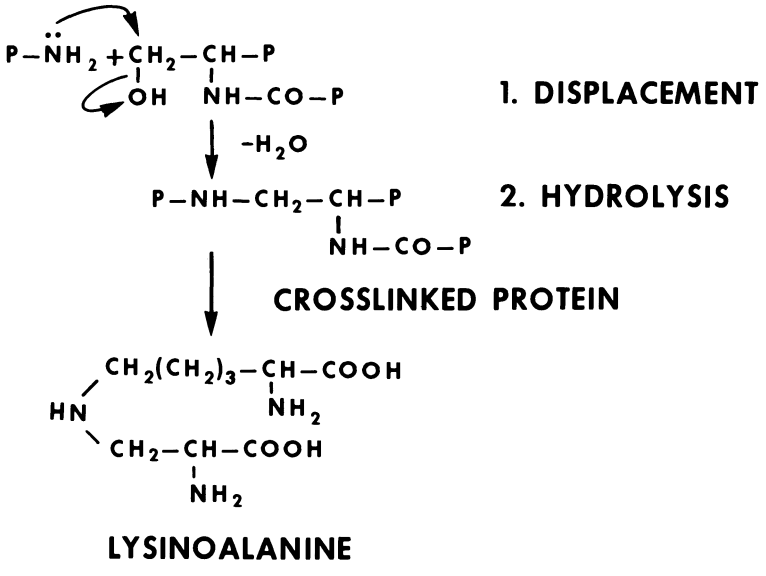


Fig. 10. Formation of a protein crosslink via substitution.

ing which derivative is formed. Not surprisingly, strong base favors elimination; polar solvents and good leaving groups favor cyclization.

INHIBITION OF CROSSLINKS

Inhibition of 3-N^6-lysinoalanine formation by added thiols or other nucleophiles such as sulfite ions can occur by at least three distinct mechanisms. First by direct competition the added nucleophile can trap dehydroalanine residues derived from protein amino acid side chains as mentioned earlier (eqs. 1, 2). Second, the added nucleophile can cleave protein disulfide bonds (eqs. 3, 4) and thus generate free SH groups, which may, in turn, combine with dehydroalanine residues as illustrated in eq. 5 (secondary competition). Third, by cleaving disulfide bonds, the added nucleophile can diminish a potential source for dehydroalanine, inasmuch as cystine residues would be expected to undergo β- elimination reactions more readily than negatively charged cysteine ($P\text{-}S^-$) or sulfo-cysteine ($P\text{-}S\text{-}SO_3^-$) protein side chains (suppression of dehydroalanine formation mechanism).

It may be possible to distinguish among these mechanisms.

Table 1

Lysine, Lysinoalanine, and Lanthionine content (μmoles/g.) of Alkali-Treated Proteins[a,b]

Protein and Treatment	LYS	LAL	LAN	Ratio of $\frac{LAL}{LAL + LYS} \times 100$
Wool: untreated	222.5	0.0	0.0	
pH 11.6	139.0	58.7	119.5	29.7
pH 11.6 + Na_2SO_3	176.9	19.1	0.0	9.73
Casein: untreated	482.7	0.0	0.0	
pH 11.6	294.0	145.2	0.0	33.06
pH 11.6 + Na_2SO_3	373.0	65.7	0.0	14.98
Lysozyme: untreated	397.3	0.0	0.0	
pH 11.6	222.2	135.2	0.0	37.83
pH 11.6 + Na_2SO_3	317.2	35.6	0.0	10.00
Trypsin inhibitor: native	319.3	0.0	0.0	
pH 11.6	129.2	235.1	0.0	64.53
pH 11.6 + Na_2SO_3	239.0	36.8	0.0	13.30

[a]Alkali treatments were carried out as follows: to 100 mg of wool top (fibers) in a round-bottom flask was added 50 ml of pH 11.6 borate buffer and, where appropriate, 1.26 g (0.01M) Na_2SO_3. The flasks were placed in a 60 C water bath for 3 hr. The soluble proteins were treated similarly, except that they were also dialyzed and lyophilized. Amino acid analyses of protein hydrolysates were carried out on a single-column Durrum Amino Acid Analyzer which resolves LAL and LAN peaks from the other amino acids under conditions given in Fig. 11. The ninhydrin color constant for LAL was determined with an authentic sample purchased from Miles Laboratories.

[b]The data show that (a) the amount of LAL formed under similar conditions varied greatly among the four proteins tested; and (b) presence of Na_2SO_3 results in a decrease in LAL content.

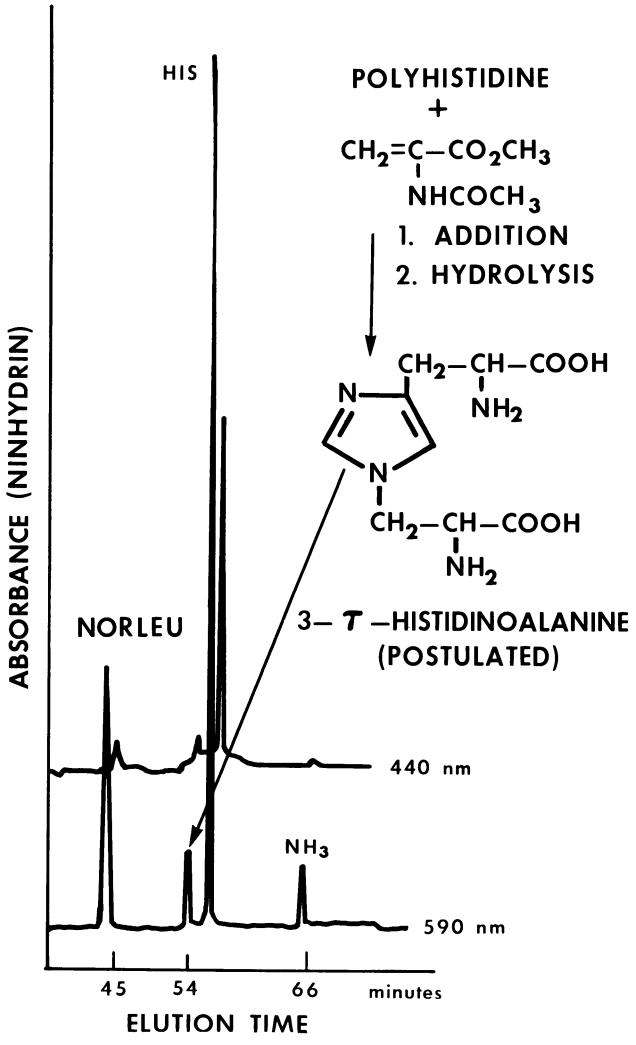


Fig. 11. Elution position of a dehydroalanine derivative of histidine on a Durrum Amino Acid Analyzer, Model D-500 under the following conditions: single column Moore-Stein ion-exchange chromatography method. Resin, Durrum DC-4A; buffer pH, 3.25, 4.25, 7.90; photometer, 440 nm, 590 nm; column, 1.75 mm X 48 cm; analysis time, 105 min. Noreleucine was used as an internal standard. 30 mg of poly-L-histidine in 30 cc 50% DMSO-50% pH 9.6 borate buffer and 70 mg of N-acetyldehydroalanine methyl ester were stirred for 80 hr. The material was dialyzed, lyophilized and hydrolyzed in 6N HCl for 24 hr.

With radioactive-labeled cysteine as the added nucleophile, for example, direct competition will give rise to labeled lanthionine, secondary competition to unlabeled lanthionine, while the suppression mechanism, to the extent it is successful, to no lanthionine. More than one of the cited pathways can, of course, operate simultaneously. we are currently attempting to differentiate among these possibilities (Table 1, Fig. 11, Finley and Friedman, 1976, 1977).

Direct Competition:

$$R\text{-}S^- + CH_2{=}C(NHCOP)\text{-}P + H^+ \rightleftharpoons R\text{-}S\text{-}CH_2\text{-}CH(NHCOP)\text{-}P \quad (1)$$

$$SO_3^{--} + CH_2{=}C(NHCOP)\text{-}P + H^+ \rightleftharpoons {}^-O_3S\text{-}CH_2\text{-}CH(NHCOP)\text{-}P \quad (2)$$

"Suppression" and First Step of Indirect Competition

$$2R\text{-}S^- + P\text{-}S\text{-}S\text{-}P \rightleftharpoons 2P\text{-}S^- + R\text{-}S\text{-}R\text{-}R \quad (3)$$

$$SO_3^{--} + P\text{-}S\text{-}S\text{-}P \longrightarrow P\text{-}S^- + P\text{-}S\text{-}SO_3^- \quad (4)$$

Indirect Competition:

$$P\text{-}S^- + CH_2{=}C(NHCOP)\text{-}P + H^+ \rightleftharpoons P\text{-}S\text{-}CH_2\text{-}CH(NHCOP)\text{-}P \quad (5)$$

NOMENCLATURE

a. 3-N^6-Lysinoalanine (LAL)

Lysinoalanine has two asymmetric carbon atoms (Figure 7). The following systematic name, originally proposed by Bohak (1964), is now widely used:

N^{ε}-(DL-2-amino-2-carboxyethyl)-L-lysine.

A more up-to-date name with R and S designations for the systematically named substituent would be:

N^6-[(2RS)-2-amino-2-carboxyethyl]-L-lysine.

D-Lysine would give rise to the following diastereoisomers: isomers:

N^6-[(2RS)-2-amino-2-carboxyethyl]-D-lysine.

Since IUPAC/IUB rule 5.1 (Eur. J. Biochem., 53, 1-14, 1975) indicates that expressing the stereochemistry of the α-carbon in amino acids by D or L and that of the chiral centers by (R) and (S)

is the more common method, this designation is used here. However, the alternate method of expressing the complete stereochemistry by (R) or (S) is also acceptable.

Note that when a series of stereoisomers derived from a single lysine, e.g. (S)-lysine, is described, the series must be a group of diastereoisomers, and not enantiomers. For an enantiomeric stereoisomer to be listed, the α-center of S-lysine would also have to be reversed. For example, (1R,2R).....(S) and (1S, 2S).....(R) lysine would be enantiomers; (1R,2R).....(S) and (1S, 2S).....(S)-lysine are diastereoisomers (Alworth, 1976). Note also, position 1 of the side chain (radical) is attached to N^6. The radical is therefore correctly named: 2-amino-2-carboxy-1-methylethyl.

In principle, alkylation of the ε-amino group of lysine side chains in proteins by dehydroalanine can produce both a mono-adduct (3-N^6-lysinoalanine) and a bis-adduct (3,3'-N^6,N^6-lysino-bis-alanine), as shown in Figure 6. The bis-adduct has three asymmetric carbon atoms, so that two sets of four enantiomeric pairs are theoretically possible. The corresponding dialkylation products from 3-methyldehydroalanine (3,3'-N^6,N^6-lysino-bis 3-methylalanine) has five asymmetric carbon atoms. A total of 32 stereoisomeric structures can be written for this compound.

Previous studies revealed that activated vinyl compounds, such as acrylonitrile, methyl acrylate, and ethyl vinyl sulfone, readily dialkylate ε-amino groups of both free and protein-bound lysine under relatively mild conditions (Friedman and Wall, 1964; Cavins and Friedman, 1967; Finley and Friedman, 1973a; Friedman and Finley, 1975a,b). Analogous dialkylation products derived from protein amino groups and dehydroalanines are therefore to be expected in alkali-treated proteins. Enzymic hydrolysis should be used in searching for such products since they may not always survive acid catalyzed protein hydrolysis.

b. 3-N^6-Lysino-3-methylalanine (LMeAL)

The compound has three asymmetric centers (Figure 7). Combination of L-lysine and 3-methyldehydroalanine may produce the following isomers:

N^6-[(1R,2R)-2-amino-2-carboxy-1-methylethyl]-L-lysine

N^6-[(1R,2S)-2-amino-2-carboxy-1-methylethyl]-L-lysine

N^6-[(1S,2S)-2-amino-2-carboxy-1-methylethyl]-L-lysine

N^6-[(1S,2R)-2-amino-2-carboxy-1-methylethyl]-L-lysine

An analogous D-lysine series is derived from D-lysine.

c. 3-N^5-Ornithinoalanines (OAL)

The following is the proposed name for the two diastereomeric reaction products of the δ-amino-group of L-ornithine and the double bond of dehydroalanine:

N^5-[(2RS)-2-amino-2-carboxyethyl]-L-ornithine.

d. Methylornithinoalanines (MeOAL)

Coupling of the amino group of L-ornithine with the double bond of 3-methyldehydroalanine would theoretically give rise to the following diastereoisomers:

N^5-[(1R,2R)-2-amino-2-carboxy-1-methylethyl]-L-ornithine

N^5-[(1R,2S)-2-amino-2-carboxy-1-methylethyl]-L-ornithine

N^5-[(1S,2S)-2-amino-2-carboxy-1-methylethyl]-L-ornithine

N^5-[(1S,2R)-2-amino-2-carboxy-1-methylethyl]-L-ornithine

D-Ornithine would produce a corresponding D-ornithine series. The δ-amino group of ornithine could also form bis-adducts analogous to those of lysine.

e. Lanthionines (LAN)

Reaction of the SH group of L-cysteine and the double bond of dehydroalanine gives rise to one pair of optically active isomers (enantiomers) and one diastereomeric meso form (Cf. Friedman and Noma, 1975).

S-[(2R)-2-amino-2-carboxyethyl]-L-cysteine, and

S-[(2S)-2-amino-2-carboxyethyl]-D-cysteine, or

(R)-L-lanthionine and (S)-D lanthionine, and

S-[(2S)-2-amino-2-carboxyethyl]-L-cysteine, or

(S)-L-lanthionine (meso-lanthionine).

The initial doubly-underlined S refers to sulfur in this series. It has no stereochemical significance.

f. 3-Methyllanthionines (MeLAN)

The following four methyllanthionine diastereomeric isomers derived from L-cysteine are theoretically possible:

(2R,3R)-2-amino-3-[(2R)-2-amino-2-carboxyethyl]thio butyric acid

(2R,3S)-2-amino-3-[(2R)-2-amino-2-carboxyethyl]thio butyric acid

(2S,3S)-2-amino-3-[(2R)-2-amino-2-carboxyethyl]thio butyric acid

(2S,3R)-2-amino-3-[(2R)-2-amino-2-carboxyethyl]thio butyric acid

g. 3-Serin-O^3-yl alanines (SAL)

Stereochemical designations of the various isomers correspond exactly to those for lanthionine except that S (sulfur) is replaced by O (oxygen). The reaction product(s) of the hydroxyl group addition of L-serine to the double bond of dehydroalanine may therefore be named:

O^3-[(2RS)-2-amino-2-carboxyethyl]-L-serine

h. 3-Methyl-3-serin-O^3-yl alanines (MeSAL)

Eight diastereoisomers are possible, corresponding to the 3-methyl-L-lanthionines. The following diastereoisomers represent the stereoisomers resulting from L_S-serine OH group attack on the double bond of methyldehydroalanine:

(3R)-O^3-[(2S)-2-amino-2-carboxyethyl]-D-threonine

(3S)-O^3-[(2S)-2-amino-2-carboxyethyl]-D-threonine

(3S)-O^3-[(2S)-2-amino-2-carboxyethyl]-L-threonine

(3R)-O^3-[(2S)-2-amino-2-carboxyethyl]-L-threonine

i. 3-Threonin-O^3-yl alanines (THRAL)

(3R)-O^3-[(2RS)-2-amino-2-carboxyethyl]-L(-)-threonine

j. 3-Methyl-3-threonin-O^3-yl alanines (MeTHRAL)

(3R)-O^3-[(1R,2R)-2-amino-2-carboxy-1-methylethyl]-L-(-)-threonine

(3R)-O^3-[(1R,2S)-2-amino-2-carboxy-1-methylethyl]-L-(-)-threonine

(3R)-O^3-[(1S,2R)-2-amino-2-carboxy-1-methylethyl]-L-(-)-threonine

(3R)-O^3-[(1S,2S)-2-amino-2-carboxy-1-methylethyl]-L-(-)-threonine

The following alternate nomenclature (Alworth, 1976) is probably also correct.

i'. Threoninoalanines (THRAL)

O-[(2'RS)-2-amino-2-carboxyethyl]-(2S,3R)-threonine

j'. Methylthreoninoalanines (MeTHRAL)

O-[(1'R,2'R)-1-methyl-2-amino-2-carboxyethyl]-(2S,3R)-threonine
(meso form)
O-[(1'R,2'S)-1-methyl-2-amino-2-carboxyethyl]-(2S,3R)-threonine

O-[(1'S,2'S)-1-methyl-2-amino-2-carboxyethyl]-(2S,3R)-threonine
(meso form)
O-[(1'S,2'R)-1-methyl-2-amino-2-carboxyethyl]-(2S,3R)-threonine

The numbers that specify the radical locants are primed and the 2S,3R positions of L-threonine root are unprimed. The prime locants clearly differentiate locants of two chiral centers in the threonine root (Alworth, 1976).

k. 3-τ-Histidinoalanines (HAL)

Two series are possible, depending on which of the two ring nitrogens are alkylated, Nτ- and Nπ-substituted histidines (See J. Biol. Chem., 247, 980, footnote 5):

τ-[(2RS)-2-amino-2-carboxyethyl]-L-histidine

π-[(2RS)-2-amino-2-carboxyethyl]-L-histidine

l. 3-τ-Histidino-3-methylalanines (HMeAL)

τ-[(1R,2R)-2-amino-2-carboxy-1-methylethyl]-L-histidine

τ-[(1R,2S)-2-amino-2-carboxy-1-methylethyl]-L-histidine

τ-[(1S,2S)-2-amino-2-carboxy-1-methylethyl]-L-histidine

τ-[(1S,2R)-2-amino-2-carboxy-1-methylethyl]-L-histidine

An analogous Nπ histidine series is also possible.

m. 3-Tyrosin-O^4-yl alanine (TyAL)

O^4-[(2RS)-2-amino-2-carboxyethyl]-L-tyrosine

n. 3-Methyl-3-tyrosin-O^4-yl alanine (MeTyAL)

O^4-[(1R,2R)-2-amino-2-carboxy-1-methylethyl]-L-tyrosine

O^4-[(1R,2S)-2-amino-2-carboxy-1-methylethyl]-L-tyrosine

O^4-[(1S,2S)-2-amino-2-carboxy-1-methylethyl]-L-tyrosine

O^4-[(1S,2R)-2-amino-2-carboxy-1-methylethyl]-L-tyrosine

o. Arginine Derivatives

Some arginine residues in proteins may be degraded to ornithine under alkaline conditions (Gilbert and O'Leary, 1975). The amino group of ornithine could then react with dehydroalanine as mentioned earlier.

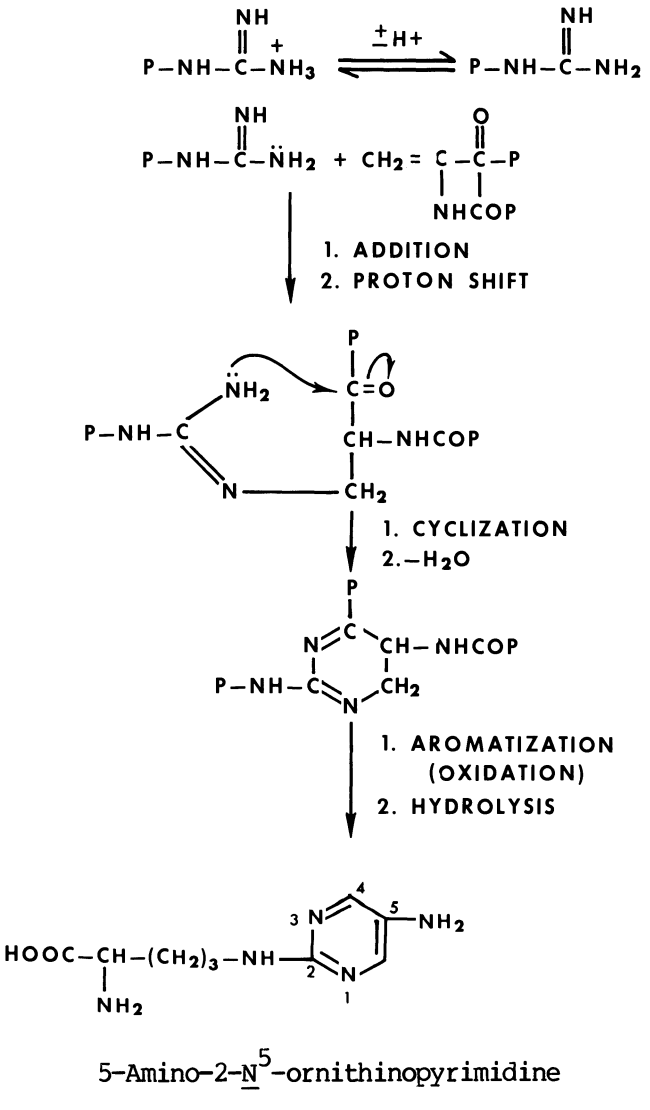
Because the guanidino group of arginine has a pKa value near 13 or higher, it is protonated under most conditions used to modify proteins. Since only the nonprotonated form can participate in nucleophilic reactions, the guanidino group of arginine is generally unreactive with double bonds. However, guanidino groups are are known to react with carbonyl compounds under strongly alkaline conditions which ionize a small fraction of the guanidino groups. Such reactions are accelerated in aprotic dipolar solvents such as dimethyl sulfoxide, which not only increases the ionization of basic groups but also enhances their nucleophilic reactivities (Friedman, 1967,1968; Whitfield and Friedman, 1973; Friedman, 1977). Since arginine residues in polyarginine appear to react with dehydroalanine (Finley and Friedman, 1976), the mechanism of such interactions, if they indeed occur, may be analogous to that postulated for dicarbonyl compounds (Whitfield and Friedman, 1972; Gilbert and O'Leary, 1975), to give a pyrimidine derivative, possibly as shown in Figure 12.

p. Tryptophan and Nucleic Acid Derivatives

The NH group of tryptophan is an extremely weak nucleophile because its unshared electron pair is delocalized by resonance with the indole ring. For this reason, it would not be expected to react with dehydroalanine. However, the α-carbon of the indole ring readily interacts with aldehyde groups, and may therefore be expected to add to the carbonyl group of dehydroalanine-derived pyruvic acid by a mechanism similar to that previously postulated for the interaction between tryptophan and glyoxalic acid (Fig. 13 and Friedman and Finley, 1975c). Morever, the indole ring is readily cleaved by radiation and oxidizing agents for form kynurenine (Friedman and Finley, 1975c; Finley and Friedman, 1973b) which has a free aromatic amino group that could be alkylated by dehydroalanine. Analogously, dehydroalanine could alkylate NH_2, NH, and OH groups of nucleic acids.

PHARMACOLOGICAL CONSEQUENCES

The stereochemical course of formation of crosslinks will un-



5-Amino-2-$\underline{N}^5$-ornithinopyrimidine

Fig. 12. Postulated mechanism for the reaction of a guanidino group of an arginine side chain and a dehydroalanine residue.

doubtedly vary from protein to protein because of constrains placed by the protein itself. Thus, the size, composition, and conformation of protein side chains as well as the number of active-hydrogen-bearing protein functional groups situated within the microenvironment near dehydroalanine and methyldehydroalanine residues will determine not only the number of crosslinks produced

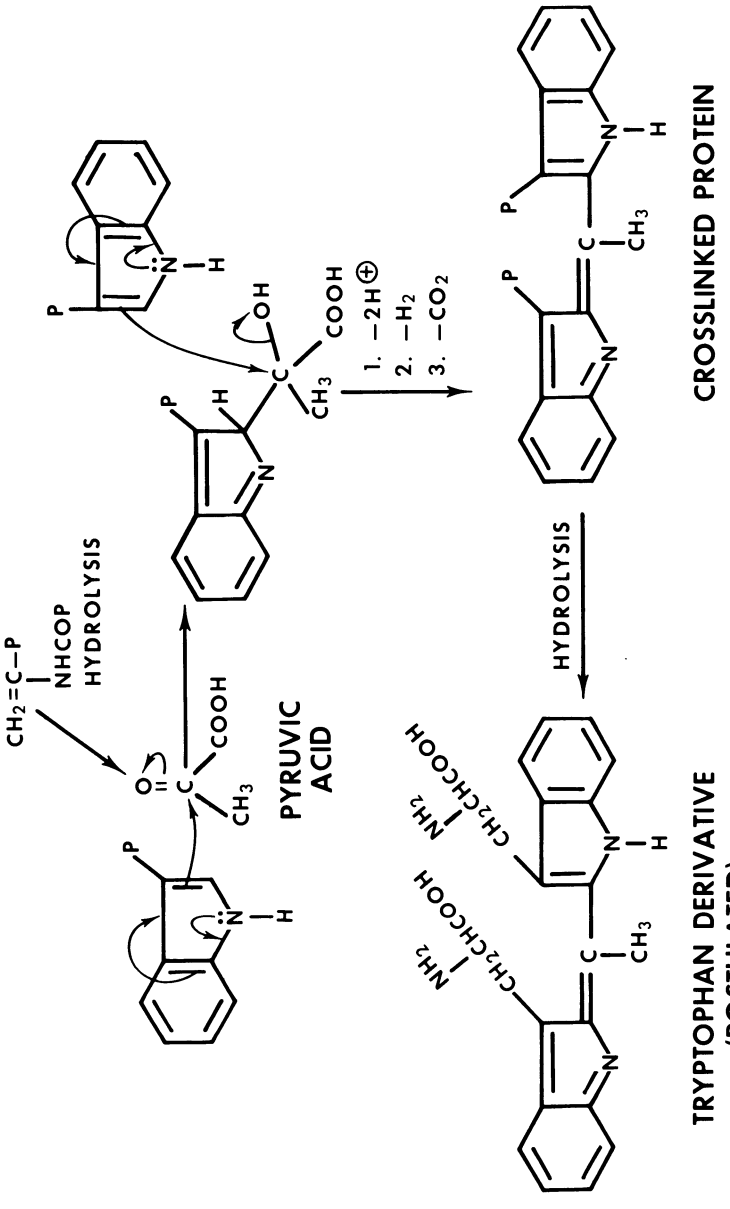
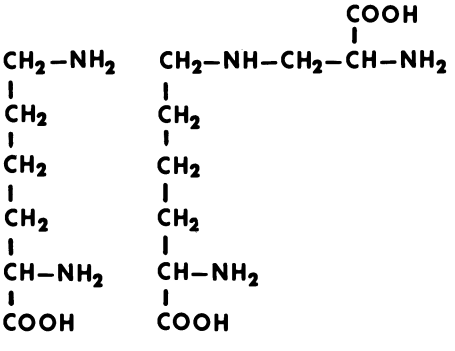


Fig. 13. Proposed mechanism of interaction of the indole ring of tryptophan and pyruvic acid. (Cf. also, Finley and Friedman, this volume).

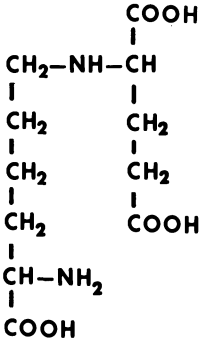
in a particular protein but also their stereochemistry. The stereochemical course of crosslink formation, in turn, undoubtedly has pharmacological consequences, since the different stereoisomers will be metabolized by different enzymes: (e.g. D-amino acid oxidases will attack D-amino acids and D-amino acid asymmetric (usually R) centers but not the corresponding L-isomers). Thus, different products may have different pharmacological (toxicological) properties. This aspect is also discussed by Gould and MacGregor (1977).

Several additional, largely speculative points (Friedman, 1977) are also germane to the present discussion. First, dehydroalanine may react with essential SH and NH_2 groups of proteins and nucleic acids in vivo. Such reactions have been postulated for peptide antibiotics that contain dehydroalanine and 3-methyldehydroalanine (Gross, 1971,1974,1977). Second, 3-N^6-lysinoalanine may exert its pharmacological effect on kidneys by undergoing a reverse Michael reaction in situ to reform dehydroalanine as the reactive alkylating agent. 3-N^6-Lysinoalanine in effect may act as a carrier of dehydroalanine to kidney target sites (Cf. MacGregor and Clarkson, 1974; Kupchan, 1974a,b; Friedman, 1973b). Third, metal ions also show strong affinity for SH and NH_2 groups (Friedman, 1974; Friedman and Masri, 1973). Therefore, they may compete with dehydroalanine for such biological nucleophiles, thus minimizing or preventing the formation of lanthionine and 3-N^6-lysinoalanine. These possibilities and the possible role of 3-N^6-lysinoalanine in metal binding in vivo deserve careful study. Fourth, 3-N^6-lysinoalanine may compete with lysine or other amino acid(s) (e.g. carnitine, Brosquist et al., 1975) during protein biosynthesis, i.e., it may act as a competitive inhibitor of protein biosynthesis. In this connection, it is noteworthy that the antimicrobial action of lysine analogs and derivatives has been ascribed to competitive inhibition of lysine incorporation into cell walls of bacteria (Perlman, 1975) and to the release of cellular constituents, respectively (Nakamiya et al., 1976). Fifth, 3-N^6-lysinoalanine may competitively inhibit lysine catabolism by reaction with α-ketoglutarate to prevent the analogous transformation of lysine to saccharopine, a key intermediate of lysine metabolism (Hutzler and Dancis, 1975; Higashino et al., 1971; Chang, 1976; Moller, 1976a, b). Note the structural resemblance between lysine, lysinoalanine, saccharopine, carnitine, and cystathionine, an intermediate in the metabolism of methionine and other sulfur amino acids (Friedman, 1973a). Sixth, the secondary amino group of 3-N^6-lysinoalanine and saccharopine may interact with nitrites both in vitro and in vivo to form toxic N-nitroso derivatives. Seventh, 3-N^6-lysinoalanine may act as a local irritant or allergen of kidney tissue. Eight, 3-N^6-lysinoalanine may exert its effect by inhibiting or affecting normal reabsorption of amino acids by kidney tubules. Finally, crosslinked amino acids other than 3-N^6-lysinoalanine may also contribute to the observed biological and nutritional effects of alkali-treated proteins.

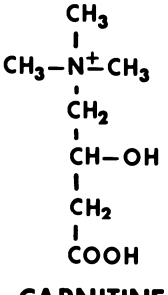


LYSINE

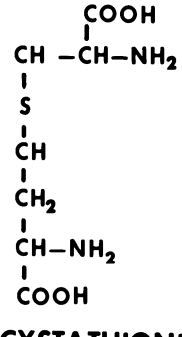
3—N^6—LYSINOALANINE



SACCHAROPINE



CARNITINE



CYSTATHIONINE

In conclusion, though very many kinds of crosslinks can theoretically be formed in proteins by heat and alkali, only very few have been satisfactorily identified and characterized. I hope this discussion will stimulate further studies and practical benefits from this still important, but incomplete research. For a discussion of α-amino group crosslinking, see Part A, p. 737.

ACKNOWLEDGMENTS

I take great pleasure in thanking W. L. Alworth, L. Chauffe, K. L. Loening, J. R. Whitaker, for their constructive comments and contributions to this manuscript, and Amy T. Noma and Linda C. Loyd for the amino acid analyses.

REFERENCES

Alworth, W. L. (1976). Private communication.

Alworth, W. L. (1972). "Steroechemistry and Its Application in Biochemistry," Wiley, Interscience, New York.

Asquith, R. S. and Otterburn, M. S. (1977). Cystine-alkali reactions in relation to protein crosslinking. This volume.

Bentley, R. B. (1970). "Molecular Asymmetry in Biology," Volume II, Academic Press, New York.

Bentley, R. (1969). "Molecular Assymetry in Biology," Volume I, Academic Press, New York.

Bohak, Z. (1964). N-Epsilon-(DL-2-amino-2-carboxyethyl)-L-lysine, a new amino acid formed on alkaline treatment of proteins. J. Biol. Chem., 239, 2878-2887.

Brooks, P. R. (1976). Reactions of oriented molecules. Science, 193, 11-16.

Brosquist, H. P., Horne, D. W. and Tanphaichitr, V. (1975). Lysine metabolism in protein-calorie malnutrition with attention to the synthesis of carnitine. In "Protein-Calorie Malnutrition," R. E. Olson (Ed.), Academic Press, New York, 49-63.

Cahn, R. S. (1964). An introduction to the sequence rule. A system for specification of the absolute configuration. J. Chem. Ed., 41, 116-125.

Cavins, J. F. and Friedman, M. (1967). New amino acids derived from reactions of ε-amino groups in proteins with α,β-unsaturated compounds. Biochemistry, 6, 3766-3770.

Chang, Y. F. (1976). Pipecolic acid pathway: the major lysine metabolic route in the rat brain. Biochem. Biophys. Res. Commun., 69, 174-180.

Finley, J. W., Snow, J. T., Johnston, P. H. and Friedman, M. (1977). Inhibitory effect of mercaptoamino acids on lysinoalanine formation during alkali treatment of proteins. This volume.

Finley, J. W. and Friedman, M. (1977). Products derived from alkali-treatment of proteins. This volume.

Finley, J. W. and Friedman, M. (1976). Products derived from

alkali treatment of proteins. 172d American Chemical Society Meeting, Abstracts, p. AGFD 46.

Finley, J. W. and Friedman, M. (1973a). Chemical methods for available lysine. Cereal Chem., 50, 101-105.

Finley, J. W. and Friedman, M. (1973b). New sweetening agents: N-formyl- and N-acetylkynurenine. J. Agric. Food Chem., 33-34.

Friedman, M. (1977). Effects of lysine modification on chemical, physical, nutritive, and functional properties of proteins, In "Food Proteins," J. R. Whitaker and S. R. Tannenbaum (Eds.), Avi, Westport, Connecticut.

Friedman, M. (Ed.) (1974). "Protein-Metal Interactions," Plenum, New York, Chapters 2, 15, 24. See also Chapter 2 of Friedman (1973a).

Friedman, M. (1973a). "Chemistry and Biochemistry of the Sulfhydryl Group in Amino Acids, Peptides, and Proteins," Pergamon Press, Oxford, England and Elmsford, New York, Chapter 5.

Friedman, M. (1973b). See Chapter 16 of Friedman (1973a).

Friedman, M. (1972). Selective chemical modification of protein sulfhydryl groups. Inta-Science Chemistry Reports, 6, 23-34.

Friedman, M. (1968). Solvent effects of reactions of protein functional groups. Quarterly Repts. Sulfur Chem., 3, 125-144.

Friedman, M. (1967). Solvent effects in reactions of amino groups in amino acids, peptides, and proteins with α,β-unsaturated compounds. J. Am. Chem. Soc., 89, 4709-4713.

Friedman, M. and Finley, J. W. (1976). Reactions of proteins with dehydroalanine. 172d American Chemical Society Meeting, Abstracts p. AGFD 45. This volume.

Friedman, M. and Finley, J. W. (1975a). Reactions of proteins with ethyl vinyl sulfone. Int. J. Peptide Protein Res., 7, 481-486.

Friedman, M. and Finley, J. W. (1975b). Vinyl compounds as reagents for determining available lysine in proteins. In "Protein Nutritional Quality of Foods and Feeds," Part 1, M. Friedman (Ed.), Dekker, New York, 503-520.

Friedman, M. and Finley, J. W. (1975c). Evaluation of methods for tryptophan analysis in proteins. In "Protein Nutritional Quality of Foods and Feeds," Part 1, M. Friedman (Ed.), Dekker, New York, 423-452.

Friedman, M. and Masri, M. S. (1973). Sorption behavior of mercuric salts on modified wool and polyamino acids. J. Applied Polym. Sci., 17, 2179-2186.

Friedman, M. and Noma, A. T. (1975). Methods and problems in the chromatographic analysis of sulfur amino acids. In "Protein Nutritional Quality of Foods and Feeds," Part 1, M. Friedman (Ed.), Dekker, New York, pp. 521-548.

Friedman, M. and Wall, J. S. (1964). Application of a Hammett-Taft relation to kinetics of alkylation of amino acids and peptide model compounds with acrylonitrile. J. Am. Chem.

Soc., 86, 3735-3741.
Gilbert, H. F., III and O'Leary, M. H. (1975). Modification of arginine and lysine in proteins with 2,4-pentanedione. Biochemistry, 14, 5194-5198.
Ginsburg, S. and Wilson, I. B. (1964). Factors affecting the competitive formation of oxazolines and dehydroalanines from serine derivatives. J. Am. Chem. Soc., 86, 4716-4720.
Gould, D. G. and MacGregor, J. T. (1977). Nutritional and biological consequences of protein crosslinking: an overview. This volume.
Gross, E. (1977). α,β -Unsaturated and related amino acids in peptides and proteins. This volume.
Gross, E. (1974). α,β -Unsaturated amino acids in peptides and proteins: formation, chemistry, and biological role. 168th Meeting of the American Chemical Society, Atlantic City, New Jersey, Abstracts p. AGFD 13.
Gross, E. (1971). Structure and function of peptides with α,β-unsaturated amino acids. Intra-Science Chem. Repts., 5, 405-408.
Hanson, K. R. (1966). Applications of the sequence rule. I. Naming the paired ligands g,g at a tetrahedral atom Xggij. II. Naming the two faces of a trigonal atom Yghi. J. Am. Chem. Soc., 88, 2731-2742.
Hanson, K. R. (1976). Concepts and perspectives in enzyme stereochemistry. Ann. Rev. Biochem., 45, 307-330.
Higashino, K., Fujioka, M. and Yamamura, Y. (1971). The conversion of L-lysine to saccharopine and α-aminoadipate in mouse. Arch. Biochem. Biophys., 142, 606-614.
Klyne, W. and Buckingham, J. (1974). "Atlas of Stereochemistry," Oxford University Press, New York.
Koshland, D. E., Jr. (1973). Contribution of orientation to the catalytic power of enzymes. Proc. Int. Conf. Theoret. Physical Biology, M. Marois (Ed.), Karger, Basel, Switzerland, 286-302.
Kupchan, S. M. (1974a). Selective alkylation: a mechanism of tumor inhibition. Intra-Science Chem. Rept., 8, 57-66.
Kupchan, S. M. (1974b). Novel natural products with antitumor activity. Fed. Proc., 33, 2288-2295.
MacGregor, J. T. and Clarkson, T. W. (1974). Distribution, tissue binding and toxicity of mercurials. In "Protein-Metal Interactions," M. Friedman (Ed.), Plenum Press, New York, 463-503.
Meloche, H. P. and Monti, C. T. (1975). Resolution of the diastereoisomers generated by the chemical reduction of pyruvate-lysine Schiff's base. Biochem. Biophys. Res. Commun., 66, 151-159.
Moller, B. L. (1976a). Conversion of saccharopine to lysine in barley. Phytochemistry, 15, 695-696.
Moller, B. L. (1976b). Lysine catabolism in barley (Hordeum vulgare L.). Plant Physiol., 57, 687-692.

Morino, Y. and Snell, E. E. (1967). The relation of spectral changes and tritium exchange reactions to the mechanism of tryptophanase-catalyzed reactions. J. Biol. Chem., 242, 2800-2809.

Nakamiya, T., Mizuno, H., Meguro, T., Ryono, H. and Takinami, K. (1976). Antibacterial activity of lauryl ester of DL-lysine. J. Ferment. Technol., 54, 369-373.

Perlman, D. (1975). Lysine and lysine analog potentiation of antibiotic and antimicrobial activity. J. Antibiotics, 2, 997-999.

Prelog, V. (1976). Chirality in chemistry. Science, 193, 17-24.

Snow, J. T., Finley, J. W. and Friedman, M. (1976). Relative reactivities of sulfhydryl groups with N-acetyl dehydroalanine and N-acetyldehydroalanine methyl ester. Int. J. Peptide Protein Res., 8, 57-64.

Spencer, H. K., Khatri, H. N. and Hill, R. K. (1976). Stereochemistry of some acetyl coenzyme A condensations. Bioorganic Chem., 5, 177-186.

Touloupis, C. and Vassiliadis, A. (1977). Lysinoalanine formation in wool after treatments with some phosphate salts. This volume.

Whitaker, J. R. and Feeney, R. E. (1977). Behavior of glycosyl and O-phosphoryl proteins in alkaline solution. This volume.

Whitfield, R. E. and Friedman, M. (1972). Chemical modification of wool with dicarbonyl compounds in dimethyl sulfoxide. Text. Res. J., 42, 344-347.

2

BIOLOGICAL EFFECTS OF ALKALI-TREATED PROTEIN AND LYSINOALANINE: AN OVERVIEW

D. H. Gould and J. T. MacGregor

Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California 94710

ABSTRACT

The renal alterations induced by alkali-treated protein and lysinoalanine are reviewed and their biological implications discussed. Alkali-treated proteins and lysinoalanine, an unusual amino acid formed in proteins during alkali treatment, have been shown to produce a renal alteration characterized by nuclear and cytoplasmic enlargement, with alterations in DNA synthesis, mitotsis and nucleoprotein. These changes are localized in the straight portion of the proximal renal tubule and have been observed in rats but not in several other species. The nephrotoxic effect of synthetic lysinoalanine has been consistently demonstrated, but the ability of alkali-treated protein to induce renal alterations is apparently modified by factors other than lysinoalanine content. Factors which may influence the development of the kidney lesions in animals fed alkali-treated protein are discussed, including nutritional factors, the chemical form of lysinoalanine in the protein, species differences, and metabolic fate. Other clinical and experimental conditions that result in similar renal alterations are presented for comparison with the lysinoalanine induced lesion, and possible functional consequences are considered.

INTRODUCTION

It has been known for many years that alkali treatment of proteins results in the decomposition of cystine, serine, phosphoserine and threonine residues, and their derivatives, with the elimination of H_2S or H_2O to form the corresponding α,β-unsaturated

amino acids (i.e.: dehydroalanine and β-methyldehydroalanine) (25, 29,30,31,36). These decompositions are accompanied by the formation of amino acids not normally found in nature (2,20,35,60), presumably via reaction of dehydroalanine residues with sulfhydryl or amino groups present in the protein. These "unnatural" amino acids include lanthionine (20), lysinoalanine (2,35), and ornithinoalanine (60). Recent evidence indicates that histidine, arginine and kynurenine derivatives may also be formed (14). The biological activities of these various "unnatural" amino acids are largely undetermined, but recent work (55) has correlated a unique morphological and biochemical lesion in the straight portion of the renal proximal tubule with the ingestion of alkali-treated proteins containing lysinoalanine (LAL). These findings have generated considerable interest in the safety of alkali-treated proteins. This discussion will focus on this particular lesion and its implications.

BIOLOGICAL EFFECTS OF ALKALI-TREATED SOYPROTEIN AND LYSINOALANINE

In 1967 Woodard and Alvarez (54) described a unique renal lesion in Sprague-Dawley rats fed an industrial grade alkali-treated soyprotein known commercially as "Alpha protein". This lesion, designated nephrocytomegaly, consisted of an increase in the size of the nucleus and often the cytoplasm of the epithelial cells of the straight portion (pars recta)[1] of the proximal renal tubules. Figure 1 illustrates this lesion. Cytophotometric studies indicated that the increase in nuclear size (karyomegaly) was accompanied by a proportional increase in the total nuclear protein. The amount of deoxyribonucleic acid (DNA) in enlarged nuclei indicated variation from two to eight times the normal content. Chromosomal protein content paralleled that of DNA in affected nuclei. Some eosinophilic, intranuclear inclusion bodies, later shown to be cytoplasmic invaginations (58), were present in both large and normal nuclei in Alpha protein-fed rats.

This lesion was morphologically similar to the renal alteration reported earlier by Newberne and Young (28), who used alkali-treated soyprotein to study the effects of diets with marginal levels of methionine and choline. These investigators attributed the lesion in the pars recta to dietary imbalances, since the severity of the lesion could be reduced by adding methionine and could be completely prevented by supplementing with choline and vitamin B_{12}, in addition to methionine (27, 28).

---

[1] It appears that the most widely accepted term for the zone of the kidney in which the pars recta predominates is the outer stripe of the outer medulla or outer medullary stripe (47, 49, 58). This zone has been referred to by some authors as the inner stripe of the cortex. We will use the term outer medullary stripe.

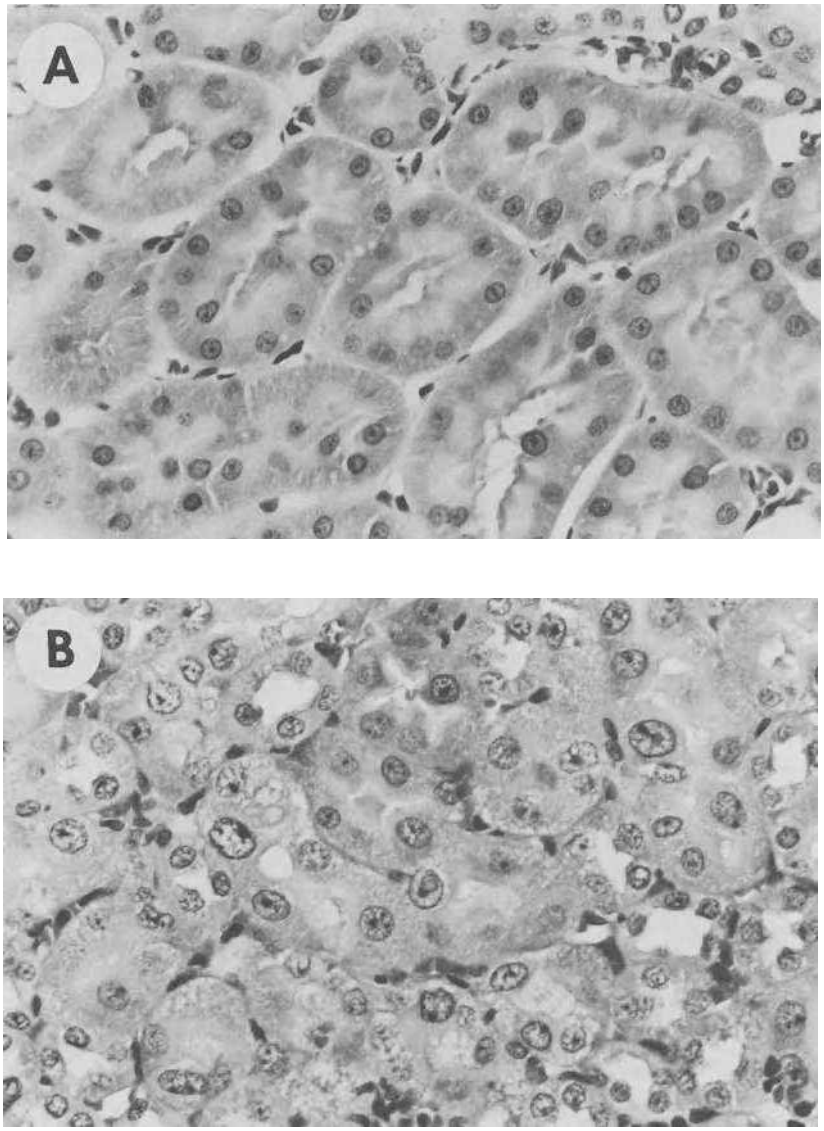


Figure 1. Photomicrograph of outer medullary stripe of kidneys from rats fed 20% soyprotein for eight weeks. Hematoxylin and eosin. (A) control, pair-fed, untreated soyprotein diet. Note uniformity of the pars recta cells. (B) alkali-treated soyprotein (2630 ppm total dietary LAL). Note the nuclear and cytoplasmic enlargement of many of the pars recta cells. Magnification both 440X.

Woodard and Alvarez (54) observed the renal lesion in the presence of levels of methionine, choline, and vitamin B_{12} that approximated those which Newberne and Young (28) and Newberne et al. (27) found to inhibit development of the lesion. Woodard and Alvarez therefore concluded that Alpha protein contained some toxic substance that exerts a selective action on the kidney.

Subsequent studies further delineated the nature of this renal lesion. Woodard (53) reported that the initial effect of Alpha protein on the kidney of Sprague-Dawley rats occurred after 1 week on the diet and consisted of an increase in the mitotic index of cells of the pars recta. By the third week the number of mitotic figures was still increased but had declined considerably from the levels observed after the first and second weeks.

After two weeks on the Alpha protein diet, an increased amount of tubular epithelial lipid was noted in all parts of the proximal tubule. This alteration varied in severity among treated animals and often persisted throughout the entire nine month experimental period.

Continuous and pulse labeling with tritiated thymidine during the second and third weeks of Alpha protein feeding revealed that increased numbers of normal and karyomegalic pars recta nuclei were synthesizing DNA. Newberne et al. (27) demonstrated a similar uptake of thymidine at six weeks but not at three weeks.

After four weeks, animals receiving the Alpha protein diet had occasional enlarged nuclei in the pars recta (53). Over the following weeks the number of enlarged nuclei increased, reaching a maximum by the eighth to tenth week. Some cells continued to increase up to nine months. Newberne et al. (27) observed nephrocytomegaly in test animals that had been fed alkali-treated soyprotein for nine months, followed for three months by supplementation with methionine, choline, and vitamin B_{12}. Woodard (53) demonstrated the presence of the lesion in animals fed alkali-treated soyprotein for nine months and Karayiannis (22) demonstrated the lesion in three animals fed alkali-treated soyprotein for eight weeks followed by commercial laboratory animal chow for four weeks.

Reyniers et al. (39) reported a number of cytochemical alterations in megalic renal nuclei from rats fed Alpha protein. The enlarged nuclei showed an increased amount of acridine orange dye binding and reduced chromatin thermal stability. According to these investigators these two findings imply that the enlarged pars recta cells are "activated" epithelial cells, i.e. cells that are stimulated to synthesize DNA. Additionally, they found that the ratio of acid dye binding by lysine and arginine residues of histone was significantly altered in enlarged nuclei. Total histone in the nucleus was proportional to the DNA content. It is of interest that Reyniers et al. (39) report that the Feulgen-DNA content of the nuclei fell into two clear DNA classes (2c and 4c) with no intermediate values, suggesting that most enlarged nuclei had completed DNA replication. In contrast, Woodard and Alvarez

(54) report many large nuclei with intermediate values.

The association between renal cytomegaly of the pars recta and the presence of LAL in the diet of rats was demonstrated by Woodard and Short (55). They observed that the nephrotoxic factor was not indigenous to soybeans, but was induced by alkali treatment. They demonstrated that intermediates in the manufacture of Alpha protein prior to the alkaline modification failed to produce the renal lesion and that edible soyprotein could be rendered nephrotoxic by alkali treatment. The common component induced by the alkali treatment and associated with the nephrotoxicity in these two diets was the unusual amino acid, LAL.

Subsequently, Woodard et al. (56, 58) provided additional evidence of the causative role of LAL. They demonstrated that synthetic LAL (N^{ϵ}-(D,L-2-amino-2-carboxyethyl)-L-lysine) induced a renal lesion in rats similar to the nephrocytomegaly observed in rats fed alkali-treated soyprotein. Animals fed semipurified diets containing 250, 500, or 1000 ppm synthetic LAL for one week exhibited alterations of the pars recta characterized by karyomegaly, tubular dilatation, loss of some tubular epithelial cells, and dimunition of eosinophilic staining properties. At 3000 ppm, the karyomegaly was induced in the proximal convoluted tubule as well as the pars recta. Both degenerative and regenerative changes were evident. Daily intraperitoneal injections of 30 mg (approximately 600 mg/Kg) of LAL produced karyomegaly, necrosis, and tubular dilatation in the pars recta.

Similar results were observed by de Groot et al. (8) with high and low levels of synthetic LAL fed to rats for four and thirteen weeks, respectively. Kidneys from the animals fed high levels of LAL (1000, 3000, 10,000 ppm) had cytomegaly, necrosis, and regeneration mainly in the pars recta. Of the lower dose groups (10, 30, 100 ppm) nephrocytomegaly was present only in the 100 ppm group.

de Groot et al. (8) investigated the effects of LAL in species other than the rat. They fed diets containing LAL to a number of animal species, including Japanese quail, mice, hamsters, rats, dogs, and Rhesus monkeys. The animals were killed after four and eight weeks of feeding, except the monkeys, which were killed after nine weeks. All animals received diets containing 1000 ppm of synthetic LAL. Two additional monkeys received a diet containing 10,000 ppm LAL provided by alkali-treated casein. In none of these animals was nephrocytomegaly detected.

CONFLICTING OBSERVATIONS ON LYSINOALANINE AND SOME POSSIBLE EXPLANATIONS

Although the findings described above indicate that lysinoalanine formed in protein during alkaline modification is the cause of alkali-treated protein-induced nephrocytomegaly, a number of divergent findings reported by different laboratories suggest that a closer examination of the relationship between dietary LAL

and the development of this lesion is necessary.

Perhaps the most striking indication of the lack of a simple, direct relationship between nephrocytomegaly and dietary LAL is the consistent failure of de Groot and coworkers (7, 8, 9) to observe nephrocytomegaly in rats fed diets containing LAL in alkali-treated soyprotein isolate. In contrast, similar diets were found by Woodard and coworkers (39, 53, 54, 55), Newberne and coworkers (27, 28) and Karayiannis (22) to consistently produce nephrocytomegaly. de Groot and coworkers do, however, observe nephrocytomegaly when free LAL is fed to rats (8, 9).

In an attempt to reconcile these apparently conflicting observations, some factors which may explain these divergent findings are discussed below.

Strain difference. The possibility of a rat strain difference in response to alkali-treated soyprotein has been raised because, in earlier work, Woodard and coworkers (39, 53, 54, 55) and Newberne and Young (28) used a Sprague-Dawley-derived strain, while de Groot and coworkers used a Wistar-derived strain (7, 50). Karayiannis (22) has shown Sprague-Dawley and Wistar rats fed alkali-treated soyprotein to be about equally sensitive to the development of nephrocytomegaly. Struthers et al. (43), however, report a greater sensitivity of Sprague-Dawley than of Wistar rats to alkali-hydrolyzed soyprotein. Similar levels of free LAL cause nephrocytomegaly in both Sprague-Dawley and Wistar rats (8, 57).

Presence of LAL-containing oligopeptides in diets. Since de Groot and coworkers have shown that oligopeptides containing LAL produced nephrocytomegaly more readily than higher molecular weight proteins with a similar LAL content (8, 9, 32), partially hydrolyzed LAL-containing proteins may be considered more likely to produce renal changes than intact proteins. The conclusion of these workers that nephrocytomegaly cannot be produced from high molecular weight proteins containing LAL does not, however, appear warranted. Since a large proportion of smaller molecular weight peptides should be removed by washing with water (32), the unwashed alkali-treated soyprotein with which de Groot and coworkers were unable to produce nephrocytomegaly should be of equal or lower molecular weight than the similarly treated, but thoroughly water-washed, alkali-treated soyprotein of roughly equivalent LAL content which was found to consistently produce severe nephrocytomegaly in other laboratories (22, 55). Thus, although it seems clear that variations in oligopeptide content of alkali-treated proteins may influence the degree of nephrocytomegaly, this factor does not appear to explain why some laboratories consistently obtain severe nephrocytomegaly from alkali-treated soyprotein isolate, while others fail to obtain the lesion from comparable protein of equal LAL content.

Nutritional factors. Since the three laboratories which report severe nephrocytomegaly in rats fed alkali-treated soyprotein have all employed diets of almost identical composition (22, 28, 55), while those employed in negative studies with similar proteins have had a somewhat different composition (7, 8, 9, 32, 50), it seems likely that dietary factors other than LAL content may affect the expression of nephrocytomegaly. Newberne and Young (28) and Newberne et al. (27) reported that the lesion could be prevented by dietary supplementation with methionine, choline and vitamin B_{12}. Woodard and Alvarez (54), however, could not confirm these findings. While the reasons for this discrepancy have not been resolved, the findings of Newberne and coworkers indicate that methionine and other nutritional factors may strongly modulate the expression of nephrocytomegaly.

In several of the negative experiments with alkali-treated soyprotein (7, 32, 50), an untreated protein source was included in the diet. Moreover, the diets which have been used to demonstrate severe nephrocytomegaly from alkali-treated proteins have generally produced poor growth, hair loss, and diarrhea, which may indicate inadequate dietary protein (22, 50, 54). It thus appears likely that an amino acid deficiency or imbalance induced by alkaline treatment of the protein may enhance the expression of nephrocytomegaly. In our laboratory, we have consistently observed renal karyomegaly in animals fed diets containing 20% alkali-treated soyprotein, providing 1400 to 2600 ppm dietary LAL, as the sole dietary protein source. We have not, however, obtained the lesion with a 12% alkali-treated soyprotein, 8% untreated lactalbumin diet, providing 1600 ppm dietary LAL, nor in 10% alkali-treated lactalbumin, 10% untreated lactalbumin diets providing 2500 ppm LAL (22). Further, diets containing 20% alkali-treated lactalbumin treated under more moderate alkaline conditions as the sole protein source produced only mild nephrocytomegaly (Fig. 2), despite a dietary LAL level of 5000 ppm (22). Additionally, it has been reported that rats fed an alkali-treated, LAL-containing confectionary product developed a more pronounced karyomegaly on a diet supplemented with 10% untreated casein than on the same diet supplemented with 20% untreated casein (32). These findings strongly suggest that the dietary amino acid source may markedly affect the development of nephrocytomegaly in rats fed alkali-treated proteins.

The diets used in the negative and positive studies differed not only in protein but also in carbohydrate source and salt concentration. Although these differences seem less likely to be involved in the conflicting results, they may play a contributing role. In some of the experiments in which nephrocytomegaly was not observed, differences in dietary LAL levels (50) or in the duration of exposure (8, 9) may explain the failure to observe nephrocytomegaly.

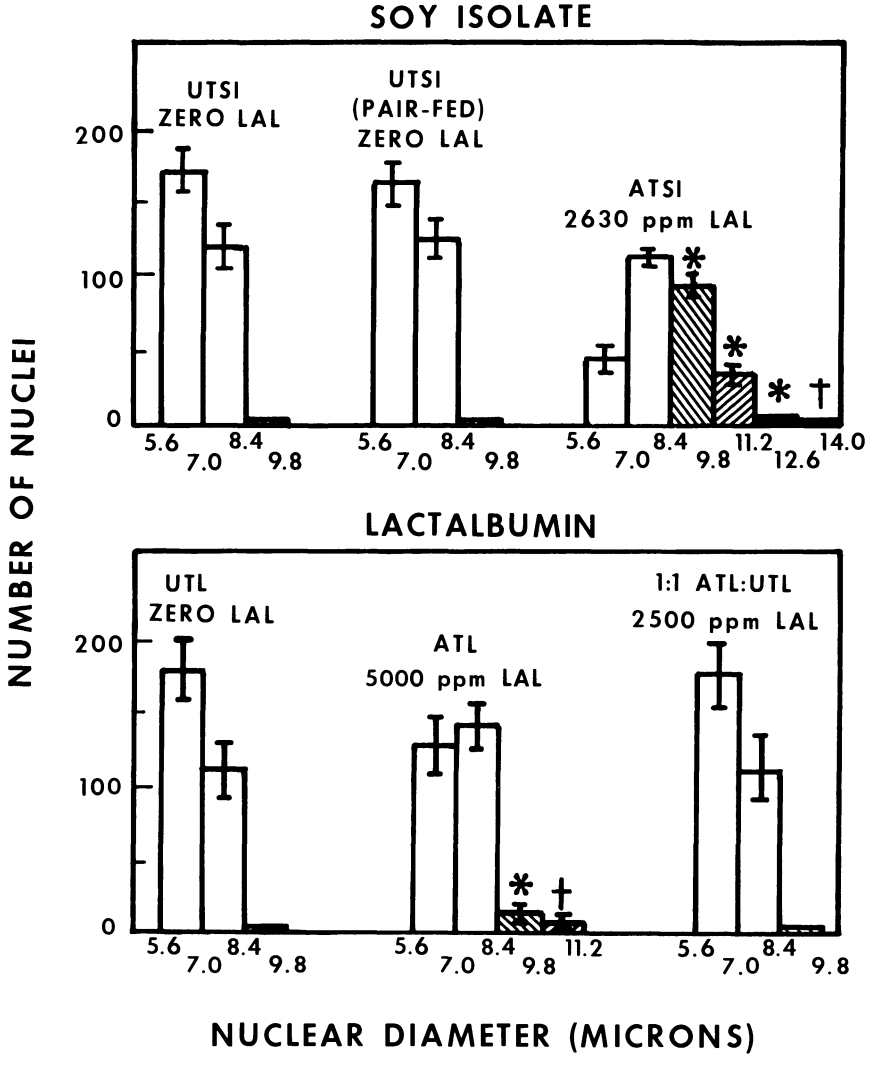


Figure 2. Size distribution of pars recta nuclei in 6 micron kidney sections from Sprague-Dawley rats fed 20% soy protein isolate (untreated, UTSI;alkali-treated,ATSI) or 20% lactalbumin (untreated, UTL;alkali-treated,ATL) for eight weeks. Data are normalized to 300 nuclei per animal. Numbers of animals per group: UTSI, 6;UTSI(pair-fed),5;ATSI,5;UTL,7;ATL,7;1:1-ATL:UTL,7. Bars indicate ± one standard error. Significant increases in the number of nuclei in size ranges above 8.4 microns, compared with the same size range in the untreated control group, are indicated; $p<.01$,*;$p<.025$,†(Nonparametric comparison by Rank-Sum Test, Wine, R.L., Statistics for Scientists and Engineers, Prentice-Hall, 1964, pp. 601-607). Data are from reference (22).

Chemical form of lysinoalanine and alkali-treated proteins. Although a knowledge of the chemical nature of the LAL formed in alkali-treated proteins is essential to a full understanding of its biological activity, little additional information has been obtained since the original descriptions of the chemistry of LAL in alkali-treated proteins by Bohak (2) and Patchornik and Sokolovsky (35). There are at least two chemical characteristics which may be of importance with respect to the biological activity of LAL.

First, LAL possesses two optically active centers, one in each of the amino acids of which LAL is comprised, thus four different optical isomers are possible (17). Bohak (2) used optically specific amino acid oxidases to determined that the LAL formed in alkali-treated ribonuclease had the configuration N^{ε}-(D,L-2-amino-2-carboxyethyl)-L-lysine. Since the mechanism of LAL formation is via dehydroalanine, in which the α and β carbons are joined by a double bond, it is to be expected that the alanyl part of the LAL, formed by the addition of the ε-amino group of lysine to dehydroalanine, would be racemic. Since the lysine moiety is present in the native protein in the L-form, the L-lysyl, D,L-alanyl form observed by Bohak is the most likely to be formed. It is perhaps for this reason that the configuration in other alkali-treated proteins has not been checked. Nonetheless, it is possible that the tendency of certain proteins to assume a particular conformation could force the α-carbon of dehydroalanine to preferentially assume one or the other optical configuration during the addition reaction to lysine. It is also noteworthy that racemization of lysine residues has been reported during relatively severe alkaline treatment of proteins (38). It is not impossible that milder treatments could result in racemization in different proteins. Since the biological activity could well depend on the optical configuration of LAL, it is of interest to determine which optical forms are obtained in various proteins under differing treatment conditions.

The second chemical characteristic which may influence the biological activity of LAL in proteins is the nature of the LAL crosslink which results when the lysyl and dehydroalanyl residues link to form LAL. Whether these two residues are located at adjacent or remote sites in the same protein molecule, or whether they are located on separate molecules and therefore result in interstrand crosslinks, may determine the extent of LAL release from the protein and perhaps even the form in which it is released. Since much higher levels of protein-bound than of free LAL are required to produce nephrocytomegaly, the extent of digestion to the free amino acid *in vivo* must be limited. Although there is evidence that severely alkali-treated proteins containing LAL have a reduced *in vivo* and *in vitro* digestibility (7, 15, 38), it is interesting to note that in moderately treated protein of high LAL yield, the overall protein digestibility *in vivo* is not diminished sufficiently to account for the poor LAL absorption (7). This may

indicate that certain regions of the protein containing LAL crosslinks are very poorly digested relative to the rest of the molecule, thus releasing large amounts of LAL containing oligopeptides in the gut. This suggestion is consistent with the in vitro finding that the LAL in alkali-treated lactalbumin is less easily converted to a dialyzable form by pepsin-pancreatin digestion than were lysine, histidine and arginine (15). Little work has been done to determine the chemical nature of the LAL which is released upon enzymatic digestion in the gut.

Although it is generally assumed that the release of free LAL from alkali-treated proteins is responsible for the development of nephrocytomegaly, it is possible that small, perhaps cyclic (formed when proximate lysyl and dehydroalanyl residues link to form LAL), peptides may be released in the gut and that these may play a role in the development of nephrocytomegaly. Patchornik and Sokolovsky (35) investigated the intramolecular location of the residues which formed LAL crosslinks in alkali-treated S-dinitrophenylated RNA and concluded that three intramolecular crosslinks were formed; two from adjacent cysteinyl-lysyl residues and one from a lysine residue in position 1 and cysteine 110, which were postulated to be spatially adjacent. Karayiannis (22), using polylysine and polyserine as model compounds, has shown that intermolecular crosslinks may also be formed during alkali-treatment of peptides, and Sternberg (41) has presented evidence that the relative formation of intra- vs inter-molecular LAL crosslinks is a function of the protein concentration during alkali treatment, with interstrand crosslinking favored at higher protein concentrations. Thus, the relative biological availability of LAL may vary both with protein source and the nature of the LAL crosslink formed under a particular set of treatment conditions. Clearly, more information on the chemical nature and biological availability of the LAL formed in various alkali-treated proteins is needed before the significance of the biological experiments performed to date can be fully evaluated.

Intestinal flora. The intestinal flora may also play a role in the disposition of LAL by the animal. Karayiannis (22) has shown that LAL may be metabolized by bacteria to serve as a source of lysine, carbon and nitrogen. Finot et al. (15) have presented evidence that LAL is oxidized to CO_2 by gut bacteria. The extent of LAL availability to the animal may vary with the intestinal flora, which may differ from laboratory to laboratory and from species to species.

Other factors. In the discussion above, we have tried to point out some factors which may influence the development of nephrocytomegaly caused by the ingestion of alkali-treated proteins. Although these factors are perhaps those most likely to influence the development of nephrocytomegaly, many chemical alterations occur in alkali-treated protein and it is possible

that some other factor associated with alkali-treated protein is involved. Dehydroalanine, for example, is known to be formed under the same conditions as LAL; indeed, its formation is a prerequisite for LAL formation. When measurements have been made (35), it has been found at levels roughly equivalent to LAL in alkali-treated protein. The potential reactivity of this compound with nucleic acid amino groups raises the possibility that it could affect nucleic acid metabolism if absorbed from the intestine. Indeed, LAL itself might serve as an intracellular source of dehydroalanine by undergoing a reverse-Michael reaction *in situ* (16). Little is known of the biological activity of dehydroalanine and other "abnormal" amino acids formed in protein during alkaline treatment, and biological studies of the effects of these compounds are needed.

METABOLIC FATE OF FREE AND PROTEIN-BOUND LYSINOALANINE

Little is known of the metabolic fate of protein-bound lysinoalanine. de Groot and Slump (7) fed rats alkali-treated soyprotein, alkali-treated casein or alkali-treated soybean meal and recovered 38-65% of the ingested LAL in the feces . With alkali-treated soyprotein, 60% was not accounted for and only 1.5% was recovered in urine. Subsequent studies with alkali-treated soyprotein and lactalbumin (8, 22) gave similar results, with slightly higher urinary recoveries (up to 6%) in some instances. Finot *et al.* (15) reported urinary recoveries of 10-25% in rats fed mildly alkali-treated soyprotein, lactalbumin and fish protein concentrate.

Finot *et al.* (15) also investigated the distribution and metabolism of ^{14}C-labeled free LAL in the rat and in three species in which nephrocytomegaly has not been observed (8, 9), the mouse, hamster, and quail. In the rat, the urinary recovery of LAL is somewhat higher and the fecal recovery lower after the administration of free LAL (8, 15) than after ingestion of protein-bound LAL, indicating that free LAL is better absorbed from the gut. Radioactivity was observed in the lumen of the cecum and intestines of rats even when labeled LAL was given intravenously. A high proportion of free LAL is oxidized to CO_2, most likely by gut bacteria (15). In addition to free LAL, more than 10 LAL metabolites were detected in rat urine (15). At least five of these metabolites appear to be acetylated LAL derivatives. In the 24 hr urine sample collected after an oral dose of 6.8 mg/kg ^{14}C-labeled LAL, 60-65% of the urinary radioactivity was associated with free LAL, 13-19% with a major, acid-stable, metabolite, and the rest with acetylated and other metabolites. The urinary metabolites found in the mouse and hamster were similar to those found in the rat, both qualitatively and quantitatively, with the notable exception that the major, acid-stable, metabolite found in rat urine was absent. This metabolite was also absent in quail urine.

Twenty-four hours after the oral administration of ^{14}C-labeled LAL, the rat kidneys contained a higher fraction of the administered dose than the mouse, hamster or quail kidneys (15). As demonstrated by autoradiography, the radioactivity in rat, mouse, and hamster kidney was localized almost entirely in the outer medulla. The total label in the rat kidney, expressed as a fraction of the administered dose, was more than ten times higher than that in hamster kidney, and 2-10 times higher than in the mouse kidney. The radioactivity in the quail kidney was distributed uniformly. Since these data were obtained from only a few animals at a single time and at different doses, the role of renal retention and metabolism, as determinants of the reported species differences in the development of nephrocytomegaly remains to be delineated.

BIOLOGICAL SIGNIFICANCE OF NEPHROCYTOMEGALY INDUCED BY LYSINO-ALANINE AND ALKALI-TREATED SOYPROTEIN

In evaluating the renal effects of LAL and alkali-treated soyprotein, two aspects must be considered; the significance of the nephrocytomegaly, particularly the karyomegaly, and the specific vulnerability of the terminal part of the proximal tubule, the pars recta.

A number of factors which affect nuclear size have been described (34). These include alterations in DNA replication and nuclear division, which can affect nuclear size by altering the chromatin content, and alterations in other components of the nucleus, such as RNA and non-histone protein. These factors are often intimately involved with the functional state of the cell. Nuclei of regenerating tissue or tissue compensating for some functional deficit frequently have nuclei substantially increased in size (45). In the alkali-treated soyprotein-fed rats studied by Woodard et al. (53, 54), most, but not all, enlarged nuclei had increased amounts of DNA, chromosomal proteins, and histones. Only total nuclear protein increased proportionally to nuclear size. It is not clear, however, that this increase is directly responsible for the increased nuclear size.

An important part of determining the significance of the renal karyomegaly induced by LAL and alkali-treated protein in rats may lie in understanding the factors that render the rat pars recta vulnerable to these agents. Lesions in LAL- and alkali-treated protein-fed rats develop preferentially in the pars recta of the proximal tubule and only after high doses of synthetic LAL is the distribution of the lesion broadened to include parts of the proximal convoluted tubule (57, 58). The morphological and functional differences between the pars recta and the convoluted portion of

the proximal tubule have been described by a number of investigators (3, 13, 21, 26, 47, 48, 49). The pars recta cells are more rectilinear, have fewer interdigitations with adjacent cells, and have fewer apical vacuoles and tubular invaginations associated with the bases of microvilli. The brush border, which is composed of microvilli, is higher in the pars recta. Mitochondria of the pars recta are smaller and more randomly distributed in the cytoplasm than those of the pars convoluta cells. Lysosomes of the pars recta generally tend to be smaller and microbodies are more abundant.

On morphological grounds it appears that endocytosis and turnover of endocytosed material is less important in the pars recta than the pars convoluta, since the pars recta has few apical vesicles and tubular invaginations, and less well developed lysosomal structures (13). The few mitochondria adjacent to the plasma membrane and the reduced interdigitation with adjacent cells suggest that the pars recta cells have limited involvement in the active transport of electrolytes. Burg (3) has in fact demonstrated a reduced capacity of the pars recta cells for water and sodium reabsorption, when compared to the convoluted portion. Pars recta cells were found to have a greater capacity to excrete p-aminohippuric acid than cells in the convoluted portion of the proximal tubules (3, 48). It has also been pointed out that pars recta cells, with their high brush border and their few basal interdigitations, have a higher proportion of the total cell surface in contact with the contents of the tubular lumen (21). Particularly relevant to this discussion is the evidence that amino acid transport mechanisms may be preferentially localized in the pars recta (21, 23, 51).

How LAL interferes with normal cell regulatory processes to produce nephrocytomegaly is unknown. Histones, which may be important in genetic regulation (1, 33), are quite rich in lysine. It is of interest that Reyniers et al. (39) demonstrated that Alpha protein-induced renal megalocytes had an altered ratio of histone lysine and arginine residues when compared to normal size nuclei. Reyniers et al. suggest that megalocytic cells in treated rats may be qualitatively different than normal cells because of differential masking and unmasking of lysine in relation to arginine. This raises the possibility that LAL acts by altering the composition of histone, thereby affecting its function. Events late in the cell cycle, such as condensation of chromatin prior to mitosis, have been shown to be associated with the methylation of histone lysine (33). It is of interest that methionine, an important dietary source of methyl groups (52), has a modulating effect on the expression of nephrocytomegaly (27, 28).

No marked disturbances in renal function have been observed in animals treated with doses of alkali-treated soyprotein or LAL that produce only nephrocytomegaly and not frank cellular degeneration. Woodard et al. (58) specifically studied renal p-aminohippuric acid excretion and could not detect a difference between treated rats

with nephrocytomegaly and normal animals. The authors point out, however, that a possible functional deficit in the pars recta could be obscured under these conditions because p-aminohippuric acid is also excreted to a limited extent in other portions of the tubule. In experiments with synthetic LAL, de Groot et al. (8) studied renal function of individual rats by determining urine glutamic-oxaloacetic transaminase (GOT) activity, specific gravity, and phenol red excretion. The only alterations observed were in the highest dose group animals (10,000 ppm), which exhibited increased activity of GOT and decreased specific gravity. Karayiannis (22) showed statistically significant elevations in blood urea nitrogen and serum glutamic-pyruvic transaminase, and a decrease in plasma globulins in rats fed alkali-treated soyprotein. These alterations do not necessarily imply a specific renal malfunction.

Possible longer term effects of nephrocytomegaly must also be considered. Renal tubular karyomegaly has been observed under a number of natural and experimental conditions. In pyrrolizidine alkaloid intoxication of pigs, renal tubular epithelial hypertrophy with karyomegaly is a prominent feature (19, 37). In two of three Basenji dogs with glucosuria associated with renal tubular dysfunction, many tubular epithelial cells had nuclei enlarged 2-4 times (10). Newberne et al. (27) described enlarged epithelial cells with bizarre nuclei in the straight portion of the proximal tubule in kidneys of rats fed aflatoxin-contaminated peanut meal. Butler (4) reported large, hyperchromatic nuclei in the renal tubules of rats treated acutely with aflatoxin B_1, while Butler and Lijinsky (6) observed large, bizarre epithelial cells with considerable variation in nuclear size in the straight portion of the proximal tubules of rats treated acutely with aflatoxin G_1. In rats treated with 4'-fluoro-4-amino diphenyl, Mathews and Walpole (24) described abnormalities in size, shape, and chromatin distribution of nuclei in tubular epithelial cells and staining alterations in the cytoplasm of cells of the outer medullary stripe that preceded the development of renal adenomas. Zak et al. (59) reported large, sometimes monstrous, nuclei in the proximal convoluted tubules of rats treated with dimethylnitrosamine at a time before the induction of renal tumors. Lead-induced renal tumors are reported to be preceded by bizarre nuclear abnormalities in the renal tubular epithelium (24, 61). Eker (11) and Eker and Mossige (12) described the familial occurrence of renal adenomas thought to be determined by a single dominant gene in rats. In some, but not all, of the rats with adenomas, there was variation in nuclear size and occasionally enlarged nuclei, especially in the outer medullary stripe. A few non-tumor-bearing siblings of tumor-bearing animals also had these lesions. Terrancini and Parker (46) observed enlarged nuclei of tubular epithelial cells of the outer medullary stripe in rats treated with S-dichlorovinyl-L-cysteine.

It is of considerable interest that many of these substances which induce karyomegaly are known to be carcinogenic. Further, many of these carcinogens are known to induce renal tumors, i.e.

aflatoxin G_1 (5), 4'-fluoro-4-amino diphenyl (24), dimethylnitrosamine (59) and lead (24, 61). These findings raise the important question of whether the nephrocytomegaly induced by LAL and alkali-treated soyprotein in rats should be considered a pre-neoplastic change.

Comparing the renal lesions in rats fed alkali-treated soyprotein with the renal lesions induced by aflatoxin-contaminated peanut meal in ducklings and rats, Newberne et al. comment that such a lesion suggests a pre-neoplastic state (27). Schoental and Magee (40) suggest that the enlarged liver cells in pyrrolizidine-treated animals represent pre-neoplastic alterations. Sugihara and Sugihara (44) comment that large, clear nuclei with enlarged irregularly shaped nucleoli are usually encountered in the proximal convoluted tubule cells in the course of dimethylnitrosamine carcinogenesis, and are therefore believed to have an intimate relationship with carcinogenesis. Arguing against the concept that enlarged renal tubular cells represent a pre-neoplastic alteration, Hard and Butler (18) comment that the enlarged nuclei induced by dimethylnitrosamine probably have little relevance to the subsequent development of renal tumors since the nuclear enlargement is widespread through all segments of the proximal tubule while tumors later develop in the first segment. In support of their conclusion, they also point out that although single injections of aflatoxin B_1 and G_1 induce renal tubular karyomegaly, subsequent kidney tumors were not observed. Nonetheless, aflatoxin G_1 does produce a high incidence of renal tumors after long-term feeding (5). Newberne et al. (27) found little or no enhancement of nephrocytomegaly in rats treated with both alkali-treated soyprotein and aflatoxin B_1, nor was there evidence of renal neoplasia. Unfortunately, the interaction of alkali-treated protein and aflatoxin G_1, which has much more marked renal effects than aflatoxin B_1 (6), has apparently not been examined. Additionally, Terrancini and Parker (46), studying rats with tubular karyomegaly induced by long-term (46 weeks) exposure to 5-dichlorovinyl-L-cysteine, found no renal tumors after necropsy at 87 weeks. Thus, although many renal carcinogens produce karyomegaly, it is not clear that karyomegaly, in itself, is necessarily precancerous. A final conclusion regarding the preneoplastic nature of the nephrocytomegaly induced by alkali-treated protein and LAL will only be possible when longer term studies than those carried out to date are available.

HEALTH IMPLICATIONS

Whether LAL, at levels which may occur in foods, could significantly affect human health is a question which cannot be unequivocally answered on the basis of the information currently available. Since the fundamental biochemical alterations leading to the development of nephrocytomegaly are not known, the functional consequences cannot be accurately predicted. While the observation that

similar nuclear alterations frequently precede tumor development is of obvious concern, this association is by no means universal. Nonetheless, any biochemical or morphological alteration of an organelle so intimately involved with cell function as the nucleus is a matter of concern.

Whether LAL or alkali-treated proteins would be expected to produce these biochemical or morphological renal nuclear alterations in the human is, however, not clear. The average human intake of LAL is probably low compared with the levels which produce nephrocytomegaly in the rat. A few commercial food products contain LAL levels near or even well above those which produce karyomegaly in rats (41, 42), but these products do not usually constitute a major fraction of the diet. A notable exception may be the relatively high LAL levels found in certain infant milk formulas (200-600 ppm), which may constitute 100% of an infant's diet. This may be compared with the rat, in which 100 ppm free LAL or alkali-treated protein providing a dietary LAL level of 1500 ppm have been shown to produce karyomegaly. Unfortunately, the relative sensitivity of man to LAL or alkali-treated protein is unknown. From the limited data available, it appears that there is a marked differential susceptibility among species to the induction of the nephrocytomegalic lesion. Of the species examined to date, the rat appears to be the most sensitive. It should be borne in mind, however, that the morphological alterations in the rat are preceded by changes in the synthesis and composition of nuclear macromolecules in the affected cell population, and it does not necessarily follow that similar biochemical changes in other species would necessarily lead to the same morphological alteration. More extensive studies of the effects of LAL and alkali-treated proteins in different species are needed to clarify the differences in species response and to permit extrapolation of the data from different species to man. Further, it is likely that a number of factors other than simple dietary LAL content determine the biological response to alkali-treated proteins. The significance of these factors, discussed in the preceding sections, needs to be resolved before the possible human health hazards can be fully evaluated.

REFERENCES

1. Berlowitz, L. and Pallota, D. (1972). Acetylation of nuclear protein in the heterochromatin and nuclear chromatin of mealy bugs. Exp. Cell Res. 71: 45-48.
2. Bohak, Z. (1964). N^{ε}-(DL-2-amino-2-carboxyethyl)-L-lysine, a new amino acid formed on alkaline treatment of proteins. J. Biol. Chem. 239: 2878-2887.
3. Burg, M. B. (1969). Transport characteristics of isolated perfused proximal tubules, in Thurau, K. and Jahrmärker, H. (Eds.), "Renal Transport and Diuretics," Springer-Verlag, N.Y., pp. 109-121.

4. Butler, W. H. (1964). Acute toxicity of aflatoxin B_1 in rats. Brit. J. Cancer 18: 756-762.
5. Butler, W. H., Greenblatt, M., and Lijinsky, W. (1969). Carcinogenesis in rats by aflatoxin B_1, G_1, & B_2. Cancer Res. 29: 2206-2211.
6. Butler, W. H. and Lijinsky, W. (1970). Acute toxicity of aflatoxin G_1 to the rat. J. Pathol. 102: 209-212.
7. de Groot, A. P. and Slump, P. (1969). Effects of severe alkali treatment of proteins on amino acid composition and nutritive value. J. Nutr. 98: 45-56.
8. de Groot, A. P., Slump, P., Feron, V. J. and Van Beek, L. (1976). Effects of alkali-treated proteins. Feeding studies with free and protein-bound lysinoalanine in rats and other animals. J. Nutr. 106: 1527-1538.
9. de Groot, A. P., Slump, P., Van Beek, L. and Feron, V. J. (1976). Severe alkali treatment of proteins, in Bodwell, C. E. (Ed.), "Evaluation of Proteins for Humans," Avi Publishing Co., Westport, CT.
10. Easley, R. J. and Breitschwerdt, E. B. (1976). Glucosuria associated with renal tubular dysfunction in three Basenji dogs. J. Am. Vet. Med. Assoc. 168: 938-943.
11. Eker, R. Familial renal adenomas in Wistar rats. (1954). Acta Path. Microbiol. Scand. 34: 554-562.
12. Eker, R., and Mossige, J. (1961). A dominant gene for renal adenomas in the rat. Nature 189: 858-859.
13. Ericsson, J. L. E. and Trump, B. F. (1969). Electron microscopy of the uriniferous tubules, in Rouiller, C. and Muller, A. F. (Eds.), "The Kidney," Vol. 1, Academic Press, N.Y., pp. 351-443.
14. Finley, J. W. and Friedman, M. (1977). Products derived from alkali treatment of proteins. This volume.
15. Finot, P. A., Bujard, E. and Arnaud, M. (1977). Metabolic transit of lysinoalanine (LAL) bound to protein and of free radioactive [^{14}C]-lysinoalanine. This volume.
16. Friedman, M. (1977). Effects of lysine modification on chemical, physical, nutritive, and functional properties of proteins, in Whitaker, J. R. and Tannenbaum, S. R. (Eds.), "Food Proteins," Avi Publishing Co., Westport, CT, Chapter 18.
17. Friedman, M. (1977). Crosslinking amino acids: Stereochemistry and nomenclature. This volume.
18. Hard, G. C. and Butler, W. H. (1971). Morphogenesis of epithelial neoplasms induced in the rat kidney by dimethylnitrosamine. Cancer Res. 31: 1496-1505.
19. Harding, J. D. J., Lewis, G., Done, J. T. and Allcroft, R. (1964). Experimental poisoning by Senecio jacobaea in pigs. Path. Vet. 1: 204-220.
20. Horn, M. J., Jones, D. B., Ringel, S. J. (1941). Isolation of a new sulfur-containing amino acid (lanthionine) from sodium carbonate treated wool. J. Biol. Chem. 138: 141-149.

21. Jacobsen, N. O. and Jorgensen, F. (1973). Ultrastructural observations on the pars descendens of the proximal tubule in the kidney of the male rat. Z. Zellforsch. 136: 479-499.
22. Karayiannis, N. (1976). Lysinoalanine Formation in Alkali Treated Proteins and Their Biological Effects, Ph.D. Thesis. University of California, Berkeley.
23. Longley, J. B. (1969). Histochemistry of the kidney, in Rouiller, C., Muller, A. F. (Eds.), "The Kidney," Vol. 1, Academic Press, N.Y., pp. 157-259.
24. Mathews, J. J. and Walpole, A. L. (1958). Tumors of the liver and kidney induced in Wistar rats with 4'-fluoro-4-amino diphenyl. Brit. J. Cancer 12: 234-241.
25. Mecham, D. K. and Olcott, H. S. (1949). Phosvitin, the principle phosphoprotein of egg yolk. J. Am. Chem. Soc. 71: 3670-3679.
26. Moffat, D. B. (1975). "The Mammalian Kidney." Cambridge University Press, N.Y.
27. Newberne, P. M., Rogers, A. E., and Wogan, G. N. (1968). Hepatorenal lesions in rats fed a low lipotrope diet and exposed to aflatoxin. J. Nutr. 94: 331-343.
28. Newberne, P. M. and Young, V. R. (1966). Effects of diets marginal in methionine and choline with and without vitamin B_{12} on rat liver and kidney. J. Nutr. 89: 69-79.
29. Nicolet, B. H. (1931). The mechanism of sulfur lability in cysteine and its derivatives. I. Some thio ethers readily split by alkali. J. Am. Chem. Soc. 53: 3066-3072.
30. Nicolet, B. H. (1932). The mechanism of sulfur lability in cysteine and its derivatives. II. The addition of mercaptan to benzoylaminocinnamic acid derivatives. J. Biol. Chem. 95: 389-392.
31. Nicolet, B. H., Shinn, L. A., and Saidel, L. J. (1942). The lability toward alkali of serine and threonine in proteins, and some of its consequences. J. Biol. Chem. 142: 609-613.
32. O'Donovan, C. J. (1976). Recent studies of lysinoalanine in alkali-treated proteins. Fd. Cosmet. Toxicol. 14: 483-489.
33. Olson, M. O. J. and Busch, H. (1974). Nuclear proteins, in Busch, H. (Ed.), "The Cell Nucleus," Vol. III, Academic Press, N.Y., pp. 211-268.
34. Palkovits, M., and Fischer, J. (1968). "Karyometric Investigations," Akademiai Kiado, Budapest.
35. Patchornik, A. and Sokolovsky, M. (1964). Chemical interactions between lysine and dehydroalanine in modified bovine pancreatic ribonuclease. J. Am. Chem. Soc. 86: 1860-1861.
36. Patchornik, A. and Sokolovsky, M. (1964). Nonenzymatic cleavages of peptide chains at the cysteine and serine residues through their conversion into dehydroalanine. I. Hydrolytic and oxidative cleavage of dehydroalanine residues. J. Am. Chem. Soc. 86: 1206-1212.
37. Peckham, J. C., Sangster, L. T., and Jones, O. H. (1974). Crotalaria spectabilis poisoning in swine. J. Am. Vet. Med.

Assoc. 165: 633-638.
38. Provansal, M. M. P., Cuq, J. L. A., and Cheftel, J. C. (1975). Chemical and nutritional modifications of sunflower proteins due to alkaline processing. Formation of amino acid cross-links and isomerization of lysine residues. J. Agric. Fd. Chem. 23: 938-943.
39. Reyniers, J. P., Woodard, J. C., and Alvarez, M. R. (1974). Nuclear cytochemical alterations in α-protein induced nephrocytomegalia. Lab. Invest. 30: 582-588.
40. Schoental, R. and Magee, P. N. (1959). Further observations on the subacute and chronic liver changes in rats after a single dose of various pyrrolizidine (Senecio) alkaloids. J. Path. Bact. 78: 471-482.
41. Sternberg, M. and Kim, C. Y. (1977). Lysinoalanine formation in protein food ingredients. This volume.
42. Sternberg, M., Kim, C. Y., and Schwende, F. J. (1975). Lysinoalanine: presence in foods and food ingredients. Science 190: 992-994.
43. Struthers, B. J., Dahlgren, R. R., and Hopkins, D. T. (1976). Biological effects of feeding graded levels of alkali hydrolyzed soy protein containing lysinoalanine [N^{ε}-(2-amino-2-carboxyethyl)-L-lysine] in Sprague-Dawley and Wistar rats. Fed. Proc. 35: 497.
44. Sugihara, R. and Sugihara, S. (1976). Electron microscopic observations on the morphogenesis of renal cell carcinoma induced in rat kidney by dimethylnitrosamine and N-(3,5-dichlorophenyl) succinimide. Cancer Res. 36: 533-550.
45. Sulkin, N. M. (1949). Cytological studies of the remaining kidney following unilateral nephrectomy in the rat. Anat. Rec. 105: 95-111.
46. Terrancini, B. and Parker, V. H. (1965). A pathological study on the toxicity of S-dichlorovinyl-L-cysteine. Fd. Cosmet. Toxicol. 3: 67-74.
47. Tisher, C. C. (1976). Anatomy of the kidney, in Brenner, B. M. and Rector, F. C. (Eds.), "The Kidney," Vol. I, W. B. Saunders, Philadelphia, PA.
48. Tune, B. M., Burg, M. B. and Patlak, C. S. (1969). Characteristics of p-aminohippurate transport in proximal renal tubules. Am. J. Physiol. 217: 1057-1063.
49. Trump, B. F. and Bulger, R. E. (1968). The morphology of the kidney, in Becker, E. L. (Ed.), "Structural Basis of Renal Disease," Harper and Rowe, N.Y., pp. 1-92.
50. Van Beek, L., Feron, V. J. and de Groot, A. P. (1974). Nutritional effects of alkali-treated soy protein in rats. J. Nutr. 104: 1630-1636.
51. Wedeen, R. P., Samuel, O. T. (1971). Intrarenal distribution of nonmetabolized amino acids in vivo. Am. J. Physiol. 220: 507-512.
52. White, A., Handler, P., and Smith, E. L. (1964). "Principles of Biochemistry," 3rd edition, McGraw-Hill Book Co., New York,

p. 505.
53. Woodard, J. C. (1969). On the pathogenesis of alpha protein-induced nephrocytomegalia. Lab. Invest. 20: 9-16.
54. Woodard, J. C. and Alvarez, M. R. (1967). Renal lesions in rats fed diets containing alpha protein. Arch. Path. 84: 153-162.
55. Woodard, J. C. and Short, D. D. (1973). Toxicity of alkali-treated soy protein in rats. J. Nutr. 103: 569-574.
56. Woodard, J. C., Short, D. D. (1975). Renal toxicity of N^{ε}-(DL-2-amino-2-carboxyethyl)-L-lysine (lysinoalanine) in rats. Fed. Proc. 34: 929.
57. Woodard, J. C., Short, D. D. (in press). Renal toxicity of N^{ε}-(DL-2-amino-2-carboxyethyl)-L-lysine (lysinoalanine) in rats. Fd. Cosmet. Toxicol.
58. Woodard, J. C., Short, D. D., Alvarez, M. R. and Reyniers, J. P. (1975). Biological effects of N^{ε}-(DL-2-amino-2-carboxyethyl)-L-lysine, lysinoalanine, in Friedman, M. (Ed.), "Protein Nutritional Quality of Foods and Feeds," Part 2, Marcel Dekker Inc., N.Y., pp. 595-618.
59. Zak, F. G., Holzner, H. J., Singer, E. J., and Popper, H. (1960). Renal and pulmonary tumors in rats fed dimethylnitrosamine. Cancer Res. 20: 96-99.
60. Ziegler, Kl., Melchert, I., and Lürken, C. (1967). N^{δ}-(2-amino-2-carboxyethyl)-ornithine, a new amino acid from alkali-treated proteins. Nature 214: 404-405.
61. Zollinger, H.-U. (1953). Durch chronische Bleivergiftung erzengte Nierenadenome und -carcinome bei Ratten und ihre Beziehungen zu den entsprechenden Neubildungen des Menschen. Virchows Arch. Path. Anat. 323: 694-710.

EDITOR'S NOTE

To help the reader better understand the preceding paper by Gould and MacGregor (1977) and the following paper by Finot et al. (1977) about structural and functional changes in kidneys of rats fed 3-N^6-lysinoalanine-containing food proteins, I invited Drs. D. H. Gould and J. T. MacGregor to prepare a cross-sectional drawing of a kidney that shows the spatial relationships of the affected pars recta to the rest of the kidney (see Figure on next page). I thank them for this figure. I also thank Robert David Wong of the Illustration Studio of the Western Regional Research Center, ARS, USDA, Berkeley, California, for his artistic skill in preparing the final drawing.

The figure was modified from Moffat (1975) and illustrates the spatial arrangement of the tubules relative to the zonation (denoted by dashed lines) of the rat kidney. Both long and short-looped nephrons are shown in this transverse section. The straight portions of the proximal tubules (PT-SP), the pars rectae, lie predominantly in the outer stripe of the outer medulla. Additional details of the morphology and function of the straight portion of the proximal tubule are disucussed by Maunsbach (1966) and Gould and MacGregor (1977).

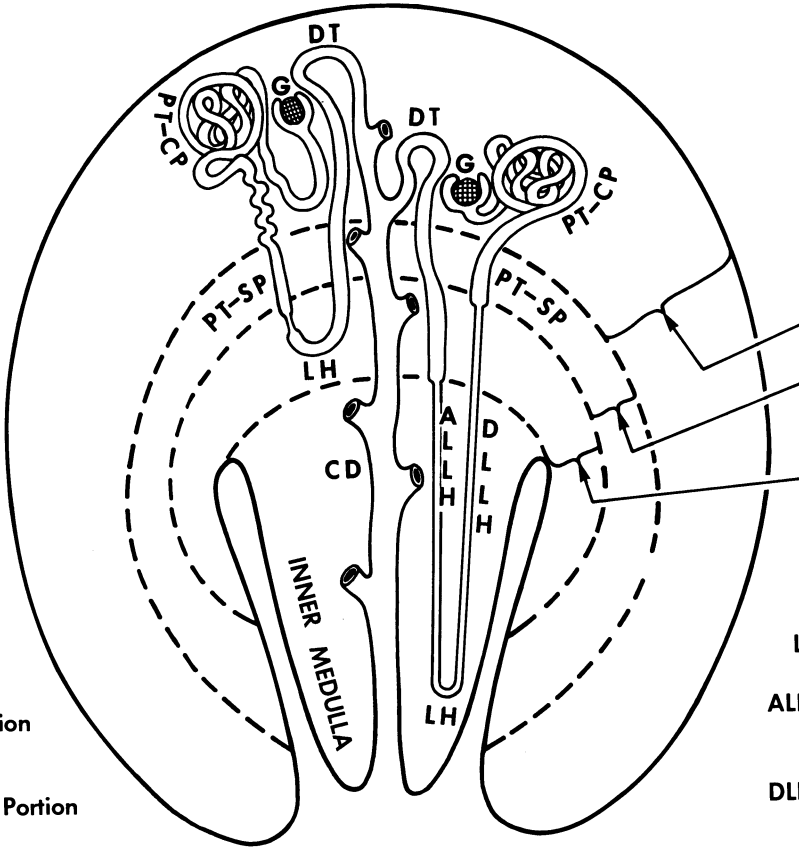
REFERENCES

Finot, P. A., Bujard, E., and Arnaud, M. (1977). Metabolic transit of lysinoalanine (LAL) bound to protein and of free radioactive ^{14}C-lysinoalanine. This volume.

Gould, D. H., and MacGregor, J. T. (1977). Biological effects of alkali-treated protein and lysinoalanine: An overview. This volume.

Maunsbach, A. B. (1966). Observations on the segmentation of the proximal tubule in the rat kidney. J. Ultrastructure Res., 16, 239-258.

Moffat, D. B. (1975). "The Mammalian Kidney". Cambridge University Press, New York, New York.



G: Glomerulus

DT: Distal Tubule

PT-CP: Proximal Tubule, Convoluted Portion

PT-SP: Proximal Tubule, Straight Portion

CD: Collecting Duct

LH: Loop of Henle

ALLH: Ascending Limb of Loop of Henle

DLLH: Descending Limb of Loop of Henle

4

METABOLIC TRANSIT OF LYSINOALANINE (LAL) BOUND TO PROTEIN AND OF FREE RADIOACTIVE $|^{14}C|$-LYSINOALANINE

Paul-André Finot, Eliane Bujard and Maurice Arnaud

Nestlé Products Technical Assistance Co. Ltd.,

CH-1814 La Tour-de-Peilz, Switzerland.

ABSTRACT

Three alkali-treated proteins (lactalbumin, fish protein isolate and soya isolate) containing respectively 1.79, 0.38 and 0.12 g of lysinoalanine (LAL) / 16 g N, were submitted to an "in vitro" enzymatic hydrolysis (pepsin then pancreatin); the higher the level of LAL present in the proteins the smaller was the proportion of LAL liberated in the dialysable fractions of the enzymatic hydrolysates. These same alkali-treated proteins were also given to rats in feeding studies. The faecal LAL varied between 30 and 50% of the ingested quantity, and the urinary LAL between 10 and 25%. The total recovery was always inferior to 100% showing that a certain proportion of LAL was modified in the organism. In the urine, LAL was partially present as free LAL and also as combined LAL, its recovery being higher after acid hydrolysis. The experiments performed with radioactive LAL confirmed the above-mentioned results and gave complementary data: LAL was partially transformed into $^{14}CO_2$; the urine contained free LAL and several metabolites, some combined LAL, probably acetyl derivatives, and some products of catabolism. The proportions of the urinary products varied widely from one animal to another. The kidneys play an important role in the chemical modification (acetylation) of LAL and in the filtration of the excretion products which is most efficient for the most acetylated catabolites of LAL. Although there is practically no difference in the pattern of the urinary catabolites in the rodents (Sprague-Dawley rat, Swiss mouse, Syrian hamster), the rat kidney retains LAL and its catabolites at levels much higher than the kidneys of other species; this retention is localized in the inner part of the cortex.

INTRODUCTION

Lysinoalanine (LAL), or N^{ε}-(DL-2-amino-2-carboxy-ethyl)-L-lysine, is the principal product of the alkaline treatment of proteins (Bohak, 1964; Patchornick and Sokolovsky, 1964; Ziegler, 1964; Asquith et al., 1969; de Groot and Slump, 1969; Woodard and Short, 1973; Provansal et al., 1975); but it is also found in trace amounts in proteins which have undergone heat-treatment only (Sternberg et al., 1975). It is formed by the reaction of the free ε-amino groups of lysyl residues with the dehydroalanyl residues resulting from the decomposition of cystine-cysteine and serine (Freimuth et al., 1974) by β-elimination.

The presence of this molecule in a food protein has both nutritional and perhaps "toxicological" implications. Firstly, the production of LAL, being at the expense of cystine and lysine, and because of the formation of cross-linkages between the protein chains, reduces the "Net Protein Utilisation" (NPU) (de Groot and Slump, 1969). Secondly, the presence of LAL in the diet induces renal lesions in rats characterized by cytomegalic alterations in the cells of the straight portion, "pars recta", of the proximal tubule (Woodard and Short, 1973).

LAL metabolism is not well known, and this work describes its liberation "in vitro" by digestive enzymes, its digestibility "in vivo", its transformation in the organism, its faecal and urinary excretion, its retention in the organism and its behaviour in the liver and kidney. Alkali-treated proteins and synthetic radioactive ^{14}C-labelled LAL were used in this work.

LIBERATION OF LAL DURING "IN VITRO" ENZYMATIC DIGESTION

Three different proteins (lactalbumin, fish protein isolate and soya isolate) which had undergone alkali treatment of varying degrees of severity and which contained varying amounts of LAL were used. The treatments were as follows: lactalbumin - 15 min. at 75°C, pH 12.5; fish protein isolate - 2 min. at 95°C, pH 11.3; soya isolate - 90 sec. at 68-70°C followed by 5 min. at 30-35°C, pH 12.6. These proteins were used in the feeding study.

The "in vitro" digestion with simultaneous dialysis of the amino acids liberated by the utilised proteolytic enzymes was done as described by Mauron et al. (1955). A sample containing 6 g protein suspended in water was incubated while stirring with 75 mg pepsin at pH of about 2 at 30°C. After 16 hrs pepsin digestion the sample was transferred to a dialysis bag, neutralized to pH 7.4 and 150 mg of hog pancreatin were added. This mixture was incubated for

24 hrs at 50°C in an apparatus which permitted the automatic adjustment of the pH, stirring, heating and the taking of dialysate fractions at regular intervals. Aliquots of each fraction were pooled and acidified to pH 4.5. The volume of the final pool was reduced by concentration in a rotary evaporator at 40°C. An aliquot of the concentrated pool was taken for the determination of the free amino acids by an automatic amino acid analyser, Beckman model 121. Another aliquot was hydrolysed in 6 N HCl and used for the determination of the amino acids liberated from the small peptides or oligopeptides which passed through the membrane. The contents of the bag were analysed after acid hydrolysis.

A comparison was made of the release of free and bound LAL with that of other basic amino acids. (See tables 1 and 2).

With proteins containing small quantities of LAL the measured values were very inaccurate, which is why the soya results have not been used, and why the apparent recovery of LAL from fish protein isolate was very far from 100%.

The following conclusions may be drawn from Tables 1 and 2.

a) In the dialysable fraction, LAL is released as free LAL, because the same amounts are found before and after hydrolysis (Table 1). The other basic amino acids are partially released in peptide form (Table 2).

b) The amount of LAL released during enzymatic hydrolysis varies from one protein to another: the protein containing the most LAL releases the smallest percentage of it (Table 1).

c) LAL is released less readily than other amino acids (Table 2).

FAECAL AND URINARY EXCRETION OF BOUND LAL BY THE RAT

Three groups of 4 or 5 Sprague-Dawley rats of about 100 g were fed for 3 days on a diet containing 20% of one of the described alkali treated proteins (lactalbumin, fish protein isolate, soya isolate). The animals were housed in individual metabolic cages from the first day of the test and the urine and faeces were collected throughout the three days of the LAL diet and also for the following 3 days, when they were receiving a LAL-free diet (containing untreated lactalbumin for the group which had had alkali-treated lactalbumin, and casein for the other two groups). The urine and faeces were collected for the 6 days and the LAL content determined in urine and faeces before and after acid hydrolysis (Table 3).

Table 1

Distribution of LAL before and after acid hydrolysis of the dialysable and non-dialysable fractions of the enzymatic hydrolysate (pepsin + pancreatin) of the alkali-treated proteins

Alkali-treated proteins	Dialysable		Non-dialysable	Total recovery
	Before acid hydrolysis	After acid hydrolysis	After acid hydrolysis	After acid hydrolysis
Lactalbumin (LAL = 1.79 g/ 16 g N)	33% *	26%	64%	90%
Lactalbumin (LAL = 1.58 g/ 16 g N)	27% **	28%	71%	99%
Fish protein isolate (LAL = 0.38 g/ 16 g N)	90% *	47%	13%	60%
	55% **	53%	15%	68%

* Values obtained with a normal run (LAL and Tryptophan not separated)

** Correct values of LAL obtained with run for physiological fluids.

From table 3 it may be seen that:

a) A considerable fraction of the LAL (30 to 50%) is found in the faeces. This faecal LAL is bound LAL, as in none of the different types of protein tested was free faecal LAL detected. Thus, free LAL is either very well digested or transformed by the intestinal flora.

b) The amount of urinary LAL is greater after acid hydrolysis, showing that some urinary LAL is combined.

c) The total recovery is always inferior to 100%, which means that LAL is either transformed by the intestinal flora or metabolised, or it is retained in the organism.

Table 2

Distribution of LAL and other basic amino acids in the dialysable and non-dialysable fractions obtained by "in vitro" digestion (pepsin + pancreatin) of alkali-treated lactalbumin

Basic amino acids liberated	Dialysable		Non-dialysable	Total recovery
	Before hydrolysis	After hydrolysis	After hydrolysis	After hydrolysis
LAL	27%	28%	71%	99%
Lysine	45%	73%	23%	96%
Histidine	28%	66%	29%	95%
Arginine	50%	66%	11%	77%

d) A higher percentage of LAL is recovered from the urine of the rats which received the least LAL-rich protein. This observation confirms that made during the enzymatic digestion of these same proteins (Table 1). However, it is difficult to say whether these differences are due specifically to the tested proteins, or whether they are also due to the level of LAL.

METABOLISM OF FREE LYSINOALANINE

The metabolism of [^{14}C]-labelled LAL was studied in order to explain the loss of LAL in the animal, to provide information on the kinetics of transformation and excretion of the LAL molecule, and to study the role of certain organs. The experiments were done with [^{14}C]-labelled lysinoalanine.

A) Synthesis of [^{14}C]-LAL or N^{ε}-(DL-2-amino-2-carboxyethyl)-[U-^{14}C]-L-lysine

The radioactive [^{14}C]-LAL was synthesized from uniformly labelled [^{14}C]-L-lysine: reaction of α-N-formyl-[U-^{14}C]-L-lysine synthesized according to Hofmann et al. (1960) with acetamino acryl-ethylester in alkaline medium (Okuda and Zahn, 1965), followed by acid hydrolysis (5 hrs in boiling 6N HCl). The radioactive LAL was

Table 3

Urinary and faecal excretion of LAL in rats fed on alkali-treated proteins

Alkali-treated proteins	Urinary LAL (% of ingested LAL) Free (Before acid hydrolysis)	Urinary LAL (% of ingested LAL) Total (After acid hydrolysis)	Faecal LAL (% of ingested LAL) (After acid hydrolysis)	Total recovery (%) (After acid hydrolysis)
Lactalbumin (LAL = 1.79 g/ 16 g N)	5.8; 7.0; 6.5; 6.3 mean=6.4	10.5; 10.7; 10.3; 10.6 mean=10.5	43; 53; 55; 53 mean=51	61.5
Fish protein isolate (LAL = 0.38 g/ 16 g N)	7.2; 7.4; 5.7; 7.4; 8.0 mean=7.1	16.9; 16.4; 16.4; 15.9; 16.5 mean=16.4	42; 35; 26; 28; 34 mean=33	49.4
Soya protein isolate (LAL = 0.14 g/ 16 g N)	20.6; 15.6 mean=18.1	23.2; 24.6; 24.2; 25.1; 25.2 mean=24.5	48; 52; 50; 44; 41 mean=47	71.5

then purified by cation-exchange chromatography (Dowex 50 w x 4, from H^+) and eluted by 3N HCl. After elimination of the HCl by vacuum evaporation, the radioactive LAL was dissolved in water and stored frozen. Its radiochemical purity was verified by autoradiography after paper electrophoresis and on an amino acid analyzer with continuous radioactive monitoring (Chroma/Cell, Nuclear Chicago). A radioactive impurity corresponding to less than 1% of the total radioactivity was present in the synthesized product: it was eluted just after the LAL on the basic amino acid column. The specific activity was 0.815 microcurie/micromole. The animals received doses of the order of 10 mg/kg.

Table 4

Radioactivity excreted in 24 hrs (expressed as % of ingested radioactivity) in the CO_2, the urine and the faeces, by 5 rats having received ^{14}C -LAL "per os"

Animal	1	2	3	4*	5*
Sex	♂	♂	♀	♂	♀
Ingested dose of LAL (mg/kg body wt.)	9.6	9.3	13	15.3	9.8
$^{14}CO_2$	17.9	14.5	13.8	24.3	10.9
Urine	40.1	41.7	26.1	34.6	64.1
Faeces	33.8	30.5	29.7	23.9	10.4
Total excretion	91.8	86.7	69.6	82.8	85.4

* For 6 days previously, animals 4 and 5 had eaten a diet of 20% casein to which 0.07% LAL (i.e. 0.35% LAL on the protein) had been added.

B) Rat Trials - Excretion Balance

The trials were made with a limited number of Sprague-Dawley rats weighing about 100 g. Five animals (3 males and 2 females) received the labelled LAL by stomach tube ("per os"), two of these animals having had for 5 days a casein-based diet with 0.07% of added LAL. Another two male rats were given labelled LAL by intravenous injection. For each experiment the animals received about 5 microcuries, corresponding to 1.4 mg of LAL, which was equivalent to the ingestion of 1 g of protein containing 0.14% of LAL (soya isolate). For all these experiments, the animals had been starved for one night and immediately after receiving the radioactive LAL, they were put in metabolic cages allowing separate collection of the expired radioactive CO_2, the urine and the faeces (Table 4). The stock diet and water were given "ad libitum".

C) Excreted Radioactivity Balance

The results of the radioactive balance, shown in table 4, lead to the following conclusions:

a) The lysine part of the LAL molecule is metabolized into $^{14}CO_2$ which is expired;

b) The proportions of radioactivity found in the urine and faeces are respectively higher and lower than the proportions of LAL found when the animals received bound LAL. Thus, free LAL is better absorbed than protein-linked LAL.

c) After 24 hrs some radioactivity remains in the organism (10-30%), the total excretion being less than 100%. This will be shown elsewhere by evaluation of residual radioactivity found in different organs.

d) At first sight, there would seem to be no great difference in LAL metabolism according to sex, nor according to whether or not the animals had previously received LAL. The observed differences are probably individual variations.

D) Expiration of $^{14}CO_2$

The expiration of $^{14}CO_2$ during the experiment arises from the destruction of the carbon chain of the lysine part of the LAL molecule, which may be caused either by the intestinal flora or by the organism. The cumulative curves of $^{14}CO_2$-appearance vary greatly from one animal to another (Fig. 1). The expiration peak occurs between 5 and 12 hours, i.e., very late, which leads one to believe that the decomposition of LAL to the stage of $^{14}CO_2$ is produced by intestinal flora. There is no absolute evidence in favour of at least partial decomposition of LAL in the organism. Although the radioactive LAL given to two rats by I.V. injection, at doses of 7.2 and 10.2 mg/kg body weight, also produced amounts of $^{14}CO_2$ comparable to those obtained from the LAL given "per os" (Table 5), the $^{14}CO_2$ does not appear earlier when the intestine is "by-passed" (Fig. 1). On the other hand, macro-autoradiographs of rats which received LAL by I.V. injection, show some radioactivity in the lumen of the caecum and of the intestines (Fig. 4). LAL and its transformation products pass from the bloodstream into the intestinal lumen where they could be degraded by the flora to $^{14}CO_2$.

Table 5

Amount of expired $^{14}CO_2$, expressed as % of given radioactivity, 8 hrs after ingestion "per os", and I.V. injection of ^{14}C -LAL

Animal	1	2	3	4	5	6	7
Sex	♂	♂	♀	♂	♀	♂	♂
Method of administration	←------ "per os" --------→					←I.V.→	
$^{14}CO_2$ expired	13.5	7.9	2.5	15.7	4.2	3.2	7.6

E) Urinary Excretion

The urinary radioactivity excretion curves (Fig. 2) show that:

a) The amount of excretion after 24 hrs varies greatly from one animal to another;

b) The excretion kinetics in the first hours are also very different from one animal to another;

c) After 24 hrs all the radioactivity is still not excreted.

These observations suggest that the organism has great difficulty in eliminating LAL and its catabolites, and that each animal adapts in its own way.

F) Residual Activity in the Organs

24 hours after ingestion of radioactive LAL, the animals were killed and the radioactivity of their organs determined by the combustion method (Oxymat-Intertechnique). The results given in Table 6 show that the highest residual radioactivity is in the kidneys. In all the animals the radioactivity in the kidneys was greater than 2% of the ingested radioactivity, and in one animal it was more than 10%. The kidney is 10 to 32 times more radioactive than the liver, per gramme of organ. This very high retention in the kidneys could explain the phenomenon of cytomegalia found by Woodard and Short (1975), which may be due to a defect in the excretion by the

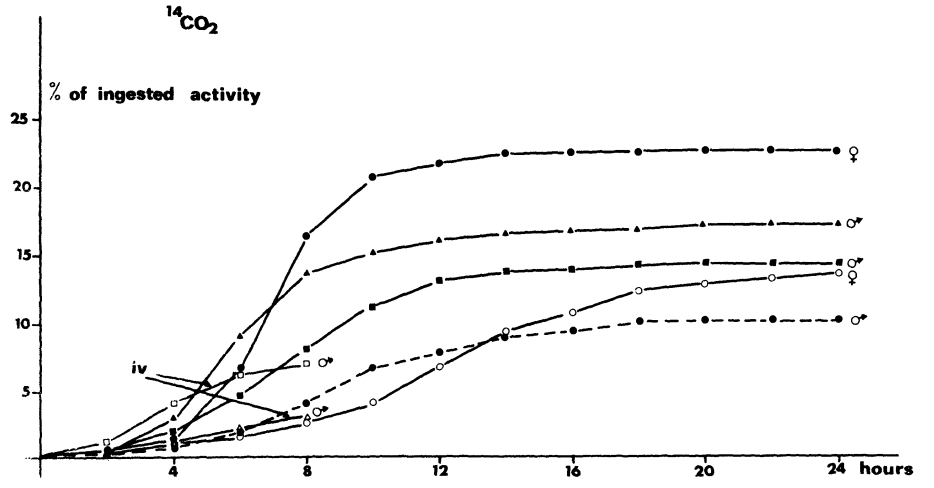


Fig. 1. Expiration of $^{14}CO_2$ after "per os" and "i.v." administration of [^{14}C]-LAL.

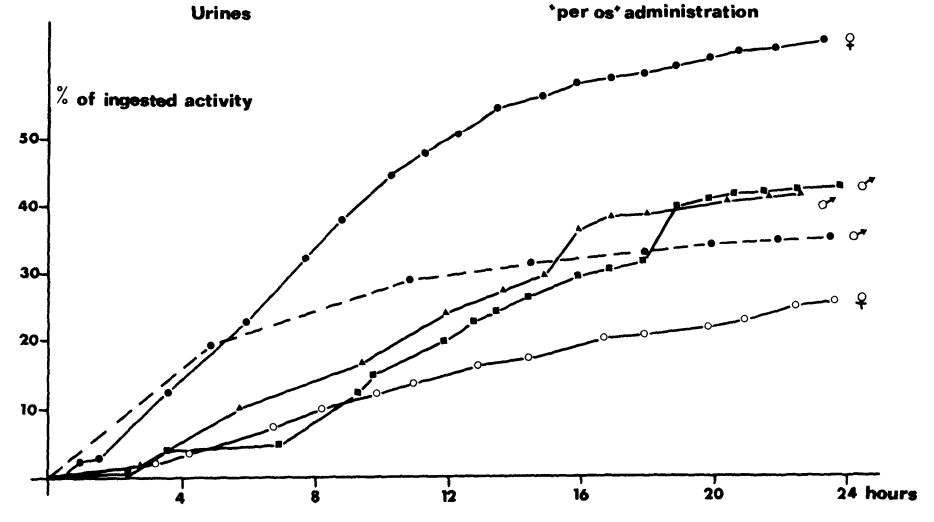


Fig. 2. Excretion of urinary radioactivity after "per os" ingestion of [^{14}C]-LAL.

Table 6

Residual radioactivity in the organs 24 hrs after "per os" ingestion of ^{14}C -LAL

Animal	1	2	3	4	5
Sex	♂	♂	♀	♂	♀
Dose (mg/kg)	9.55	9.33	13	15.3	9.8
% of ingested radioactivity in					
Brain	0.03	0.02	0.05	0.03	0.02
Lung	0.07	0.1	0.2	-	0.07
Liver	2.0	1.5	2.3	1.7	1.1
Kidney	2.9	4.9	11.3	2.3	2.3
% of ingested radioactivity per g of organ in					
Kidney	2.1	3.5	7.3	1.4	1.1
Liver	0.2	0.15	0.23	0.15	0.09
Ratio $\frac{\text{Kidney}}{\text{Liver}}$	10	23	32	9.3	12

kidney of LAL or of its transformation products, which could be harmful. The composition of the residual radioactive products of the kidney is discussed in the following section on urinary catabolites.

G) Study of Urinary Catabolites

The urinary catabolites were separated on a Beckman 121 amino acid analyzer, with continuous recording of the radioactivity by an anthracene scintillation cell (Chroma/Cell, Nuclear Chicago).

More than 10 different radioactive catabolites were detected by this system. Some were present in trace amounts, others in substantial quantities (Fig. 3). All of the catabolites are more acid in character than LAL. Certain of them arise from the decomposition of the molecule starting from the lysine residue, with formation of $^{14}CO_2$ which is then expired. In no case can the products formed by this metabolic path give back LAL after acid hydrolysis. Now, after acid hydrolysis of urine, the proportion of radioactive LAL is always increased, and similarly, in the previous experiments with bound LAL, the urinary LAL was always greater after hydrolysis than before (Table 3) . Thus this phenomenon may be explained by the presence of combined LAL. Very probably, it is in the acetylated form, as acetylation is the classic detoxification process of amino derivatives by the kidneys. For example, Smith (1972) has shown that methionine sulphoxide and methionine sulphone were excreted in the urine in their acetylated form.

In order to compare the products thus formed with the urinary catabolites which disappear during acid hydrolysis, some [^{14}C]-LAL was partially acetylated. The acetylation was carried out as follows. 0.5 µcurie of [^{14}C]-LAL, mixed with 2.5 mg of unlabelled LAL, was dissolved in 0.5 ml of 1N NaOH and a small excess of acetic anhydride was added to the solution which was then shaken at ambient temperature for one hour.

The acetylated products formed during this reaction were separated on an amino acid analyser in the same way as for the urine samples, but with simultaneous recording of the ninhydrin reaction and of the radioactivity (Fig. 3).

Four peaks were resolved for the acetylated products; three of them were very acid and were eluted before aspartic acid, the fourth was eluted in the isoleucine zone. Each one corresponded to a catabolite present in the untreated urine, but absent from hydrolysed urine (Fig. 3). They correspond respectively to peaks 3, 4, 5 and 9 of rat urine, which are thus very likely all acetylated forms of LAL.

As LAL has three amino groups, there must exist 3 monoacetylated, 3 diacetylated and 1 triacetylated forms, i.e., 7 acetyl-LAL derivatives in all. Peak 3, which is most acid and reacts hardly at all to ninhydrin is the most acetylated form, probably the triacetyl derivative. The 3 other peaks (4, 5 and 9) react very differently to ninhydrin. For the same radioactivity expressed in DPM, i.e., the same quantity of LAL, peaks 4 and 5 are respectively 3 and 18 times less sensitive to ninhydrin than peak 9. This observation provides some information about the structure of each of these products: the tri-N-acetyl derivative could be peak 3, the di-N-acetyl

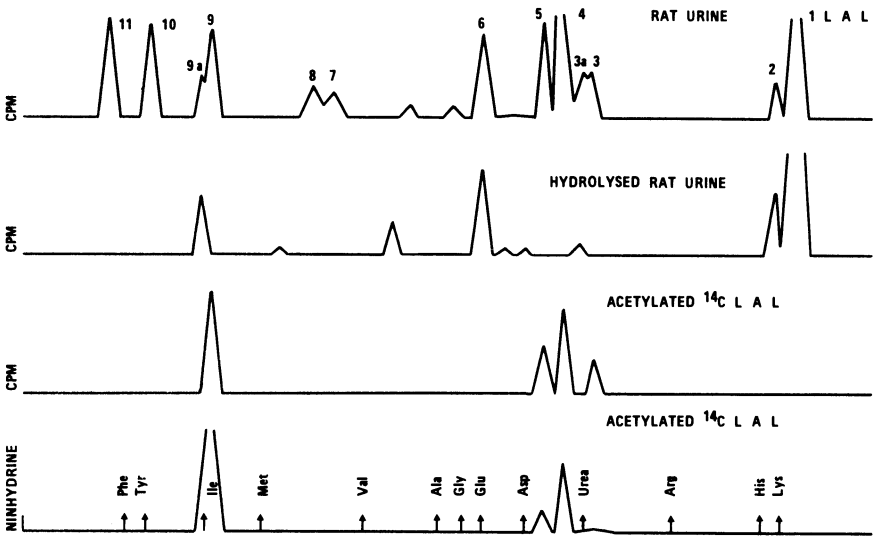


Fig. 3. Pattern of the radioactive urinary catabolites of [^{14}C]-LAL before and after acid hydrolysis as compared to the acetylated products of [^{14}C]-LAL.

derivatives, peaks 4 and 5, and the mono-N-acetyl derivatives, peaks 9 and 11. Peak 11 which disappeared after acid hydrolysis was eluted at the same position as the product obtained by acetylation of the copper-complex of LAL which corresponds to the ε-N-acetyl-LAL. This acetyl derivative did not appear during the "in vitro" acetylation of LAL but could be formed "in vivo". In which organs are the urinary catabolites synthesized? The liver has no effect on LAL; in fact, perfused "in vitro" in rat liver for 4 hours, [^{14}C]- LAL underwent no chemical change. On the other hand, the kidney probably plays an essential rôle in LAL transformation. Intestinal flora can also play a part in the formation of certain catabolites, especially in the formation of $^{14}CO_2$ and non-acetylated catabolites. Now, the most important catabolites of the urine have been recovered from the faeces, particularly the acetylated catabolites (i.e. peaks 3, 4, 5, and 9 + 9a), and as it is hardly likely that the flora is responsible for the formation of acetyl-LAL, it may be assumed that these derivatives, synthesized in the kidney, are later excreted in the intestinal lumen, to be further transformed to $^{14}CO_2$.

The composition of the radioactive products remaining in the kidneys 24 hrs after ingestion of [^{14}C]-LAL has been compared to

Table 7

Radioactivity distribution of each catabolite, expressed in % of the radioactivity found in the kidneys 24 hrs after the ingestion of ^{14}C -LAL, and the last urine of a rat

Catabolites	LAL	11	9+9a	4	5	3+3a
Probable degree of acetylation	0	monoacetyl		diacetyl		triacetyl
Kidney (K)	52.2	2.1	30.4	6.7	2.3	0.9
Urine (U)	11.8	0.4	13.4	39.6	14.2	16
$\frac{K}{U}$	4.4	5.25	2.77	0.17	0.16	0.06

the composition of the radioactive products present in the last urine passed by the rat. The percentage of each radioactive product found in the last urine is quite different from the percentage of the same radioactive products found in the kidneys. In order to see which of these products was the least easily excreted, we calculated the following ratio: percentage of each urinary product / the corresponding percentage for the kidney; these ratios (kidney/urine, table 7) show that:

a) LAL and catabolites 9 + 9a were the most important components in the kidney.

b) Catabolite 4 was the main component in the urine.

c) The ratio K/U, (% radioactivity in kidneys / % radioactivity in urine), varied widely from one catabolite to another. It was greatest for LAL itself and decreased through 11, 9 + 9a, 4 and 5 (which were the same) to 3 + 3a, which shows that the more the LAL was acetylated, the better it was excreted.

d) Catabolite 11 appears to have a K/U ratio of the same order as LAL, but it was only weakly present and the measurements on it were much less accurate.

e) The urine of rat 3 (tables 4 and 6), which had 11.3% of the ingested radioactivity in its kidneys, contained a very low level

of LAL (≃ 12%), indicating a serious defect in the excretion of free LAL.

COMPARISON OF LAL METABOLISM IN DIFFERENT SPECIES

In answer to the question: are the anomalies observed in rat kidney species-specific? (De Groot et al., 1975; O'Donosan, 1976), some metabolic transit assays with [^{14}C]- LAL were made on other species: Swiss mouse, Syrian hamster and Japanese quail (two animals per experiment).

The animals, starved for one night, were given the following doses of [^{14}C]-LAL by stomach tube (20 mg/kg for the mice, 6 mg/kg for the hamsters and 10 mg/kg for the quails) except for the rats which received a dose of ^{14}C -LAL (6.8 mg/kg) added to their only meal. The animals were then placed in metabolic cages for 24 hours. At the end of the experiment one of each pair of animals was utilized to localize the radioactivity by autoradiography. For this purpose, the animals were anesthetized with diethyl ether, killed by quick dipping in liquid nitrogen, and then frozen at -70°C. Whole body sections (20 microns) were done with a Leitz microtome at -20°C. Autoradiographies were obtained by applying these sections against Kodirex films. The exposure lasted 10 to 30 days. The second animal was utilized to measure the radioactivity in the organs.

A) Study of the Radioactivity Balance

Comparative measures were made on:

a) the rate of transformation to $^{14}CO_2$. All the animals broke down LAL and eliminated it partially as $^{14}CO_2$ (table 8).

b) The urinary and faecal excretion. Some differences were found in urine only; urinary excretion is important in rats and mice, and weak in hamsters (table 8).

c) The residual radioactivity in the organs. This is the same in the liver for the four species, while in the kidneys, it is greatest in the rat, less in the mouse and least in the hamster and the quail (table 8 and 8a).

As is shown on the autoradiography of sections of the whole animals, the radioactivity is also incorporated in the salivary and lachrymal glands, in the bones and in the intestinal walls (Fig. 4).

Table 8

Distribution of radioactivity (expressed in % of the ingested radioactivity), 24 hours after ingestion of [^{14}C]-LAL, in the rat, mouse, hamster and quail

	Rat	Mouse	Hamster	Quail
Animals in experience	5	2	2	2
Dose (mg/kg)	9.3 to 15.3	20	6	10
CO_2	10.9 to 24.3	15.5-10.4	3.8-27.3	3.3- 6.2
Urine	26.1 to 64.1	35.5-52.3	22.2- 5.5	47.4-49.8
Faeces	10.4 to 33.8	12.0-22.3	7.4-31.4	
Liver	1.1 to 2.3	1.26	2.34	1.57
Kidneys	2.3 to 11.3	1.15	0.26	0.29

Table 8a

Residual radioactivity (% of ingested activity / g of organ) in the liver and kidneys of various animals

Organ	Rat	Mouse	Hamster	Quail
Kidneys (K)	1.1 - 7.35	1.26	0.31	0.46
Liver (L)	0.1 - 0.23	0.39	0.53	1.20
$\frac{K}{L}$	9 - 33	3.2	0.59	0.38

Table 9

Distribution of the catabolites of [^{14}C]- LAL in the urine collected over 24 hrs from the rat, the mouse, the hamster and the quail

Catabolite	Rat	Mouse	Hamster	Quail
LAL	60.0-64.7	68.2-73.5	86.1-72.9	6.3-14.8
3+3a	5.2- 3.4	2.6- 2.2	0.7- 3.3	0 - 0
4	1.0- 2.5	2.8- 5.1	0.7-12.4	39.1-33.1
5	0 - 0	4.6- 2.2	1.7- 1.1	0 - 0 (11.5)*(11.7)*
6	19.1-13.4	0 - 0	0 - 0	0 - 0
9+9a	6.9- 7.8	7.2- 7.2	5.6- 6.1	43.1-40.4
10	1.3- 1.0	0 - 0	0 - 0	0 - 0
11	3.0- 2.4	1.4- 2.1	4.6- 3.3	0 - 0

* product eluted between peak 5 and peak 6

B) Comparison of the Urinary Catabolites

The proportions of the urinary catabolites were determined in the 24-hour accumulated urine. Very slight differences in the composition and the proportions of the catabolites in the three rodents were found, but the pattern in the quail was dissimilar (Table 9).

For the rodents, LAL remained the most important compound in the urine (60 to 86%). Peak 10 is the sole catabolite found only in the rat urine in very small quantities and not present in the kidney; thus it is unlikely to have a toxic effect.

Compared to the rodents, the pattern of the catabolites excreted by the quail was quite different; the level of LAL was very low while peaks 4 and 9 were very high.

Thus it would seem that there are no fundamental differences of either structure of relative proportion in the different urinary catabolites of LAL in the rodents. The only difference is that, in the rat, the kidneys eliminate LAL and its transformation products less easily than in the other species.

C) Localization of the Radioactivity Remaining in the Kidneys of the four Species

Fig. 5 reproduces the autoradiographies of the kidneys of the four different species. In the rat kidney the remaining radioactivity is localized in the inner cortex, which includes the "pars recta" of the proximal tubule in the cells of which Woodard and Short (1973) have characterized the cytomegalic alterations. There is therefore a high probability that this cytomegalia is induced by the accumulation of LAL and its catabolites. The kidney of the hamster also concentrates the radioactivity in the inner cortex but at a lower concentration (Tables 8 and 8a). In the mouse kidney, the radioactivity is also concentrated in the inner cortex; fig. 5 shows a section through the outer and the inner cortex of the mouse kidney. The concentration of radioactivity in the inner cortex is clearly shown. In the quail kidney, the radioactivity is uniformely distributed.

CONCLUSION

This study of LAL metabolism complements those which have been carried out on the nutritional and toxicological implications of the presence of LAL in food proteins. It has been shown that LAL was partially released by the enzymes of the digestive tract and then absorbed by the intestine. The non-absorbed part was partially degraded by the intestinal microflora to CO_2 : the absorbed part was eliminated in the urine, largely as free LAL in the case of rodents (rat, mouse, hamster) but also in the form of acetlyated derivatives. The composition and proportion of urinary catabolites appear not to vary much among the rodents, but the quail, on the contrary, excreted little free LAL.

In the rat, free LAL was excreted more slowly than the acetylated derivatives, and the more acetylated the molecule the better it was excreted. Those derivatives which were excreted with difficulty were concentrated in the internal cortex of the kidney, in the zone where Woodard and Short (1973) observed nephrocytomegalia. The same type of concentration, although less pronounced, was also

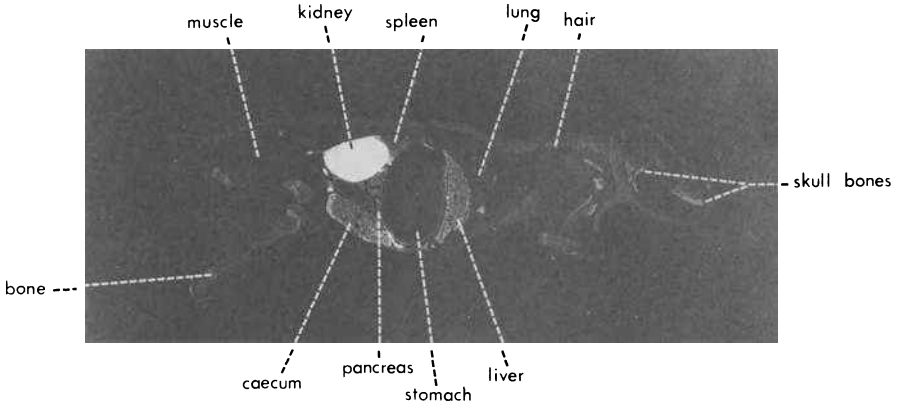


Fig. 4. Autoradiography of a whole section of a rat, 8 hrs after intravenous injection of [^{14}C]-LAL.

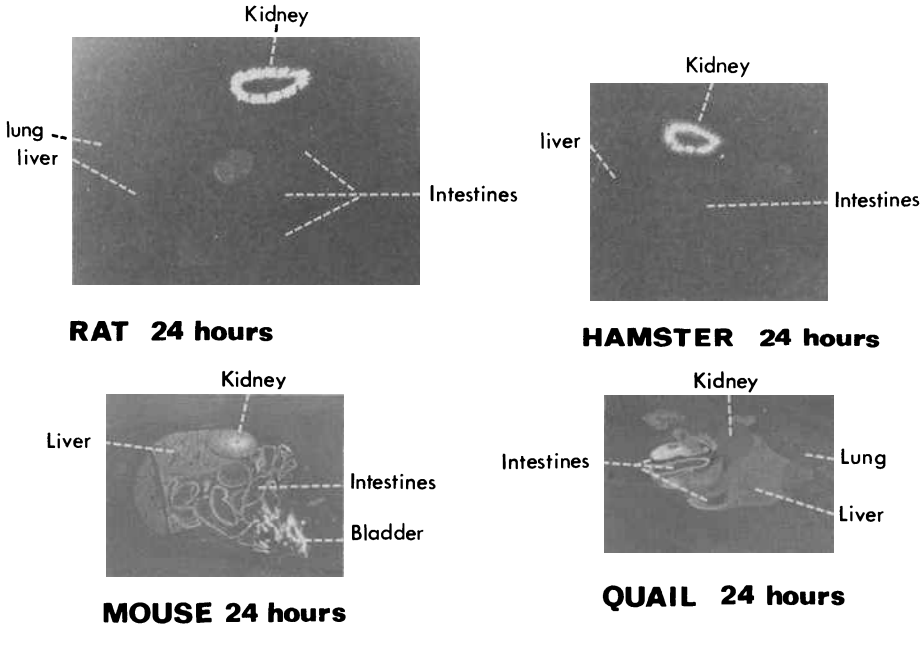


Fig. 5. Autoradiography of whole sections of rat, hamster, mouse and quail, 24 hrs after oral ingestion of [^{14}]C -LAL.

observed in the hamster and mouse, but not in the quail. These observations tend to support the hypothesis that the appearance of nephrocytomegalia, observed in rats receiving LAL in their diet, depends on the species studied

ACKNOWLEDGEMENTS

The authors wish to thank Dr. O. de Rham for the preparation of alkali-treated lactalbumin, Dr. G. Philippossian for the synthesis of lysinoalanine and Mrs R. Madelaine, Miss E. Magnenat, Mr. R. Deutsch, and Mr. A. Isely for their skilful technical assistance without which this work could not have been done.

REFERENCES

Asquith, R.S., Booth, A.K. (Miss), and Skinner, J.D. (1969). The formation of Basic Amino Acids on Treatment of Proteins with Alkali. Biochem. Biophys. Acta,181, 164-170.

Bohak, Z. (1964). N-Epsilon-(DL-2-Amino-2-Carboxy-ethyl)-L-Lysine, A new Amino Acid Formed on Alkaline Treatment of Proteins. J. Biol. Chem., 239, 2878-2887.

De Groot, A.P., and Slump, P. (1969). Effects of Severe Alkali Treatment of Proteins on Amino Acid Composition and Nutritive Value. J. Nutr., 98, 45-56.

De Groot, A.P., Slump, P., van Beek, L., and Feron, V. (1975). Abstract, 35th Annual Meeting Institute of Food Technologists, 1975, p. 162.

Freimuth, U., Schlegel, B., Gahner, E., and Nötzold, H. (1974). Die Bildung von Lysinoalanin in Alkalibehandelten Milchproteinen. Die Nahrung, 181, K5-K8.

Hormann, K., Sutz, E., Spühler, G., Yajima, H., and Schwartz, E.T. (1960). Studies on Polypeptides. XVI The preparation of Nε-Formyl-L-Lysine and its application to the Synthesis of Peptide. J. Amer. Chem. Soc., 82, 3727-3732.

Mauron, J., Mottu, F., Bujard, E., and Egli, H.R. (1955). The availability of Lysine, Methionine and Tryptophan in Condensed Milk and Milk Powder. "In Vitro" Digestion Studies. Arch. Biochem. Biophys., 59, 433-451.

O'Donovan, O.J. (1975). Recent Studies of Lysinoalanine (LAL) in Alkali-Treated Proteins. Miles Laboratories, Inc., Food Research Institute Meeting, 1975.

Okuda, T., and Zahn, H. (1965). Synthese von N^{ε}- 2-Amino-2-Carboxy-Aethyl-L-Lysin einer neuen Aminosäure aus alkalibehandelter Wolle. Chem. Berichte, 1164-1167.

Patchornik, A., and Sokolovsky, M. (1964). Chemical Interactions between Lysine and Dehydroalanine in Modified Bovine Pancreatic Ribonuclease. J. Am. Chem. Soc., 86, 1860-1861.

Provansal, M.M.P., Cuq, J-L.A., and Cheftel, J.C. (1975). Chemical and Nutritional Modifications of Sunflower Proteins due to Alkaline Processing. Formation of Amino Acid Cross-Links and Isomerization of Lysine Residues. J. Agr. Food Chem., 23, 938-943.

Smith, R.C. (1972). Acetylation of Methionine Sulfoxide and Methionine Sulfone by the rat. Biochem. Biophys. Acta, 261, 304-309.

Sternberg, M., Kim, C.Y., and Schwende, F.J. (1975). Lysinoalanine: Presence in Foods and Food Ingredients. Science, 190, 992-994.

Woodard, J.C., and Short, D.D. (1973). Toxicity of Alkali-Treated Soyprotein in Rats. J. Nutrition, 103, 569-574.

Woodard, J.C., and Short, D.D. (1975). Renal Toxicity of N^{ε}-(DL-2-Amino-2-Carboxyethyl)-L-Lysine (Lysinoalanine) in Rats. Federation Proceedings, 34, No 4022.

Ziegler, K.L. (1964). New Cross-links in Alkali-treated Wool. J. Biol. Chem., 239, PC 2713-2714.

5

LYSINOALANINE FORMATION IN PROTEIN FOOD INGREDIENTS

M. Sternberg and C. Y. Kim

Miles Laboratories, Inc.
1127 Myrtle Street
Elkhart, Indiana 46514

Home cooking or industrial processing of foods causes a multitude of changes of appearance, taste and texture, related to chemical modification and interaction of food components. In the days before the emergence of food science there was little concern about delayed consequences of food processing on human health as long as the immediate effect was improved palatability or digestibility. The advent of industrial processing and mass distribution as well as the emergence of engineered foods imposed on manufacturers the responsibility of maintaining wholesomeness and safety of foods. Peculiar food practices from the past such as lye treatment of fish or corn, cannot be confined anymore to small populations but may spread to larger groups. For these reasons, the discovery of lysinoalanine in proteins of foods and food ingredients was received with mixed feelings.

Lysinoalanine (LAL) was first found in acid hydrolysates of proteins subjected to severe alkaline treatment (Figure 1). Cystinyl or glycosidically bound seryl residues in proteins undergo a β-elimination reaction, forming an unsaturated intermediary compound (Figure 2) called dehydroalanine. This compound combines with free ε amino groups of lysyl residues (Figure 3) giving a lysinoalanyl residue in the protein structure. If the protein is hydrolyzed in standard conditions with hydrochloric acid, free LAL (Figure 4) can be separated. LAL has a unique structure among the common amino acids, containing two carboxyl, two primary amino and one secondary amino functional groups.

Except for two peculiar antibiotics, cinnamycin and duramycin, in which Erhard Gross has found lysinoalanine as a natural constituent, never has lysinoalanine been discovered in nature, either

FIGURE 1

OCCURRENCE OF LAL IN TREATED PROTEINS

Source	Reference
1. Ribonuclease, Phosvitin, Bovine Plasma Albumin, Papain, Lysozyme, α-chymotrypsinogen.	Z. Bohak, J. Biol. Chem. 1964, 239, 2878.
2. Ribonuclease - dinitrophenylated reduced.	A. Patchornik and Sokolovsky, M., J. Am. Chem. Soc. 1964, 86, 1860.
3. Wool.	Kl. Ziegler, J. Biol. Chem., 1964, 239, PC 2713. M. C. Corfield and Wood, C., Biochem. J., 1967, 103, 15 C. R. S. Asquith and Garcia-Dominguez, J. Soc. Dyers, Colour, 1968, 84, 1211. P. Mellet, Text. Res. J. 1968, 38, 977.
4. Soybean, Casein, Ground nut meal, Sesame, Brewer's yeast.	A. P. DeGroot and P. Slump., J. Nutr., 1969, 98, 45.
5. Lysozyme, Myoglobin, RNase, Insulin.	R. S. Asquith et. al., Biochim. Biophys. Acta 1969, 181, 164.
6. Cartilage glicoprotein.	A. H. Whiting, Biochem. Biophys. Acta 1971, 243, 332.
7. Cinnamycin, Duramycin.	E. Gross et. al. ACS 166th Natl. Mtg. Abstract #81, 1973, Chicago.
8. Soybean Protein.	K. Saio and M. Murase, J. Food Sci. & Technology, 1975, 22, 30.
9. Sunflower Protein Isolate.	M. M. P. Provansal et. al., J. Agric. Food Chem., 1975, 23, 938.
10. Home Cooked and Commercial Foods and Ingredients.	M. Sternberg et. al., Science, 1975, 190, 992.

Figure 2

Formation of Dehydroalanine

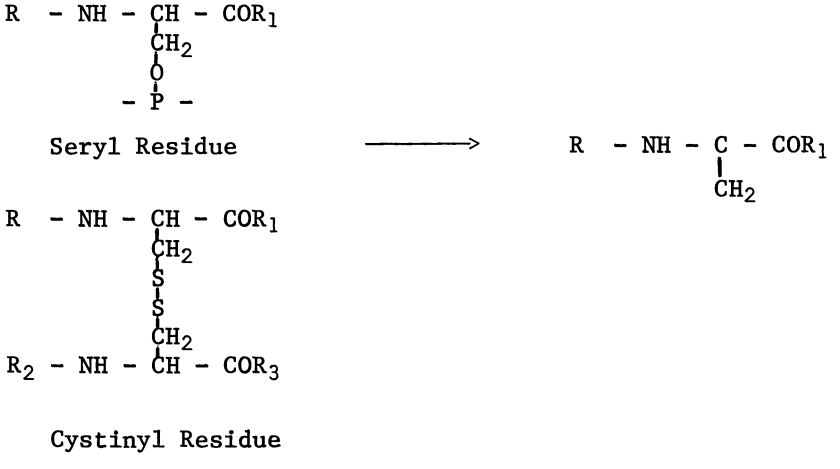


Figure 3

Formation of Lysinoalanine from Dehydroalanine

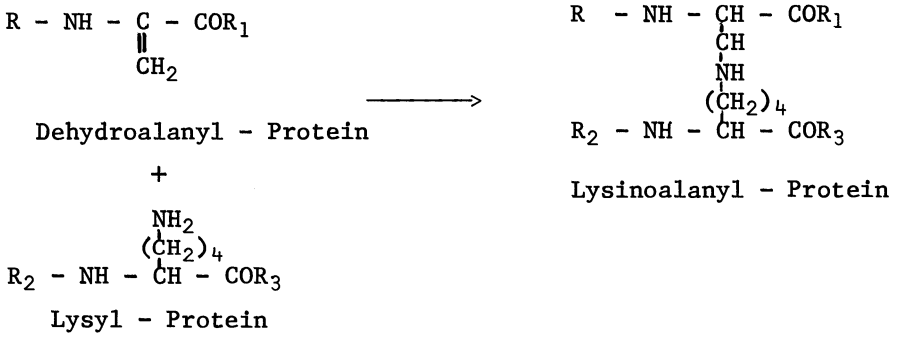
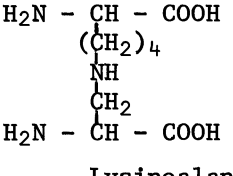


Figure 4



N^{ϵ} - (DL - 2 - Amino - 2 carboxyethyl) - L - Lysine

free or as part of a protein structure, unless that protein was treated with alkali or heated for a sufficient time.

LAL can be estimated by a variety of methods (Figure 5) including ion exchange chromatography, electrophoresis, gas chromatography and TLC.

The biological significance of the presence of LAL in food proteins was approached from the point of view of its nutritional consequences. De Groot and Slump from the TNO (CIVO) Research Institute in Holland noted that severe alkali treatment conditions of soya protein, reduces the net protein utilization and digestibility of the treated protein. Work by Woodard and col. described the occurrence of a unique histologic lesion called nephrocytomegalia, in the kidneys of rats fed an alkali treated industrial grade soya protein. These histologic changes, characterized by cytoplasmic and nuclear enlargement of the tubular epithelial cells in the pars recta, were attributed by Woodard to the presence of lysinoalanyl residues in the alkali treated protein. Later, De Groot and co-workers, found that only synthetic LAL or protein hydrolysates containing free LAL or oligopeptides with LAL, can induce renal changes in rats. They did not see cytomegalic changes when laboratory animals were fed diets of intact protein containing structurally bound LAL. The renal changes caused by free LAL seemed, however, to be species specific to the rat. Mice, hamsters, Japanese quail, dogs rabbits and monkeys failed to exhibit renal cytomegalic effects when fed synthetic LAL.

Figure 5

LAL Estimation

	Method	Reference
1.	Ion exchange chromatography	Z. Bohak, J. Biol. Chem., 1964 239, 3878.
		A. Robson et. al., J. Chromatog, 1967, 31, 284.
		A. P. De Groot and P. Slump, J. Nutrition 1969, 98, 45.
2.	Electrophoresis	J. J. Garcia Dominquez, Bull, Inst. Text. Fr. 1967, 21, 91.
		R. S. Asquith et. al., Biochim. Biophys. Acta., 1969, 181, 164.
3.	Gas chromatography	A. H. Whiting, Biochim. Biophys. Acta, 1971, 243, 332.
4.	Thin layer chromatography	M. Sternberg et. al., J. Food Science 1975, 40, 1168.

In view of these considerations we were interested to examine foods and common food protein ingredients to determine how widespread is LAL. In spite of the fact that LAL formation in proteins was strictly correlated with alkaline treatment, we decided to include in our survey not only foods exposed to alkali but other foods too.

To our surprise we found LAL widely distributed in proteins from home cooked foods, commercial food preparations, and many food ingredients (Figures 6, 7 & 8). Some of the food proteins included in the figures such as egg white, tortillas or hominy were at one point exposed to alkali but many were not. The one factor which caused the formation of LAL in proteins which were not exposed to alkali was heat.

The effect of heat on food proteins has been reported to cause a decrease of the nutritional value due to racemization and aminocarbonyl reaction. To these should be added the formation of LAL in sensitive proteins at pH levels which are not necessarily alkaline.

Purified proteins which may occur in food systems yielded LAL by heating, yet their sensitivity was markedly different. We shall now report the formation of LAL in a few proteins used as food ingredients.

Figure 6

Home-Cooked Foods

Name	Origin	LAL ppm/protein
Frankfurter	As purchased, before heating	None
Frankfurter	Boiled	50
Frankfurter	Fried	50
Frankfurter	Oven-baked	170
Frankfurter	Charcoal-broiled	150
Chicken thigh	Raw	None
Chicken thigh	Charcoal-broiled	150
Chicken thigh	Retorted	100
Chicken thigh	Cooked in microwave oven	200
Chicken thigh	Oven-baked	110
Chicken thigh	Retorted in gravy	170
Pan scrapings	Pan-frying of sirloin steak	130
Egg white	Fresh	None
Egg white	Boiled 3 minutes	140
Egg white	Boiled 10 minutes	270
Egg white	Boiled 30 minutes	370
Egg white	Pan-fried 10 minutes at 150°C	350
Egg white	Pan-fried 30 minutes at 150°C	1,100

Figure 7

Commercial Food Preparation

Name	Origin	LAL ppm/protein
Corn chips	Commercial sample	390
Pretzels	Commercial sample	500
Hominy	Commercial sample	560
Tortillas	Commercial sample	200
Taco shells	Commercial sample	170
Milk, infant formula	Commercial sample, manufacturer A, batch 1	330
Milk, infant formula	Commercial sample, manufacturer B, batch 1	550
Milk, infant formula	Commercial sample, manufacturer A, batch 2	150
Milk, infant formula	Commercial sample, manufacturer A, batch 3	640
Milk, infant formula	Commercial sample, manufacturer B, batch 2	510
Milk, infant formula	Commercial sample, manufacturer C	490
Milk, evaporated	Commercial sample, manufacturer D	860
Milk, evaporated	Commercial sample, manufacturer E	590
Milk, skim, evaporated	Commercial sample, manufacturer D	520
Milk, condensed	Commercial sample, manufacturer F	540
Milk, condensed	Commercial sample, manufacturer G	360
Simulated cheese	Commercial sample, manufacturer H	1,070

Figure 8

Food Ingredients

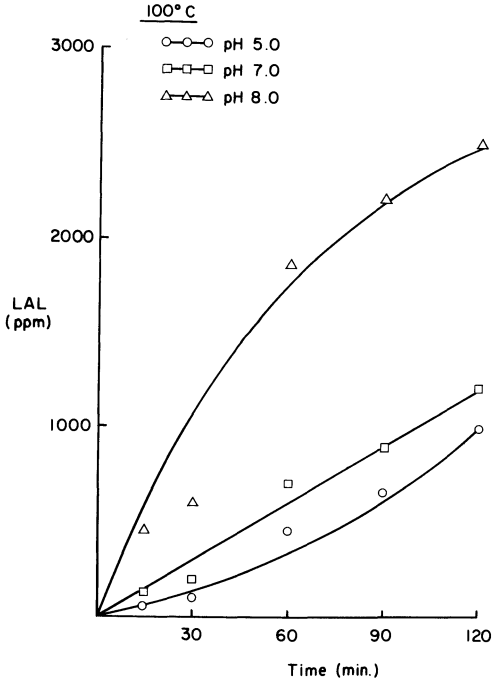
Name	Origin	LAL ppm/protein
Egg white solids, dried	Commercial sample, manufacturer I	1,820
Egg white solids, dried	Commercial sample, manufacturer J	1,530
Egg white solids, dried	Commercial sample, manufacturer K	490
Egg white solids, dried	Commercial sample, manufacturer L	160
Calcium caseinate*	Commercial sample, supplier M	1,000
Calcium caseinate	Commercial sample, supplier N	370
Sodium caseinate	Commercial sample, supplier O	600
Sodium caseinate	Commercial sample, supplier P	6,900
Sodium caseinate	Commercial sample, supplier Q	1,190
Sodium caseinate	Commercial sample, supplier R	430
Sodium caseinate	Commercial sample, supplier S	800
Acid casein	Commercial sample, supplier T	140
Acid casein	Commercial sample, supplier U	190
Acid casein	Commercial sample, supplier V	70
Masa harina	Commercial sample	480
Hydrolyzed vegetable protein	A total of 18 commercial samples of different batches from five manufacturers	40–500
Whipping agent	Commercial sample, manufacturer's type 1	6,500
Whipping agent	Commercial sample, manufacturer's type 2	50,000
Soya protein isolate	A total of 45 commercial samples of different batches from two manufacturers	0–370
Yeast extract	Commercial sample	120

*The identity of the manufacturer was not determined; therefore, the term supplier was used instead.

Our study of LAL formation in casein was performed with laboratory prepared casein. The reason for not using commercial preparations of casein was the relative scarcity of commercial casein or sodium caseinate which was free of LAL. We prepared the casein from fresh skimmed milk by precipitation at the isoelectric point with hydrochloric acid or by precipitation with calf rennet. After thorough washing with distilled water, the casein preparations were dried by lyophilization. The amount of LAL formed is directly dependent on temperature, pH and time of exposure to these factors. No significant difference was noticed between the acid and enzyme precipitated casein (Figures 9, 10). We found that at neutral pH, and even at acid pH, LAL is generated in casein if sufficient heating is applied. Over 2000 ppm LAL are formed in 30 min. at 120° C pH 8.0, which are conditions frequently used in food processing. The sensitivity of casein to LAL formation at pH 8, explains our find-

Figure 9

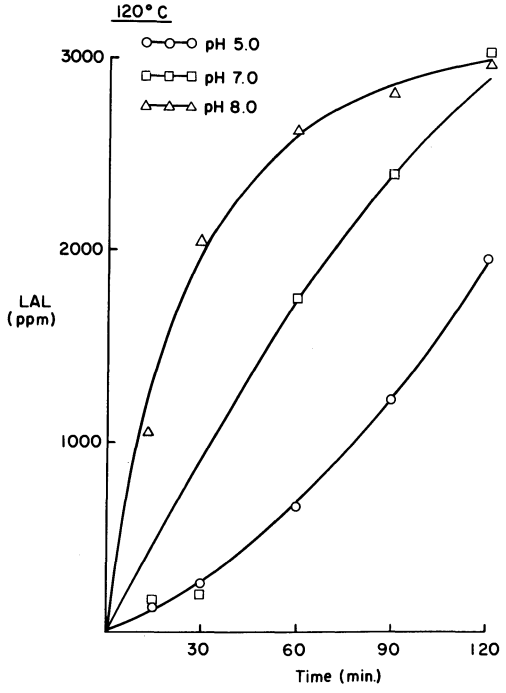
Rate of LAL Formation



5% (w/v) of the acid precipitated or rennin coagulated casein.

Figure 10

Rate of LAL Formation



5% (w/v) of the acid precipitated or rennin coagulated casein.

ing of LAL in most commercial sodium caseinates, in which it is probably generated during the alkali neutralization and drying steps of the manufacturing process.

There is a dependence between concentration of the casein solution and amount of formed LAL (Figure 11). The fact that the amount of LAL increases as the concentration of protein is increased may indicate the formation of intermolecular crosslinkings of LAL, additional to the expected intramolecular LAL.

Wheat gluten, seems less susceptible than casein to the formation of LAL, but is more sensitive than corn gluten, both to heat and alkali. Heating wheat gluten as a 6.6% suspension in water for 4 hours at 100° C caused the appearance of detectable amounts of LAL starting from pH 5 (Figure 12). Wheat or corn flour foods which

Figure 11

Formation of LAL vs Concentration of Casein

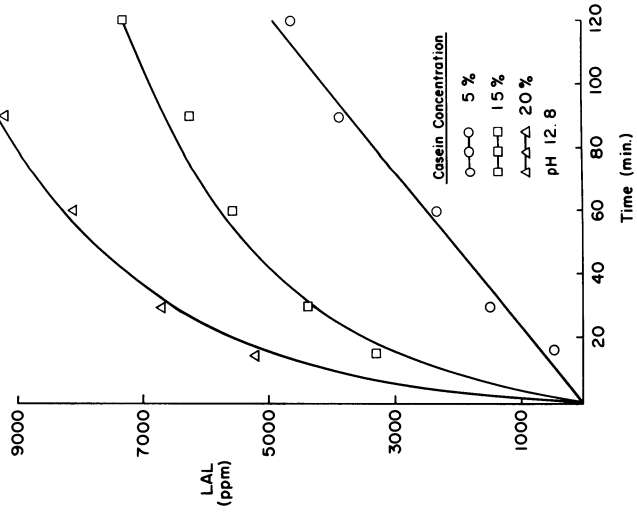
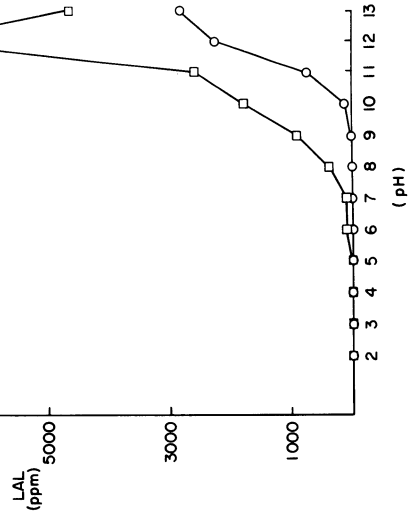


Figure 12

Formation of LAL in Wheat or Corn Gluten in Various pHs



were alkali treated, such as tortilla, hominy or pretzels, do indeed contain LAL. Our trials to determine LAL in bread, however, were not conclusive.

To prevent the appearance of LAL in a given food ingredient one may select a combination of pH and heating time conditions, which will minimize chances of its formation. Another way to decrease the possibility of having LAL in a protein, is treatment with cysteine (Figure 13). Addition of 5% cysteine to casein effectively prevents the appearance of LAL at pH not greater than 7, if the heating time does not exceed 60 minutes. Likewise, 3% cysteine added to fresh egg white will prevent LAL formation if boiling time does not exceed 30 minutes (Figure 14).

Figure 13

Effect of 5% Cysteine on LAL Formation in Casein

pH	Time (min) at 120° C.	Lysinoalanine (ppm) Cysteine Treated	Untreated
5.0	15	0	140
5.0	30	0	280
5.0	60	0	680
5.0	90	0	1240
5.0	120	0	1960
7.0	15	0	1030
7.0	30	0	2020
7.0	60	0	2610
7.0	90	55	2810
7.0	120	240	2810

5% (w/v) of acid casein concentration

Figure 14

Cysteine treatment of
fresh egg white
boiled 30 minutes

% Cysteine	LAL ppm
0	1780
1	850
3	0

Figure 15

Disulfide Interchange and Reduction

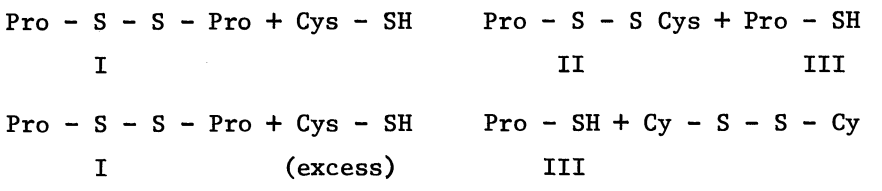


Figure 16

Effect of 3% Cysteine on Soya Globulin

Time	LAL (no cysteine)	LAL (cysteine added)	Δ LAL
10	720	180	540
20	1160	270	890
30	1780	360	1420
40	2130	470	1660
50	2490	520	1970
60	3070	600	2470

Soya globulin 18% suspension, cysteine added at pH 6.5. Alkali treatment at pH 12.5 at 20° C.

Finley* independently noticed that chemical agents, including cysteine capable to reduce disulfide bonds, are able to prevent the formation of LAL if added during the alkaline treatment of proteins. He suggested that the dehydroalanine formed by alkaline treatment combines with cysteine to yield lanthionine rather than combine with free NH_2 groups of lysyl residues to generate LAL. In our experiments we acted on the protein with cysteine prior to alkali or heat treatment ultimately causing the reduction of disulfide bonds (Figure 15) and formation of a mercaptide ion which is less susceptible to β elimination. We found that no significantly measurable lanthionine does form in the presence of cysteine while lysinoalanine formation is decreasing in the same cysteine treatment by a factor of between four to five fold (Figure 16).

*Finley, J. W., Snow, J. T., Johnston, P. H. and Friedman, M. 36th IFT Meeting Program paper #236, 156 (1976).

The work which was presented here shows that LAL is widespread in many common cooked or processed foods and protein ingredients. Whatever the biological effect, humankind has been exposed for a very long time to this amino acid. The selection of adequate processing parameters or pretreatment with cysteine will reduce significantly the amount of LAL, yet it is doubtful that this amino acid could be totally eliminated from our diet.

6

INHIBITORY EFFECT OF MERCAPTOAMINO ACIDS ON LYSINOALANINE FORMATION DURING ALKALI TREATMENT OF PROTEINS

John W. Finley, John T. Snow, Philip H. Johnston and Mendel Friedman

Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California 94710

ABSTRACT

Alkali treatment of food proteins converts some amino acid residues to the unnatural amino acid lysinoalanine which has been found to cause kidney damage when fed to rats. Formation of lysinoalanine was essentially prevented when isolates of soy protein and casein were exposed to alkali in the presence of thioalamino acids such as cysteine. The results suggest that added thiols minimize the formation of potentially toxic lysinoalanine.

INTRODUCTION

Alkali treatment of food proteins has been used since ancient times (Katz et al., 1974) and is used increasingly in the food industry for several purposes including preparation of textured vegetable proteins (Hamdy, 1974), destruction of aflatoxin in peanut proteins (Goldblatt, 1969), and peeling of fruits and vegetables (Gee et al., 1974; Hart et al., 1974). Alkali and heat convert some of the amino acid residues in proteins to unnatural amino acids and cause racemization of the amino acid residues (Provansal et al., 1975; Asquith and Otterburn, 1977). One of these unnatural amino acids, lysinoalanine, has been found to cause kidney lesions when fed or administered intraperitoneally to rats (Gould and MacGregor, 1977; Woodard et al., 1974). Similar lesions were obtained by Woodard and associates when they fed alkali-treated soy protein to rats. These results suggest that lysinoalanine, which was shown to be present in the alkali-treated proteins, may have caused the lesions. However, De Groot and Slump (1969) report no kidney lesions, and they did observe a lowering in net protein utilization (NPU) when alkali-

treated soy proteins were fed to rats. Recently Sternberg and associates (Sternberg et al., 1975; Sternberg and Kim, 1977) reported that many kinds of protein-containing foods that had been heated contain small amounts of lysinoalanine. These authors suggest that since lysinoalanine appears to be ubiquitous and is formed under usual conditions of cooking and baking, it may not be harmful. However, we feel that until the safety of lysinoalanine has been established, it may be wise to try to limit its formation in alkali processed foods. In this report we offer a method to minimize lysinoalanine formation.

RESULTS AND DISCUSSION

Lysinoalanine and other crosslinked amino acids (Finley and Friedman, 1977) presumably arises from the reaction of the ε-amino group of lysine side chains and other functinal groups with the double bond of a dehydroalanine residue. Dehydroalanine residues have been postulated as products from many different base-catalyzed elimination reactions of protein disulfide and other residues. Rate studies show that, depending on the pH of the reaction, sulfhydryl groups in mercaptoamino acids such as penicillamine or cysteine react about 6 to 1300 times faster than the ε-amino groups of lysine with vinyl compounds such as N-acetyl dehydroalanine methyl ester (Friedman and Wall, 1964; Friedman et al., 1965; Snow et al., 1976; and Tables 1 and 2). It may be, therefore, possible to prevent the formation and/or to trap dehydroalanine residues formed during alkali-treatment of proteins by adding thiols such as cysteine and reduced glutathione. The added thiols may suppress lysinoalanine formation by reducing cystine to cysteine residues which do not readily undergo elimination to dehydroalanine side chains and/or by combining with dehydroalanine residues.

To test whether the kinetic data with model compounds can be extrapolated to proteins, we added several mercaptoamino acids (0.1 g mercaptoamino acid per 10 g of protein) to samples of soybean protein isolates, which were then treated with alkali to induce lysinoalanine formation. Table 3 shows that this procedure strikingly inhibits lysinoalanine formation.

Two observations are noteworthy. First, penicillamine, which is a sterically hindered mercaptoamino acid, and therefore, reacts much more slowly than cysteine with the double bond of dehydroalanine or with protein disulfide bonds (Snow et al., 1976) was a much less effective inhibitor of lysinoalanine formation. Second, in contrast to reduced glutathione, oxidized glutathione increases the amount of lysinoalanine formed. Since it contains a disulfide bond, the oxidized glutatione apparently acts as an additional source of dehydroalanine (Whiting, 1971).

Since the alkali treatment used in the experiments mentioned above (10% protein solution in 0.1 M NaOH for 8 hr at 60°C) is much more severe than that used in the usual food processing,

Table 1

Comparison of Reaction Rates of N-Acetyl Dehydroalanine Methyl Ester with the ε -NH_2 Groups of α-N-Acetyl-L-Lysine and SH Groups of L-Cysteine and DL-penicillamine and Thiosalicylic acid

Reactant	pH	pK	$k_{2\ obs}$ $M^{-1}\ min^{-1}$	$k_{(anion)}$[a] $M^{-1}\ min^{-1}$
α -N-acetyl-lysine	9.5	10.53	0.71	11.6
L-cysteine		8.15		358.7[b]
DL-penicillamine	8.0	7.90	5.08	9.14
Thiosalicylic acid	6.4	7.79	1.76	45.7

[a] Rates of addition of the ε-amino group of α -N-acetyl-L-lysine to N-acetyl dehydroalanine methyl ester were measured by ninhydrin reaction previously described (Friedman and Wall, 1964).

Rates of reaction of SH groups in the three other compounds were measured by nuclear magnetic resonance (Snow et al., 1976).

The second-order "anion" (inherent) rate constants (k_A - for NH_2 groups and k_S- for S^- groups) can be determined directly at a pH about two units above the pK values of the NH_3^+ and SH groups, respectively, or calculated by means of the following equation: $k_{(anion)} = k_2 (1 + H^+/K)$.

Comparisons of nucleophilic reactivities of ionized amino and thiol groups with dehydroalanine are valid only when done in terms of "anion" (and not observed) rate constants (Friedman et al., 1965; Snow et al., 1976).

[b] Calculated value (Snow et al., 1976).

Table 2. Calculated second-order rate constants (k_2 in M^{-1} min^{-1}) for reaction of N-acetyl-dehydroalanine methyl ester with the ε-NH_2 group of N-α-acetyl-L-lysine and with the SH groups of cysteine, penicillamine, and thiosalicylic acid at various pH's[a].

pH	α-N-acetyl-L-lysine	L-cysteine	DL-penicillamine	Thiosalicylic acid	Rate ratio: SH cysteine / ε-NH_2 lysine
7.0	5.14×10^{-3}	25.62	1.02	6.38	5000
8.0	6.48×10^{-2}	148.8	5.09	28.3	2300
9.0	0.763	314.4	8.47	43.0	410
10.0	2.58	344.6	9.07	45.4	133
11.0	8.66	357.2	9.13	45.7	43
12.0	11.22	358.6	9.14	45.7	34

[a] The rate constants were calculated from the "anion" rate constants with the equation shown in the footnote of Table 1. Note that the calculated reactivity ratio of SH of cysteine to ε-NH_2 of lysine varies inversely with pH from 5000 to 34 in the pH range 7-12. These data suggest that cysteine will be a much more effective inhibitor of lysinoalanine formation near neutrality than in strongly alkaline media.

Table 3

Lysinoalanine Content of Soy Isolate Treated in the Presence of Various Mercaptoamino Acids

Mercaptoamino acid added (0.1 g/10 g soy isolate)	Lysinoalanine content[a] g/16 g N	Ratio to control X100
None (control)	0.51	100
DL-Penicillamine	0.29	57
L-Cysteine	0.04	8
Propionylmercaptoglycine	0.02	4
Glutathione (reduced)	0.04	8
Glutathione (oxidized)	1.24	243

[a] Lysinoalanine was determined by ion-exchange chromatography with an amino acid analyzer as described by Spackman (1963). Lysinoalanine appears as a discrete peak immediately before lysine. A ninhydrin color constant of 1.59 was used to calculate the lysinoalanine content from the elution record. This value was obtained with a synthetic sample of lysinoalanine.

Table 4

The Effect of Adding Cysteine on Lysinoalanine Content of Proteins Prepared at High pH

Preparation	Source	Lysinoalanine content g/16 g N
Soy isolate[a]	Soy flour	0.087
Soy isolate	Soy flour plus cysteine	0.001
Commercial soy isolate		0.027
Sodium caseinate[b]	Skim milk	0.160
Sodium caseinate	Skim milk plus cysteine	0.006
Commercial sodium casseinate		0.21

[a] Soy isolates were prepared from soy flour in the presence and absence of cysteine during isolation: 100 g soy flour at pH 9.0 in 1 liter water plust 0.5 g cysteine was left standing for 1 hr. The mixture was then centrifuged at 5000 rcf for 20 min, and the supernatant adjusted to pH 4.5. The precipitated protein was filtered, then washed 3 times with distilled water, freeze-dried. A soy isolate control without cysteine was prepared similarly. A 10 mg sample was hydrolyzed in 6N HCl for 20 hr at 100°C and analyzed for lysinoalanine with an amino acid analyzer.

[b] Sodium caseinate was prepared as follows: Casein was precipitated from 1 liter skim milk and 0.5 g cysteine was added per liter skim milk at pH 4.6, the mixture was centrifuged, and the filtered precipitate was redissolved by adding N NaOH to pH 8.7 at room temperature in water. The casein was then reprecipitated at pH 4.6, centrifuged, redissolved at pH 8.5, and freeze-dried. A sodium caseinate control without cysteine was prepared similarly.

we carried out additional studies under milder conditons. Results of these experiments are comapred in Table 4 (0.5 g cysteine 100 g soy flour) with those obtained from a commercial protein isolate. Similar results with sodium caseinate derived from skim milk are also shown (0.5 g of cysteine per liter of skim milk). In both cases, protein isolates prepared in the absence of cysteine contain considerable lysinoalanine, as did the commercial sample. However, addition of cysteine decreased the lysinoalanine content of the experimental samples.

In conclusion, our studies show that lysinoalanine formation can be significantly reduced during alkali treatment of proteins by adding mercaptoamino acids such as cysteine and reduced glutathione. If lysinoalanine is determined to be toxic or affect the digestibility of foods and feeds that are processed under alkaline conditions, the suggested strategy may permit alkali treatment of proteins. However, the safety of the thiol-treated protein products should be evaluated before this method is adopted.

REFERENCES

Asquith, R. S. and Otterburn, M. S. (1977). Cystine-alkali reactions in relation to protein crosslinking. This volume.

Finley, J. W. and Friedman, M. (1977). Products derived from alkali treatment of proteins. This volume.

Friedman, M. and Wall, J. S. (1964). Application of a Hammett-Taft relation to kinetics of alkylation of amino acid and peptide model compounds with acrylonitirle. J. Am. Chem. Soc., 86, 3735-3741.

Friedman, M., Cavins, J. F., and Wall, J. S. (1965). Relative nucleophilic reactivities of amino groups and mercaptide ions in addition reactions with α,β-unsaturated compounds. J. Am. Chem. Soc., 87, 3572-3582.

Gee, M., Huxsoll, C. C., and Graham, R. P. (1974). Acidification of 'dry' caustic peeling waste by lactic acid fermentation. American Potato J., 51, 126-131.

Gould, D. H. and MacGregor, J. T. (1977). Biological effects of alkali-treated protein and lysinoalanine: an overview. This volume.

de Groot, A. P. and Slump, P. (1969). Effects of severe alkali treatment of proteins on amino acid composition and nutritive value. J. Nutr., 98, 45-56.

Katz, S. H., Hediger, M. L., and Valleroy, L. A. (1974). Traditional maize processing techniques in the New World. Science, 184, 765-773.

Hamdy, M. (1974). The nutritional value of vegetable protein. Chem. Technol., 616-622.

Hart, M. R., Graham, R. P., Williams, G. S., and Hanni, P. F. (1974). Lye peeling of tomatoes using rotating rubber discs. Food Technol., 28, 38-48.

Provansal, M. M. P., Cug, J. L. A., and Cheftel, J. C. (1975). Chemical and nutritional modification of sunflower proteins due to alkaline processing. Formation of amino acid crosslinks and isomerization of lysine residues. J. Agric. Food Chem., 23, 938-943.

Snow, J. T., Finley, J. W., and Friedman, M. (1976). Relative reactivities of sulfhydryl groups with N-acetyldehydroalanine and N-acetyldehydroalanine methyl ester. Int. J. Peptide Protein Res., 8, 57-64.

Spackman, D. H. (1963). Accelerated systems for automatic analysis of amino acids. Fed. Proc., 22, 244.

Sternberg, M. and Kim, C. Y. (1977). Lysinoalanine formation in protein food ingredients. This volume.

Sternberg, M., Kim, C. Y., and Schwende, F. J. (1975). Lysinoalanine: presence in foods and food ingredients. Science, 190, 992-994.

Whiting, A. H. (1971). Isolation of lysinoalanine from the protein-polysaccharide complex of cartilage after alkali treatment. Biochim. Biophys. Acta, 243, 332-336.

Woodard, J. C., Short, D. D., Alvarez, M. R., and Reyniers, J. P. (1975). Biological effects of N^{ε}-(DL-2-amino-2-carboxyethyl)-L-lysine, lysinoalanine. In "Protein Nutritional Quality of Foods and Feeds," Part 2, M. Friedman (ed), Marcel Dekker, New York, pp. 595-618.

7

CYSTINE-ALKALI REACTIONS IN RELATION TO PROTEIN CROSSLINKING

Raymond S. Asquith and Michael S. Otterburn

Department of Industrial Chemistry

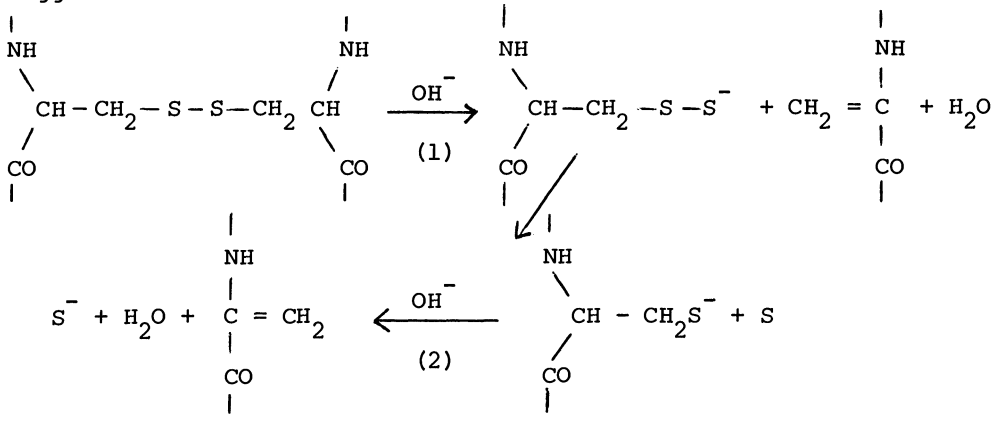
The Queen's University of Belfast, BELFAST, N. Ireland

ABSTRACT

The formation of lysinoalanine, lanthionine and β-aminoalanine in proteins, through the alkaline degradation of cystine residues to dehydroalanyl residues, is discussed. The formation of these amino-acids, during the dry heating of proteins, is also reported. It is shown that, during alkaline degradation of protein-bound cystine, amino or thiol-containing compounds present in the alkaline liquor can compete with protein-bound amino and thiol groups for addition to the dehydroalanyl residues formed as intermediates in the reaction. Results showing the relative importance of such competitive reactions are given. Evidence given indicates the one protein-bound cystine residue can, on alkaline degradation, yield two dehydroalanyl residues. The extent of addition of a series of alkylamines, to the transient dehydroalanyl residues, is shown to be dependent on the structure of the pendant alkyl group. Addition is governed by two factors, activity of the addendum, and the positioning of possible addenda in relation to the protein-bound dehydroalanyl residues, when the latter are formed. Dyeing experiments with alkylamine-treated wools show that the pendant alkyl groups influence dyeability. The results indicate that hydrophobic interactions in the modified protein have as great an effect on dyeability as do covalent crosslinks and ionic groups. Dye uptake is determined by the shape and size of the pendant alkyl groups introduced during the protein modification, rather then the enhanced basicity caused by introduction of the basic sidechains. The possibility of introducing hydrophobic sidechains into proteins, as amide derivatives of protein-bound lysine residues, by simple dry heating, is speculated upon, in relation to dry-heating of a fat-containing protein.

INTRODUCTION

Changes in the covalent crosslinking of keratin have been extensively examined in relation to alkaline processing of wool. From early mechanochemical studies (Astbury and Woods, 1934) it was postulated that the action of steam or alkaline solutions on wool fibres resulted in the degradation of cystine disulphide bonds which crosslinked the protein chains. Further, subsequent to this breakdown, rebuilding of new crosslinks occurred (Speakman, 1936). The identification of lanthionine in alkali-treated wool (Horn, Jones and Ringel, 1941) established the structure of one such new crosslink, already postulated by Speakman in 1936, as arising from the reaction. Indirect evidence (Asquith and Speakman, 1952) indicated that lysine was also involved in other new crosslinks formed. Numerous possible crosslinks involving lysine have been suggested, and it was not until 1964 that the true nature of the lysine-containing crosslink was determined by the identification of lysinoalanine in alkali-treated proteins (Bohak, 1964; Patchornik and Sokolovsky, 1964; Ziegler, 1964). Numerous mechanism by which lanthionine and lysinoalanine can be formed, during the alkaline degradation of cystine, have been proposed. The majority of such mechanisms require the first formation of an intermediate, dehydroalanine. Though much indirect experimental evidence supported this postulate, the formation remained hypothetical for some time. Asquith and Carthew (1972a) synthesised a dehydroalanyl-containing peptide, dehydroglutathione, in a pure form. Using this compound as an electrophoretic standard, they were able to identify and estimate it directly during the alkaline decomposition of oxidised glutathione. To account for the fact that during this decomposition reduced glutathione (cysteine-containing) and dehydroglutathione (dehydroalanine-containing) were not produced in equal amounts, the latter being in considerable excess, they suggested that the decomposition of one cystine link could proceed by two stages, resulting in the formation of two dehydroalanyl residues:
The stages of the reaction, based on previous mechanisms, were suggested to be:



Subsequent formation of lysinoalanine and lanthionine in the protein then occurs by addition reactions to the dehydroalanyl residues. The identification of β-aminoalanine in hydrolysates of alkali-treated wool (Asquith and Dominguez, 1968) indicated that these 'internal' addition reactions within the protein were specific cases of a general type of addition. Such reactions could also occur when a cystine-containing protein is treated with alkaline solutions, or steam, in the presence of an amino or thiol-containing compound. (Aromatic amines are inert.) When any protein, containing cystine, is treated with alkaline solutions, containing thiols or amines, we are not considering two or three competitive internal reactions in isolation, but a series of competitive addition reactions. The types and amounts of final products formed will depend on a series of parameters, such as:

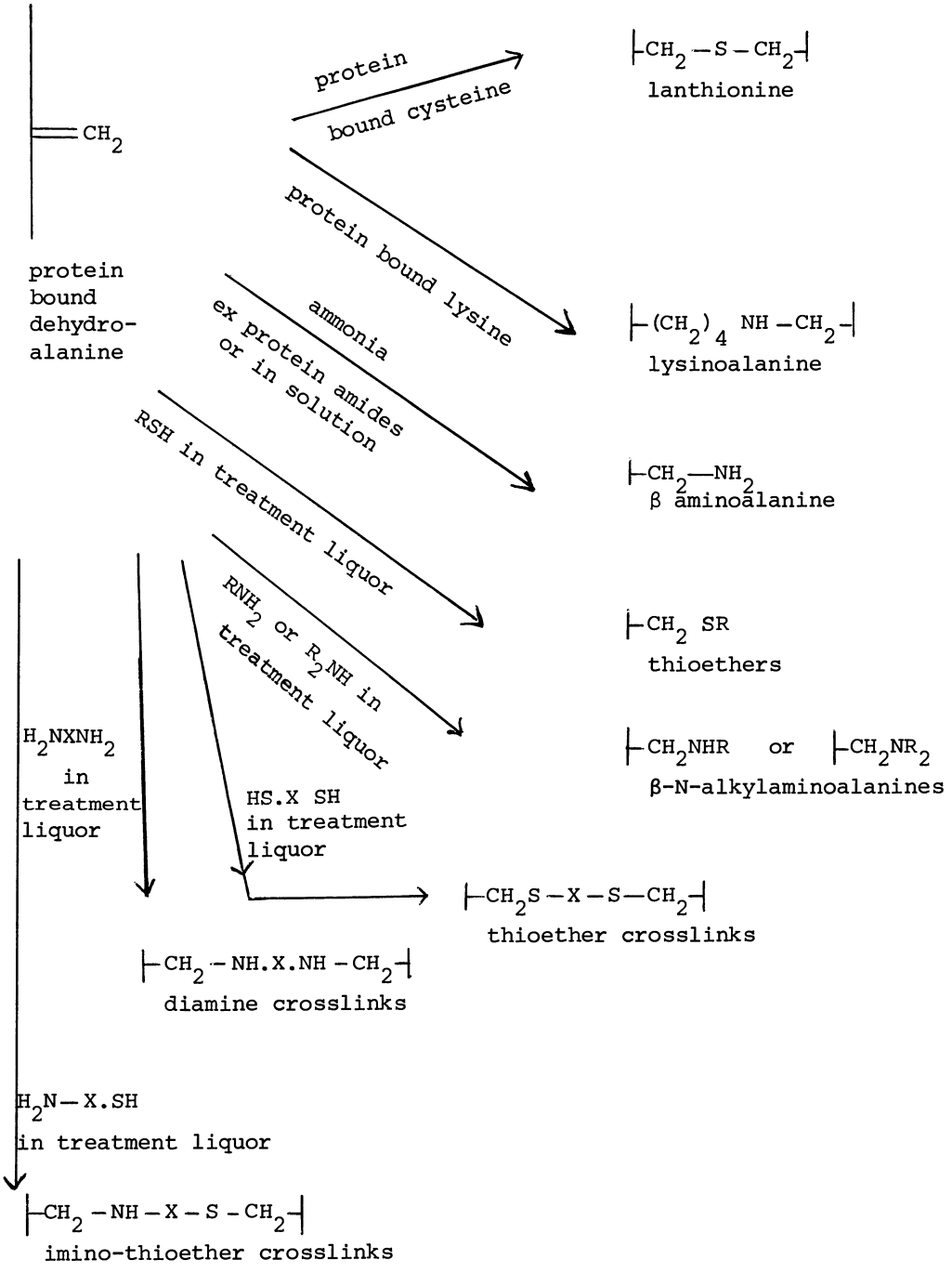
(a) The differing reactivities to addition to the dehydroalanyl residues of the thiols and amines present in the protein (internal reactions) and those present in the alkaline reaction liquor.

(b) The proximity of protein-bound cysteine thiol groups and lysine ε-amino groups to the dehydroalanyl residues formed within the protein.

(c) The concentration of thiol or amino-containing compounds in the alkaline treatment liquor.

(d) With heterogeneous systems, such as keratin in alkaline solutions, the reaction products will be influenced by the ease of penetration into the fibre of the soluble thiols or amines.

Treatment of proteins with alkaline solutions or steam may, therefore, lead to crosslinking by lanthionine and lysinoalanine, by difunctional amines, dithiols, or amino-thiols present in the treatment liquor. Alternatively, monofunctional thiols or amines with alkyl sidechains, present in the reaction medium, may react such that their sidechains enhance crosslinking by secondary valency forces. Such secondary forces may be sufficiently powerful as to alter the properties of the protein. Certainly in relation to nutrition this latter possibility cannot be ignored. Thus poly-phenol/protein interactions materially alter the nutritional properties of the protein although only secondary valency forces are involved (Synge, 1975). The various types of reaction which may occur can be summarised as:


protein bound dehydro-alanine
protein bound cysteine
lanthionine
protein bound lysine
lysinoalanine
ammonia ex protein amides or in solution
β aminoalanine
RSH in treatment liquor
thioethers
RNH2 or R2NH in treatment liquor
β-N-alkylaminoalanines
H2NXNH2 in treatment liquor
HS.X SH in treatment liquor
thioether crosslinks
diamine crosslinks
H2N—X.SH in treatment liquor
imino-thioether crosslinks

The present paper discusses various reactions of protein-bound cystine in alkali-containing solutions. Using wool keratin as a substrate, it indicates how such pretreatments modify fibre properties, as instanced by dyeability.

'Internal' Crosslinking by Lanthionine and Lysinoalanine

A comprehensive study (Garcia-Dominguez, 1968) was made of the reactions of wool keratin with sodium hydroxide solutions at various temperatures, the amounts of lanthionine, lysinoalanine and β-aminoalanine formed being determined. The results are summarised in Table 1.

Obviously these addition reactions will be primarily controlled by the rate of formation of dehydroalanyl residues, but within this constraint only at low temperatures is the extent of addition of the ε-aminogroup of lysine comparable with the extent of addition of the cysteine thiol group. At higher temperatures thiol addition, as would be expected from the known ease of addition to double bonds, far exceeds amine addition (Friedman, 1973; Friedman, Cavins and Wall, 1965; Cavins and Friedman, 1968). It is difficult to explain why amine addition should be so ready at low temperatures. A very tentative explanation could be that at these temperatures,

TABLE 1

Effect of Temperature of Treatment of Wool Keratin (with 0.1 N Sodium Hydroxide for 30 mins) on the Formation of Dehydroalanine Addition Products

Temperature of Treatment (°C)	% Lanthionine formed	% Lysinoalanine formed	% β-Amino Alanine formed
Control	Nil	Nil	Nil
0	0.15	0.21	-
10	0.29	0.31	trace
20	0.46	0.32	trace
30	1.05	0.49	0.10
40	2.39	1.14	0.14
50	3.40	1.69	0.17
55	3.96	1.88	-
60	4.25	2.01	0.23
70	5.25	2.34	0.26
80	-	-	0.17

where the protein structure is least disturbed (cf. ribonuclease), certain lysine groups are in close proximity to the dehydroalanine sites, when these are formed. The addition of ammonia (ex. hydrolysis of asparagine and arginine residues) is negligible at low temperatures, reaching a maximum at 70° and then declining at higher temperatures. There are more constraints on this addition, however, than in the other cases. Firstly at low temperatures, the hydrolysis of amide sidechains to release the necessary ammonia is slower, whilst at high temperatures the free ammonia can escape from the open reaction vessel (cf. dry heat reactions). Even accepting these additional constraints, the competitive addition of ammonia for dehydroalanyl residues is low.

With other proteins (Asquith, Booth and Skinner, 1969) alkaline treatments with sodium hydroxide solution also results in much greater quantities of lysinoalanine being formed than β-amino alanine. Table 2 compares the relative quantities for a series of proteins treated with sodium hydroxide solution at different temperatures.

With the exception of lysozyme at higher temperatures, ammonia (ex amide sidechains) addition is negligible as compared with lysinoalanine formation. The varied amounts of the latter formed in the different proteins are interesting. Thus myoglobin contains no cystine, yet amounts of lysinoalanine, comparable with those formed from insulin, are produced. This supports previous suggestions (Ziegler, 1967; Mellet and Louw, 1965; Robson and Zaida, 1967) that dehydroalanyl residues can be formed in proteins by alkaline decomposition of serine.

TABLE 2

Amounts of β Aminoalanine and Lysinoalanine found in Proteins after treatment with 0.1 N Sodium Hydroxide for 30 Minutes (expressed as μ moles g^{-1} Protein) at different Temperatures (Lanthionine not determined)

Protein	β Aminoalanine Content			Lysinoalanine Content		
	20°C	40°C	60°C	20°C	40°C	60°C
Lysozyme	nil	nil	32.0	4.5	90.1	210.5
Myoglobin	nil	trace	trace	14.6	17.3	51.2
Ribonuclease	8.2	8.2	nil	96.1	131.8	210.0
Insulin	nil	trace	trace	4.7	40.0	60.0
β Lactoglobulin	nil	trace	4.0	11.1	71.6	82.3
Wheat gluten	nil	nil	nil	nil	12.7	15.1

The structure of the proteins markedly affects the formation of lysinoalanine. Thus lysozyme and ribonuclease contain similar numbers of cysteinyl and lysyl residues per molecule, and it is not surprising therefore that similar amounts of lysinoalanine are produced from them at higher temperatures of treatment where damage to the molecular structure will be greatest. At low temperatures of reaction, only small quantities of lysinoalanine are produced from lysozyme and much larger quantities from ribonuclease, indicating that in ribonuclease some of the lysyl residues are closely aligned to dehydroalanyl sites in the molecular configuration. It is not surprising that at low temperatures of reaction, lysinoalanine formation in insulin is negligible, the one lysyl residue being far removed from the cystine bridges. At higher temperatures partial protein hydrolysis occurs (electrophoretic patterns of insulin after alkaline treatment at 60° showed that hydrolysis was more marked than with any of the other proteins examined). Such hydrolysis enables the peptides formed to react with the dehydroalanyl residues.

In these reactions only the species present in the protein can react with dehydroalanyl residues. Theoretically addition of water to the double bond could occur, though by analogy with other compounds such as vinyl-containing reactive dyes, this addition would be relatively slow. It may account in part for the small increases in serine content sometimes observed in alkali-treated proteins.

Lysinoalanine and Lanthionine Formation by Dry Heating of Proteins

It was observed (Asquith and Otterburn, 1969, 1970) that wool, on dry heating, was rendered considerably more insoluble in standard solubility tests. Analysis of hydrolysates of the heated wools proved that lysinoalanine lanthionine and β-aminoalanine were all formed during the heating process, but in insufficient amounts to explain the reduced solubility of the fibre. Subsequently it was shown (Asquith and Otterburn, 1971) that the major cross-linking reaction was the enhanced formation of isopeptide links. Nevertheless this work clearly showed that cystine degradation (possibly also serine) could give rise to dehydroalanyl residues in anhydrous conditions. It is interesting to note that (Table 3) when the amounts of lysinoalanine and β-aminoalanine are compared with the amounts found in alkali-treated wool (Table 1) the proportion of β-aminoalanine is far greater. (As sealed vessels were used ammonia formed would not escape.) In general, the amounts of both products are insignificant in comparison with the amounts of lanthionine formed. Only at very high temperatures of heating (where lanthionine itself is subject to partial decomposition) do lysinoalanine and lanthionine quantities compare.

TABLE 3

Formation of Lysinoalanine and Lanthionine on Heating Dry Wool Keratin for 48 hrs.

Temperature of Treatment °C	β-Aminoalanine Content (μ moles g^{-1})	Lanthionine Content (μ moles g^{-1})	Lysinoalanine Content (μ moles g^{-1})
Control	nil	nil	nil
60	3.4	17.0	trace
100	6.7	32.0	1.2
140	8.6	43.0	4.4
160	18.0	44.2	4.8
*180	11.0	11.1	6.9

* General decomposition of aminoacids is occurring at this temperature.

A study of the effects of dry heat on some edible proteins was also made. It can be seen that when these are heated under comparable conditions (Table 4) the amounts of lanthionine formed have no simple relationship with the amount of cystine decomposed. Presumably the formation of lanthionine is considerably influenced by the configuration of the protein chains. It could be that the mechanism of formation is different to that postulated for alkaline degradation. Further, only in the case of wool is any lysinoalanine formed, none being detectable on dry heating of the other proteins. Some absorbed water is present in the proteins during dry heating; therefore the lanthionine formation may be confined to dehydroalanyl residues formed by the reaction of this moisture.

Competitive Additions for Dehydroalanyl Residues in Alkaline Media

The preceding reactions have dealt solely with cases where no extraneous amine or thiol is present, in contact with the proteins, in the alkaline media. When a soluble reactive thiol or amino compound is present, this compound may compete for the dehydroalanyl sites giving thioethers or N-substituted β-aminoalanine derivatives respectively.

TABLE 4

Formation of Lanthionine on Dry Heating of Proteins
(Results expressed in gms/16 gms Protein N)

Protein	Time of Heating (hrs)	Temperature	Lanthionine Formed
Lactalbumin	27	85	0.4
	57	115	1.2
Casein	27	115	0.3
Defatted Chicken Muscle	27	121	Nil
Bovine Plasma Albumin	8	121	1.3
	27	121	1.4
Zein	57	115	0.5
Lysozyme	27	121	1.7
Egg Albumin	57	115	1.5

TABLE 5

Amounts of β-Aminoalanine and Lysinoalanine found in Proteins after Treatment with 3.5 M Ammonia Solution for 30 mins (Expressed as μ moles g^{-1}) at different Temperatures (Lanthionine not Determined)

Protein	β-Aminoalanine Content			Lysinoalanine Content		
	20°C	40°C	60°C	20°C	40°C	60°C
Lysozyme	trace	24.0	127.0	3.2	70.0	95.0
Myoglobin	nil	nil	trace	6.2	12.0	10.0
Ribonuclease	trace	6.0	6.2	25.0	75.0	155.0
Insulin	trace	49.0	67.0	trace	6.4	6.5
β-Lactoglobulin	trace	8.8	20.1	8.5	19.0	20.0
Wheat gluten	trace	40.0	81.0	1.5	3.2	8.4

The simplest case of such competitive reactions is when sodium hydroxide is replaced by ammonia as the reactive alkaline species. Table 5 shows the amounts of lysinoalanine and β-aminoalanine formed, using proteins already examined (cf. Table 2) for reaction with sodium hydroxide. Unfortunately fine comparisons between Tables 2 and 5 cannot be made as no attempt was made to control pH values. Nevertheless gross comparison shows that the amounts of β-aminoalanine formed in ammonia-treated proteins is considerably higher, at higher temperatures of treatment than is the case with sodium hydroxide-treated proteins. With insulin, β-aminoalanine is particularly readily formed, and it would appear that with the other proteins the ammonia is competing directly with lysine for addition. At lower temperatures, where the lysine is presumably more favourably placed to react with certain dehydroalanyl residues, the competitive addition of ammonia is unsuccessful. At higher temperatures, its successful addition would seem to depend on the ability of the protein to remain in a structure favouring lysinoalanine formation. (Compare particularly β-lactoglobulin, wheat gluten and ribonuclease.)

Using wool keratin as a model, Asquith and Carthew (1973) attempted to assess the competition for dehydroalanyl residues, resulting from alkaline treatments in the presence of ammonia and thioglycollic acid separately, and also together in the reaction medium. They standardised the pH value of the media used at pH 10 (using either sodium borate or ammonia as the source of hydroxyl ions). Time of treatment (24 hrs.) and temperature of treatment (60°) were also standardised, hence allowing for more fine comparison than in the works reported above.

The presence of a thiol-containing compound adds a further variable to the reaction, i.e. reduction of the disulphide bond:

$$\underset{\text{cystine}}{R-S-S-R} + HSCH_2COOH \longrightarrow R-S-SCH_2COOH + \underset{\text{cysteine}}{R-SH}$$

This reaction could be expected to proceed at least as rapidly as the decomposition of cystine to dehydroalanine and the cysteine formed can itself decompose. Early work on the alkaline decomposition of cystine (Elliott et. al., 1960) showed that the presence of cysteine tended to catalyse the degradation of cystine. The results obtained by Asquith and Carthew are summarised in Table 6.

In the absence of added thioglycollic acid, the presence of ammonia in the alkaline medium reduces the amount of lysinoalanine formed and seems to inhibit lanthionine formation. No explanation of this latter observation is possible, but it is interesting to note that the fall in lysinoalanine content is reflected by the rise in β-aminoalanine content. Even when small amounts of

TABLE 6

Amounts of dehydroalanine addition compounds formed when wool keratin is treated with alkaline solutions of 2% borax (pH 10) or 3.5 M ammonia (pH 10) for 24 hrs. at 60° in the presence of various concentrations of thioglycollate.

Alkaline Medium 2% Borax

Amounts of Addition Compounds formed

% Thioglycollate present in Treatment Liquor	S-Carboxymethyl Cysteine	Lanthionine	Lysinoalanine	β-Amino Alanine
0	nil	203	24.0	nil
0.1	26.5	241	9.5	7.9
0.2	33.6	296	3.6	6.4
0.3	47.6	293	1.6	nil
0.4	65.5	248	trace	nil
0.5	150.0	197	trace	nil

Alkaline Medium 3.5 M Ammonia

Amounts of Addition Compounds formed

S-Carboxymethyl Cysteine	Lanthionine	Lysinoalanine	β-Aminoalanine
nil	131	12.0	13.7
19.6	240	4.7	10.0
42.6	245	4.5	8.0
57.0	251	trace	trace
65.0	240	trace	nil
92.0	228	trace	nil

thioglycollate ion (0.1%) are introduced into the reaction medium, considerable amounts of S-carboxymethyl cysteine are formed, and both the lysinoalanine and β-aminoalanine contents fall in the presence of ammonia. Surprisingly the β-aminoalanine content rises under these conditions in the absence of ammonia. As the thioglycollate content of the reaction medium increases, both lysinoalanine and β-aminoalanine contents of the treated fibres decline to zero, the presence of ammonia failing to halt the decline of the latter. This clearly establishes the expected fact that thiol addition is far more ready than amine addition under these conditions. More interesting is the fact that both lanthionine and S-carboxymethyl cysteine contents rise as the thioglycollate content of the reaction medium increases (up to 0.3%). Only at concentrations of added thioglycollate over 0.3% does this thiol group actively compete with the cysteine present in the fibre for dehydroalanyl residues. At lower concentrations of thioglycollate, in support of earlier findings (Elliott et. al., 1960) it would seem that the additional thiol groups present (thioglycollate/cysteine) actively catalyse the formation of dehydroalanine.

The results suggest that:

(a) In the presence of ammonia, the ammonia competes partially successfully with the ε-aminogroup of lysine in additions to dehydroalanyl residues.

(b) In the presence of a thiol, the decomposition of cystine is catalysed, followed by enhanced formation of thioethers. Only at high concentrations of added thiol is lanthionine formation partially inhibited.

(c) Thiol addition is far more rapid under these conditions than is amine addition. Lysinoalanine and β-aminoalanine formation is easily inhibited.

Competitive Additions for Dehydroalanyl Residues by Monofunctional Amines

The reaction of ammonia with keratin fibres results in only small quantities of β-aminoalanine being formed. 'Internal' additions yielding lanthionine and lysinoalanine occur preferentially. Nevertheless the introduction into the fibre of this basic aminoacid residue should alter, at least to a slight extent, the properties of the keratin fibre; for example, enhanced basicity should enhance acid dye absorption. Severe treatments with ammonia do render the fibre distinctly more basic but fibre damage is so severe as to render the wool useless for industrial processing. Alternative amines of the type RNH_2 were, therefore, examined as such amines are more basic than ammonia, and hence their dehydroalanyl adducts could be expected

to ionise more readily than β-aminoalanine residues. Early dyeing studies (Asquith and Skinner, 1970; Garcia-Dominguez, Miro and Fuentes, 1974) of amine-treated wools indicated that alkaline application of aliphatic amines as a pretreatment enhanced the dye uptake of the pretreated fibres to differing extents, dependent on the length of the aliphatic sidechain (R), methylamine being most effective. The β-N alkylaminoalanine residues so formed should not have, it was argued, significantly different pK values, and hence it was probable that the reason for the differing dye-uptakes was a function of the differing extents of addition of the various amines to the dehydroalanine residues.

The syntheses of some β-N-alkylaminoalanines have been reported (Asquith and Carthew, 1972b) and further examples have since been made in our laboratories and are reported here. These synthetic products have been used as standards for high voltage electrophoretic estimation of these residues in hydrolysates of amine-treated wools. For convenience the properties of the entire series are reported here in Table 7.

It can be seen that the melting points of the straight-chain compounds follow the expected changes of a homologous series, and also the incremental decrease in electrophoretic mobility is reasonably constant indicating that there is little change in pK value of the basic sidechains from one member of the series to another. Any changes in protein properties, arising from introduction of these groups into the fibre cannot be ascribed to differing basicities of the sidechain alkylamino groups.

TABLE 7

β-N-Alkylaminoacids ($RNHCH_2-\underset{NH_2}{\overset{COOH}{CH}}$) and their Electrophoretic Mobilities on Paper

R	M.P. (°C)	R_M relative to Glycine	Incremental Decrease in R_M value
[1]Me	-	1.20	-
Et	154-5	1.13	0.7
n-Pr	165-6	1.07	0.6
n-But	177-8	1.00	0.7
n-Pentyl	183-4	0.95	0.5
[2]n-Hexyl	177-9	0.91	0.4
[2]n-Heptyl	180-2	0.86	0.5
[2]Isopropyl	173-4	0.99	-
[2]Isobutyl	167-8	0.94	0.5
[2]Isopentyl	170-1	0.88	0.6

[1] Kindly supplied by Professor J. J. Garcia-Dominguez
[2] Synthesis details not yet reported

The alkylamines have very similar pK values (see Table 9) and hence can act as their own source of hydroxyl ions as alkaline liquors used for pretreating the fibres, all maintaining the same pH value. The amounts of reaction products were therefore assessable under standard comparative conditions of treatment. Such assessment (Asquith, Carthew, Hanna and Otterburn, 1974) revealed the following findings:

(1) To account for the amounts of degradation products found when wool is treated with ethylamine (or butylamine) two degradation steps from cystine are required to produce sufficient dehydroalanine, i.e.

(a) cystine (1 mole) $\longrightarrow$ dehydroalanine (1 mole) + cysteine (1 mole);

(b) cysteine (1 mole) $\longrightarrow$ dehydroalanine (1 mole).

Table 8 shows the total cystine and cysteine degradation products present in wool after treatment with ethylamine for various periods of time. The final columns show the amounts of cystine (515 μ moles g^{-1}) present in untreated wool, and the calculated amounts which would be required to account for the found degradation products if only reaction (a) above was occurring and also if reactions (a) and (b) are occurring. Obviously if only (a) occurs the cystine required would be far in excess of the amount actually present in the fibre. Thus a similar type of decomposition is occurring as was observed with glutathione previously (Asquith and Carthew, 1972a).

(2) Whilst, as shown earlier, the presence of ammonia in an alkaline treatment liquor does not entirely eliminate lysinoalanine formation, the presence of ethylamine (or butylamine) inhibits it completely, none being detected in the treated wools.

(3) n-Butylamine reacts more readily than does ethylamine with dehydroalanyl residues. Further it competes more successfully with cysteine (precusor to lanthionine) in additions to these sites. Thus when wool samples are treated with 1 M solutions of the amines under the same conditions (2 hrs. at 60°) ethylamine treatment yields 424 μ moles g^{-1} wool of β-N-ethylaminoalanine and 138 μ moles g^{-1} wool of lanthionine, whereas n-butylamine yields 600 μ moles g^{-1} wool of β-N-(n-butyl)-aminoalanine and only 85 μ moles g^{-1} wool of lanthionine.

In view of the finding (3) a series of both straight and branched chain amines was used to treat wool fibres under identical conditions. The pH values of the treatment liquors (with the exception of ammonia) did not vary by more than 0.1 units. The

TABLE 8

Amounts of Degradation Products of Cystine (Including Undegraded Cystine) in Wool Formed by Treatment with 1-M Ethylamine for Different Times at 60° (Amounts in μ moles g^{-1} Wool)

Time (min)	N-Ethyl-Aminoalanine	Lanthionine	Residual Cystine	Cysteine (½ Cystine)	Total Cystine required based on Equation (a)	Total Cystine required based on Equations (a) plus (b)
0	0	0	495	40	515	515
30	206	80	274	28	574	471
60	286	101	157	20	554	511
120	424	138	119	18	690	478
180	460	144	105	19	719	489
240	480	150	66	19	706	466

TABLE 9

Amounts of β-N-Alkylaminoacids formed by a Series of Amines on Treating Wool with 1M Solutions of the Amines (RNH_2) at 60° for 30 mins.

Amine Sidechain	pK Value of Amine Addendum	Amount of β-N-Alkyl-Aminoacids formed in Wool (μ moles g^{-1})
H	9.27	trace
Methyl	10.65	131
Ethyl	10.63	206
n-Propyl	10.63	230
n-Butyl	10.58	293
n-Pentyl	10.61	338
iso-Propyl	10.63	135
iso-Butyl	10.48	187
iso-Pentyl	10.60	229

amounts of β-N-alkylaminoalanines determined in hydrolysates of the treated wools are given in Table 9.

With the straight chain amines, extent of reaction with dehydroalanyl residues increases progressively as the length of the alkyl chain increases. The branched chain amines do not lie in this series. The branching results in a lower amount of the β-N-alkylaminoalanine being formed than with the corresponding straight chain analogue. Nevertheless, in the branched chain series, the amines show progressively increased reaction as the size of the sidechain increases. The explanation of these peculiar changes in reactivity are not yet clear but the following postulates have been considered:

(1) Differences in hydroxyl ion concentration within the wool samples during treatment results in enhanced degradation of the cystine residues therein. As the treatment liquors are maintained at the same pH value, this could only arise if the amines increase the swelling of the fibre to differing extents allowing easier penetration by the hydroxyl ions. Studies of the amounts of cystine degraded with ethylamine and n-butylamine respectively do not support this postulate. Figure 1 shows that the rates of cystine degradation with these amines are very similar.

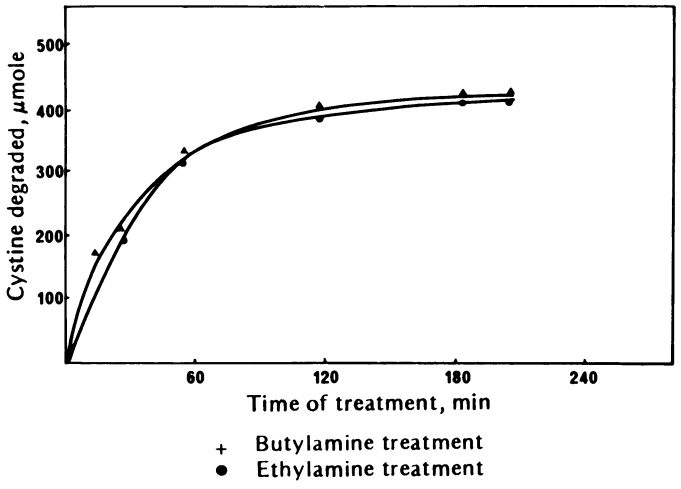


Figure 1. Degradation of cystine in wools on treatment with 1M solutions of ethylamine and n-butylamine respectively.

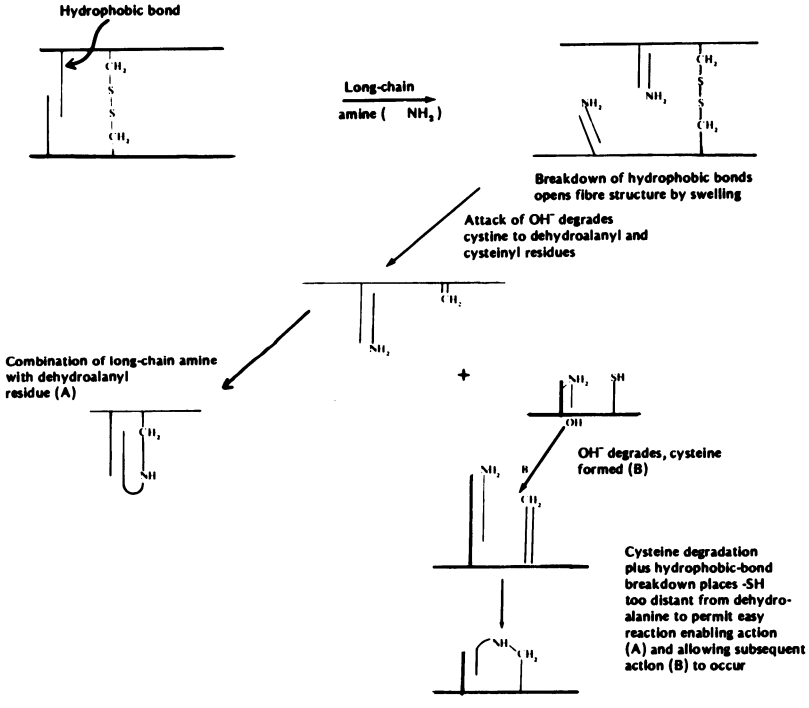


Figure 2. Postulated scheme for the addition of alkylamines to dehydroalanyl residues in wool.

(2) The increase in size of the alkyl sidechains, by breaking internal hydrophobic interactions, causes increased swelling of the fibre. The free amines are absorbed into the protein in increasing amounts as the chain length or size of the alkyl sidechain increases. This increased swelling will result in dehydroalanyl residues, as formed, separating from cysteine or lysine residues present, thereby inhibiting easy reaction. The larger the concentration of amine within the fibre the greater the possibility of its reaction with dehydroalanyl residues.

Based on the second postulate a scheme is proposed for the straight chain amines (Figure 2) in which the amines first break up internal hydrophobic interactions allowing swelling and easier penetration. Molecules of the free amines then align themselves, in some way, by hydrophobic interaction, with the hydrophobic sidechains of such amino acids as valine and leucine. The amino-groups of the amines are then in close proximity to the dehydroalanyl residues, as these are formed by hydroxyl ion attack on the cystine.

Such a postulate contains many assumptions, in particular that hydrophobic aminoacid residues are close to cystine residues. However, the fact that branched chain amines react to a less extent with dehydroalanyl residues than do the corresponding straight chain analogues does suggest that ease of diffusion of the amine into the wool may be a defining factor. Until more data, such as swelling of the fibre, is available the precise nature of the reaction-controlling steps must remain uncertain.

The fact that amines show different reactivities, when reacting with a cystine-containing protein may, however, explain other phenomena observed in industrial processes. Thus it has been long known that in the unhairing of hides with lime old used lime liquors are more effective than fresh liquors. McLaughlin et. al. (1928) showed that this was due to the formation of amines, by bacterial action, during unhairing. Similar effects could be obtained by addition of methylamine to the fresh lime liquors (Moore and Koppenhoefer, 1933).

Dyeing Properties of Amine-Modified Wool Fibres

The investigations here reported have, at first sight, little connection with the nutritional and biochemical aspects of protein crosslinking. Indeed, monofunctional amine reactions cannot introduce covalent crosslinks into proteins. Such covalent cross-links as lanthionine, lysinoalanine and isopeptides do have a considerable effect on both nutritional values and biochemical activity of the proteins containing them. The importance of isopeptides, subsequent to their identification in keratin

(Asquith et. al., 1970), in relation to nutritional properties and metabolism has been described (Hurrell et. al., 1976). The alkaline reaction of alkylamines with proteins does introduce not only basic sidechains but also aliphatic hydrophobic groups into the protein structure. Such groups by inter-attraction do introduce a loose form of intermolecular bonding into the protein. This bonding materially affects the dyeing properties of amine-treated wool keratin, and hence may have other significant effects on biochemical properties of the protein.

Wool keratin fibres, treated with different amines, such that the amounts of the different β-N-alkylaminoalanyl residues present were known (see Table 9) were dyed with different classes of dyes. The rates of exhaustion of the dyes from solution were determined.

As the pK values of the basic sidechains, introduced into the fibre, are very similar (Table 7) it was expected that the number of these groups introduced into the fibre, rather than the nature of the inert alkyl sidechain thereon, would determine the rate of dye-uptake, at least when using simple acid dyes. Thus, as n-pentylamine introduces many more basic groups into the fibre than does methylamine, the former should enhance dyeability more than the latter. Treatment of the wool with the intermediate straight chain amines should result in progressively increased dyeability of the treated fibres, as the chain length of the amines used increases. Figure 3 shows the rates of exhaustion of CI Acid Red 57 on wools treated with different straight chain amines, the dyeings being carried out from aqueous solution.

The order of dyeability obtained is exactly the reverse of that predictable from the different numbers of basic groups introduced into the fibres.

Low molecular weight reactive dyes are first attracted into wool fibres by the same acid/base interactions as for acid dyes. Subsequently the reactive dyes bind covalently (fixation) to reactive sites in the fibre. Exhaustion rates, therefore, follow a similar pattern to those observed for the acid dye, but measurements of fixation rates show increased differences on the pre-treated wools. The rates of fixation of Procion Red MX.2B (a dichloro-S-triazinyl dye) are given in Figure 4.

Two possible explanations for these effects can be suggested, but as these must be inter-related it would be difficult to distinguish the contribution of each.

(1) The increasing size of the alkyl sidechains inhibits penetration of the dyes by steric effects.

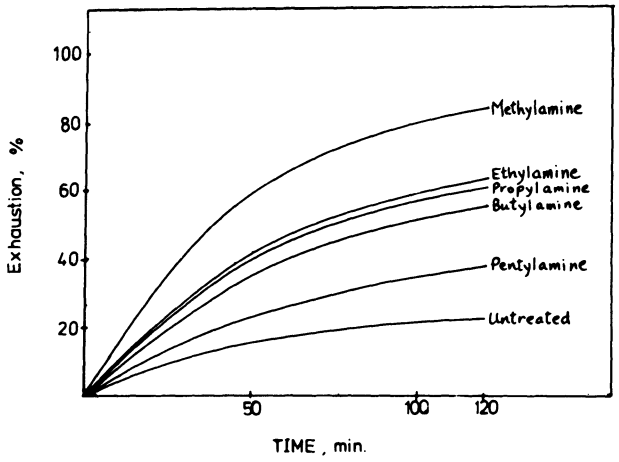


Figure 3. Rates of exhaustion of C.I. Acid Red 57 on wools pre-treated with straight-chain alkylamines as indicated. Temperature of dyeing 25°C.

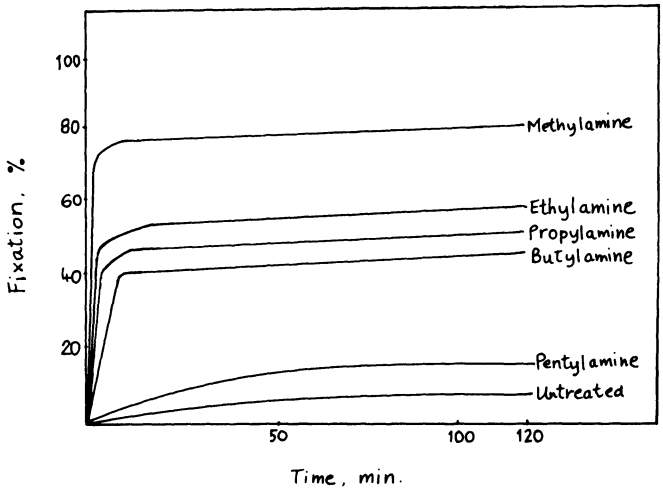


Figure 4. Fixation curves of Procion Red MX 2B, from aqueous dye-baths, on wools pre-treated with straight-chain alkylamines. Temperature of dyeing 25°C.

(2) The introduced alkyl sidechains, by hydrophobic interactions between themselves and native hydrophobic sidechains of the wool, yield a form of crosslinking within the protein structure which prevents penetration of the dye into the fibres.

Aqueous solutions of n-propanol are known to disrupt hydrophobic interactions in proteins. Dyeings of the pre-treated wools were therefore carried out under the same conditions as previously but replacing the water of the dyebaths by a 1:1 n-propanol-water mixture. Both with CI Acid Red 57 exhaustion and Procion Red MX.2B fixation the presence of the n-propanol caused a complete reversal of the orders of exhaustion and fixation respectively. The results for CI Acid Red 57 are given in Figure 5.

The differently pre-treated wools now dye in the order expected from their different basicities. The lower final exhaustion figures in all cases, as compared with dyeings from aqueous dyebaths, may be due to suppression of the ionisation of the dye in the mixed solvent. It, therefore, appears that the presence of n-propanol in the dyebath, by reducing hydrophobic interactions within the fibre structure, permits the basicity of the treated fibres to determine the dyeing rates, unimpeded by the steric/hydrophobic interaction effects of the introduced hydrophobic sidechains.

Wools, pre-treated with branched chain aliphatic amines, generally follow the same pattern in relation to dye uptake from aqueous solutions. In Figure 6 the rate of exhaustions of CI Acid Red 57 on three wool samples, treated with different branched chain amines, are given. For comparison the exhaustion curve for n-pentylamine-treated wool is included. Here again the fibres, treated with isopentylamine, which inserts most basic groups into the fibre (Table 9), dye least readily. Further a comparison of Figure 6 with Figure 3 shows that both isopropylamine and isobutylamine treatments are more effective in enhancing dyeing than are n-propylamine and n-butylamine respectively. Yet the latter amines introduce more basic residues into the fibres (see Table 9).

The fixation values for the reactive dye Procion Red MX.2B (Figure 7) conform to a similar general pattern. It is interesting to note, however, that fixation on wools pre-treated with branched chain alkylamines are very similar to the fixation observed on wools pre-treated with the corresponding straight chain isomers. This would suggest that the introduced basic sidechains, whilst altering the exhaustion of acid and reactive dyes, do not themselves react to any extent covalently to fix the reactive dyes.

When the wools, pre-treated with branched chain alkylamines, are dyed with CI Acid Red 57 from a mixed solvent bath (1:1 n-propanol/water), the reversal in ease of dyeing, found with the

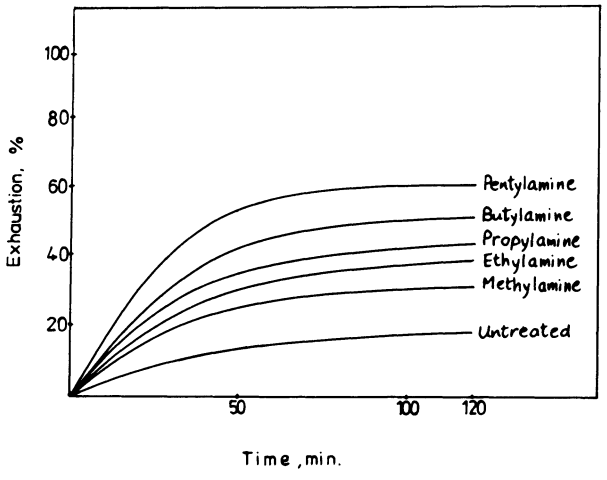


Figure 5. Exhaustion curves of C.I. Acid Red 57, from 1:1 n-propanol/water mixtures, on wools treated with straight-chain alkylamines. Temperature of dyeing 25°C.

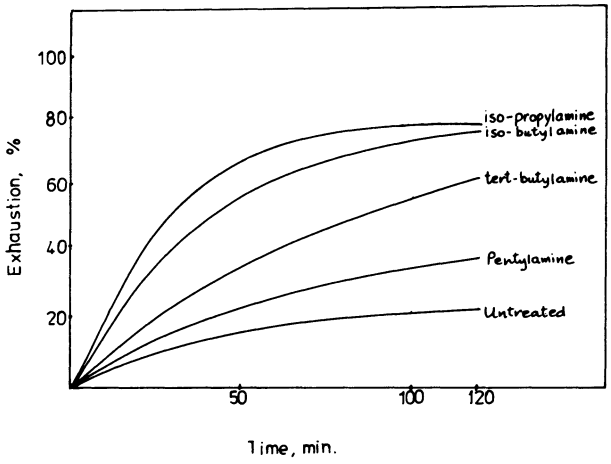


Figure 6. Exhaustion curves of C.I. Acid Red 57, from aqueous solutions, on wools treated with branched-chain alkylamines. Temperature of dyeing 25°C. (n-pentylamine-treated wool included for comparison.)

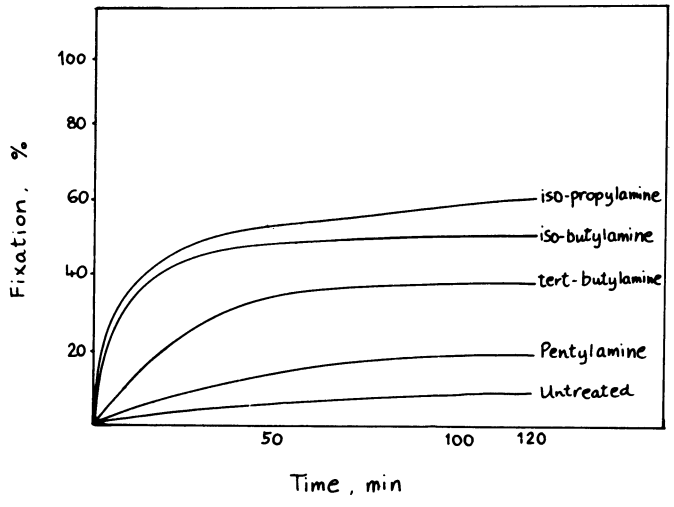


Figure 7. Fixation curves of Procion Red MX 2B, from aqueous solutions, on wools treated with branched-chain alkylamines. Temperature of dyeing 25°C (n-pentylamine-treated wool included for comparison).

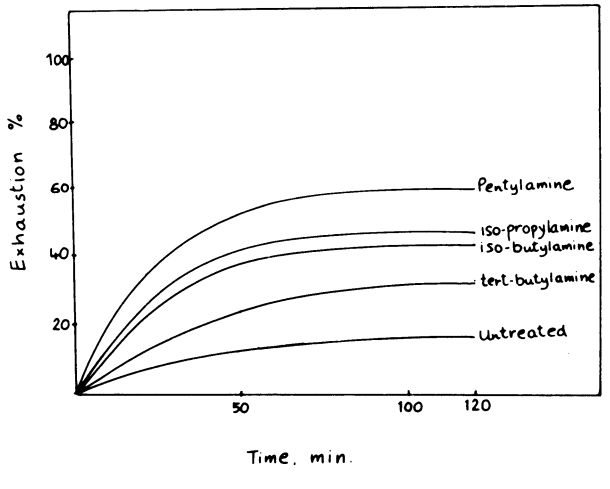


Figure 8. Exhaustion curves of C.I. Acid Red 57, from 1:1 n-propanol/water mixtures, on wools treated with branched-chain alkylamines. Temperature of dyeing 25°C (n-pentylamine treated wool included for comparison).

wools, pre-treated with straight chain alkylamines, is not observed (Figure 8). By comparison with the n-pentylamine-treated fibres, there is no change in order of dyeability. Similar findings were observed for the fixation of the reactive dye.

The reasons for this could be that n-propanol is incapable of dispersing hydrophobic interactions between the branched hydrophobic sidechains, or the steric effects of these sidechains are such as to preclude penetration of the dye molecules sufficiently closely into the fibre to give firm ionic bonding.

Overall the results show that the dyeing properties of the proteins are profoundly influenced, not only by the introduction of basic sites into the fibre, but also, and to a greater extent, by the size and shape of pendant alkyl sidechains attached to the bound basic groups. It seems reasonable to suggest that hydrophobic sidechains, introduced into proteins by dehydroalanyl additions, could equally influence such properties of the protein as enzyme digestibility and nutritional value in the case of edible proteins. It is also interesting to speculate whether, if such sidechains are introduced into the protein by other mechanisms than dehydroalanyl additions, they will affect the biochemical properties to different extents. The final part of this paper indicates that such sidechains can be introduced as addendums to the lysine ε-amino groups by simple heating reactions.

Hydrophobicity in Relation to Protein Digestibility

It must be stressed that the suggestions implied in this section are purely speculative, but the previous work reported here suggests that it would be unwise to assume that the blocking of active sites in a protein, by different alkyl-containing compounds, would result in the treated proteins having similar properties such as digestibility irrespective of the size and shapes of the alkyl groups introduced.

Sinclair (1975) observed that when chicken muscle protein (15% moisture content) was heated in sealed vessels at 121^{o} in the presence of natural fat, the ε-aminogroups of lysine therein, available for reaction with 1:fluoro-2:4 dinitrobenzene (FDNB) decreased considerably more than when the fat-free protein was similarly heated. Further, the nutritional value of the heated protein tended to fall in relation to the amounts of ε-aminogroups of lysine inactivated. Lysinoalanine was not formed and the amounts of isopeptide links formed decreased in the presence of fat, rather then increasing. The inactivation of ε-aminogroups of lysine to FDNB in the heated, fat-containing protein could not therefore be ascribed to either isopeptide link on lysinoalanine formation (see

Table 10). Similar effects were observed when fat-free chicken muscle protein was heated in the presence of methyl stearate.

It would seem possible, therefore, that in the presence of fatty acid esters an alternative condensation to the formation of isopeptide crosslinks is occurring on heating, i.e.

$$\underset{\text{protein bound lysine}}{\text{Protein}\text{-}(CH_2)NH_2} + \underset{\text{fatty acid ester}}{RCOOR_1} \xrightarrow{\text{heat}} \underset{\text{amide sidechain on the lysine residue}}{\text{Protein}\text{-}(CH_2)_4NHOCR} + R_1OH$$

Such a link would be broken on acid hydrolysis of the heated protein returning free lysine, as was found.

Subsequently keratin has been treated with solutions of free fatty acids in water or aqueous ethanol, air dried and then heated at 121° for 24 hours in open vessels. An increase in the amounts of ε-aminogroups of lysine inert to FDNB reaction is observed (Table 11).

The proportion of lysine residues affected depends in part on the fatty acid used. It seems probable that in these cases dehydration reactions are occurring, analogous to isopeptide formation, i.e.

$$\underset{\text{Lysine/fatty acid salt}}{\text{Protein}\text{-}(CH_2)_4NH_3^+ \; {}^-OOCR} \xrightarrow{\text{heat}} \text{Protein}\text{-}(CH_2)_4NHOCR + H_2O$$

As yet there is no direct evidence to support such ester formation (i.e. isolation of lysine esters from treated proteins). Such sidechains have previously been introduced into wool fibres by treatment with reactive acid chlorides or anhydrides and it has been shown that the size and shape of the sidechains has a profound effect on the physical properties of the fibre, notably in the ability of the fibres to 'set', i.e. maintain a strained configuration (Milligan and Wolfram, 1972).

The few results available lead us to speculate on whether, during the heating of edible proteins in the presence of fats, fatty acids, or fatty acid esters, such reactions occur. Further, if so, whether the differing types of hydrophobic sidechains, so introduced, will influence the digestibility of the protein to differing extents by hydrophobic interaction or steric effects. By analogy with the findings of Davies, Laird and Synge (1975), that oxidation products of δ-diphenols can couple with aminogroups of plant proteins and thus damage the nutritional availability of lysine, these reactions, though forming less stable bonds with

TABLE 10

Influence of Fatty Esters on Isopeptide Formation in Heated Chicken Muscle* (g/16 gN)

Chicken Muscle Protein	Asp-lys	Glu-lys
Purified	0.8	0.9
Containing natural fat	0.3	0.2
Containing 5% methyl stearate	0.3	0.2
Containing 15% methyl stearate	0.3	0.3

* All samples heated at 121°C for 27h

TABLE 11

Effect of Heating Wool, pre-treated with 0.7 M Fatty Acid Solutions in Water, or Alcohol/Water for 24 hrs. at Room Temperature. Time of Heating 24 hrs. (in Open Vessels at 121°)

Pre-treatment Acid	Solvent	Lysine rendered unavailable to FDNB (μ moles g^{-1})
Acetic	Water	12.0
Propionic	Water	28.0
n-Butyric	Water	32.5
n-Butyric	50:50 Ethanol/ Water	45.0
n-Octanoic	50:50 Ethanol/ Water	70.0

lysine, could have marked and different effects on the easy availability of lysine. Enzymic hydrolysis of the heated proteins could, by analogy with the dyeing studies reported, reduce enzyme penetration of the edible proteins and hence inhibit, *in vivo* hydrolysis.

CONCLUSIONS

The study of dehydroalanine additions in proteins has opened up a wide field of chemical reactions. A vast number of possible addition reactions remain to be studied. Both aromatic and aliphatic thiol derivatives can be obtained by such additions, whilst amine addition is apparently limited to those containing an aliphatic bridging group, e.g. RCH_2NH_2. Properties of proteins studied suggest that aliphatic sidechains introduced into the protein by dehydroalanyl addition or other mechanisms influence the subsequent properties considerably.

REFERENCES

Asquith, R. S., Booth, A. K. and Skinner, J. D. (1969). The formation of basic amino acids on treatment of proteins with alkali. *Biochim. Biophys. Acta*, 181, 164.

Asquith, R. S. and Carthew, P. (1972a). An investigation of the mechanism of alkaline degradation of cystine in intact protein. *Biochim. Biophys. Acta*, 278, 8.

Asquith, R. S. and Carthew, P. (1972b). Synthesis and PMR properties of some dehydroalanyl derivatives. *Tetrahedron*, 28, 4769.

Asquith, R. S. and Carthew, P. (1973). The competitive reaction of dehydroalanyl residues formed during the alkaline degradation of wool cystine. *J. Textile Inst.*, 64, 10.

Asquith, R. S. Carthew, P., Hanna, H. D. and Otterburn, M. S. (1974). The covalent reactions of primary amino sidechains and disulphide bonds in keratin, Pt. 1. *J. Soc. Dyers Colourists*, 90, 357.

Asquith, R. S. and Garcia-Dominguez, J. J. (1968). New amino acids in alkali treated wool. *J. Soc. Dyers Colourists*, 84, 155.

Asquith, R. S. and Otterburn, M. S. (1969). Basic amino acids in heated keratin. *J. Textile Inst.*, 60, 208.

Asquith, R. S. and Otterburn, M. S. (1970). Self-crosslinking in heated keratin. *J. Textile Inst.*, 61, 569.

Asquith, R. S. and Otterburn, M. S. (1971). Self-crosslinking in keratin under the influence of dry heat. *Applied Polymer Symposium*, 18, 277.

Asquith, R. S., Otterburn, M. S., Buchanan, J. H., Cole, M., Fletcher, J. C. and Gardner, K. L. (1970). The identification

of N^{ε}-(γ-L-glutamyl)-L-lysine crosslinks in native wool keratins. Biochim. Biophys. Acta, 221, 342.

Asquith, R. S. and Skinner, J. D. (1970). Modification of keratin and other related proteins by alkali. Textilveredlung, 5, 5406.

Asquith, R. S. and Speakman, J. B. (1952). Chemical mechanism of permanent set. Nature, 170, 798.

Astbury, W. T. and Woods, H. J. (1934). X-ray studies on the structure of hair, wool and related fibres, Pt. II. Phil. Trans. Roy. Soc., A, 232, 333.

Bohak, Z. (1964). N^{ε}-(DL-Z-amino-Z-carboxyethyl)-L-lysine, a new amino acid formed on alkaline treatment of proteins. J. Biol. Chem., 239, 2878.

Cavins, J. F. and Friedman, M. (1968). Specific modification of protein sulfhydral groups with α,β-unsaturated compounds. J. Biol. Chem., 243, 3357.

Davies, R, Laird, W. M. and Synge, R. L. M. (1975). Hydrogenation as an approach to study of reactions of oxidizing polyphenols with plant proteins. Phytochem., 14, 1591.

Elliott, R. L., Asquith, R. S. and Hobson, M. A. (1960). Some observations on the degradation of cystine in ethylamine solution. J. Textile Inst., 51, T692.

Friedman, M. (1973). The chemistry and biochemistry of the sulfhydryl group in amino acids, peptides and proteins. Pergamon Press, Oxford, England and Elmsford, New York. Chapter 4.

Friedman, M., Cavins, J. F. and Wall, J. S. (1965). Relative nucleophilic reactivities of amino groups and mercaptide ions with α,β-unsaturated compounds. J. Am. Chem. Soc., 87, 3572.

Garcia-Dominguez, J. J. (1968). M.Sc. Thesis, University of Bradford (U.K.).

Garcia-Dominguez, J. J., Miro, P. and Fuentes, A. A. (1974). Reaction of wool with alkylamines; possible technique for producing differential-dyeing wool. J. Soc. Dyers Colourists, 90, 105.

Horn, M. J., Jones, D. B. and Ringel, S. J. (1941). Isolation of a new sulfur-containing amino acid (lanthionine) from sodium carbonate-treated wool. J. Biol. Chem., 138, 141.

Hurrell, R. F., Carpenter, K. J., Sinclair, W. J., Otterburn, M. S. and Asquith, R. S. (1976). Mechanisms of heat damage in proteins, Pt. 7: The significance of lysine containing isopeptides and of lanthionine in heated proteins. Brit. J. Nutr., 35, 383.

McLaughlin, C., Heighberger, J. and Moore, W. M. (1928). J. Amer. Leather Chemists Assoc., 23, 318.

Mellet, P. and Louw, D.F. (1965). The modification of silk fibroin by alkali. Chemical Communications, 17, 396.

Milligan, B. and Wolfram, L. J. (1972). The setting of wool. Enhancement by introduction of bulky groups. J. Textile Inst., 63, 515.

Moore, W. M. and Koppenhoefer, R. L. (1933). J. Amer. Leather Chemists Assoc., 28, 245.

Patchornik, A. and Sokolovsky, M. (1964). Chemical interactions between lysine and dehydroalanine in modified bovine pancreatic ribonuclease. J. Amer. Chem. Soc., 86, 1860.

Robson, A. and Zaida, Z. H. (1967). The formation of lysinoalanine during the treatment of silk fibroin with alkali. J. Text. Inst. Trans., 58, 267.

Sinclair, W. J. (1975). Ph.D. Thesis, The Queen's University of Belfast.

Speakman, J. B. (1936). The reactivity of the sulphur linkage in animal fibres. Pt. I: The chemical mechanism of permanent set. J. Soc. Dyers Colourists, 52, 335.

Synge, R. L. M. (1975). Interactions of polyphenols with proteins in plants and plant products. Qualitas Plantarum, 377, 24.

Synge, R. L. M. (1976). Damage to nutritional value of plant proteins by chemical reactions during storage and processing. Qualitas Plantarum. (In press).

Ziegler, K. (1964). New cross-links in alkali-treated wool. J. Biol. Chem., 239, P.C. 2713.

Ziegler, K. (1967). N-δ-(2-amino-2-carboxyethyl)-ornithine, a new amino-acid from alkali-treated proteins. Nature, 214, 404.

8

NEW AMINO ACID DERIVATIVES FORMED BY ALKALINE TREATMENT OF PROTEINS

John W. Finley and Mendel Friedman

Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California 94710

ABSTRACT

Intense heat treatment of proteins at high pH favors formation of transient, reactive intermediates derived from serine and cystine. The postulated dehydroalanine intermediate reacts further with the ε-amino groups side chains and sulfhydryl groups of cysteine residues to form derivatives of lysinoalanine and lanthionine. Recent studies indicate that besides these crosslinked products, several others are formed, by reaction of histidine, arginine, and possibly other residues. For this reason, caution should be exercised in assigning the prelysine peak(s) of an amino acid chromatogram to lysinoalanine. These results suggest that unnatural amino acid derivatives, other than lysinoalanine, may also contribute to the toxic effects reported from studies in which alkali-treated protein was fed to rats.

INTRODUCTION

Alkaline treatment of foods and food proteins has been used since ancient times. For example, Central American Indians have treated corn with alkali for many generations (Katz et al., 1974). This treatment, by making the vitamin niacin more nutritionally available, may be important in controlling pellagra in these areas. Advanced technology has made alkaline treatment of food proteins, as in texturizing soy protein, even more widely used.

Although such treatments commonly impart desirable textural and functional properties, feeding alkali-treated proteins appears to induce kidney damage in rats (Woodard et al., 1975; Gould and MacGregor, 1977). These treatments differ, in part, from the liming of corn by being more severe. In addition, they are not

carried out on intact plant cells as is the case with corn.

Sternberg and associates (Sternberg et al., 1977; Sternberg and Kim, 1975) found that the unnatural amino acid lysinoalanine is present in many food products heated under alkaline conditions. Their work suggests that the amount of lysinoalanine present in foods may be an excellent index of overprocessing. We found that lysinoalanine formation can be largely prevented when isolates of soy and casein are exposed to alkali by including mercapto amino acids such as cysteine (Finley et al., 1977).

In the course of these studies, we observed several unknown peaks in amino acid chromatograms of alkali-treated proteins. In this paper, we report preliminary attempts to characterize these peaks.

EXPERIMENTAL

Alkaline treatments were carried out by suspending 10 g of soy isolate (Promine D, Central Soya, Chicago, Illinois) in 100 ml of 0.01N NaOH and heating for 8 hr in a water bath at 65°C. Lysinoalanine was prepared by treating the copper complex of lysine and α-alanine with N-acetyldehydroalanine methyl ester, then with H_2S to remove the copper, and finally chromatography with 4N HCl on a Dowex 50W X 8 ion-exchange resin (Fig. 1). In a similar manner the copper complexes of the following amino acids were treated with N-acetyldehydroalanine methyl ester: arginine, histidine, ornithine, tryptophan, and kynurenine. Similarly, 100 mg of each of polylysine, polyarginine, polyhistidine, polyornithine, and polytryptophan (Sigma) were treated with a ten-fold excess of N-acetyldehydroalanine methyl ester at pH 10 in a 50% aqueous-ethanol-triethylamine buffer for 48 hr, dialyzed, and hydrolyzed at 110°C for 20 hr in 6N HCl. Amino acid analysis was carried out according to Spackman (1963) on a Beckman 120 amino acid analyzer.

RESULTS AND DISCUSSION

Intense alkali treatment of proteins is believed to form transient dehydroalanine residues that can react with the ε-amino group of lysine, the δ-amino group of ornithine, the imidazole group of histidine, and the guanidino group of arginine to form crosslinked amino acids, as illustrated in Figure 2. In studies of the extent of lysinoalanine formation in various food products under alkaline conditions, we noted the presence of several unknown peaks on chromatograms of amino acid hydrolysates. These peaks were especially pronounced with alkali-treated soy protein (Figure 3). For the analysis shown, about five times the usual sample was applied to enhance the peaks.

We speculate that the unknown products are due to adding active-hydrogen-bearing amino acid residues to the double bond of dehydroalanine residues derived from cystine and serine residues, as illustrated in Figure 2.

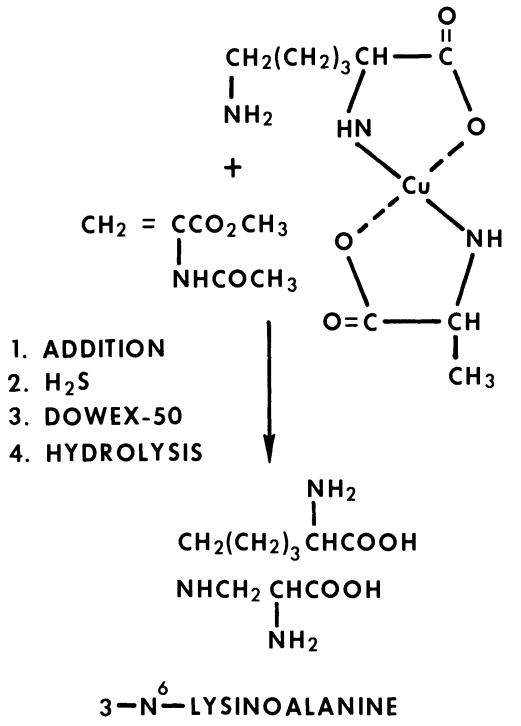


Fig. 1. Reaction of the copper complex of lysine and α-alanine with N-acetyldehydroalanine methyl ester followed by removal of copper with H_2S, purification of the reaction mixture in 4N HCl on a Dowex 50 X 8 ion-exchange column, and hydrolysis to liberate lysinoalanine.

Table 1 compares the elution times of the new peaks with those observed when copper complexes of selected amino acids or polyamino acids are treated with N-acetyldehydroalanine methyl ester. Striking correspondences for several derivatives suggest that the new peaks may be derived from arginine, histidine, and possibly tryptophan, in addition to lysinoalanine, already known.

Ornithine is eluted almost in the same position as lysine. In addition, the peak due ornithinoalanine is very close to that for lysinoalanine (they appear as a double peak). For this reason, it is quite possible that reported analyses for lysinoalanine may include measurable amounts of ornithinoalanine, which may be derived from alkaline decomposition of arginine to ornithine. Current work is directed at unequivocal identification of the postulated amino acid derivatives as well as factors leading to their formation.

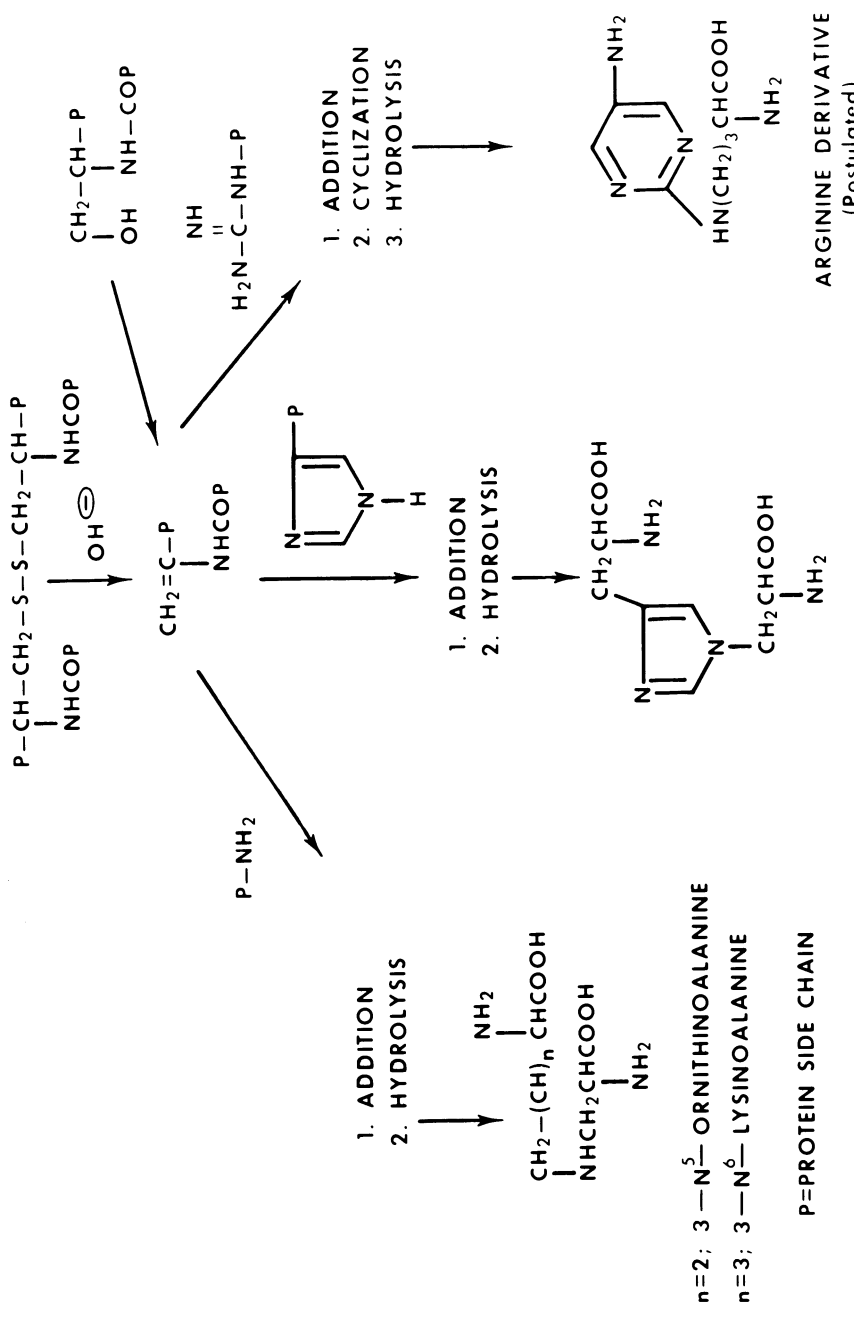


Fig. 2. Postulated formation of crosslinked amino acios.

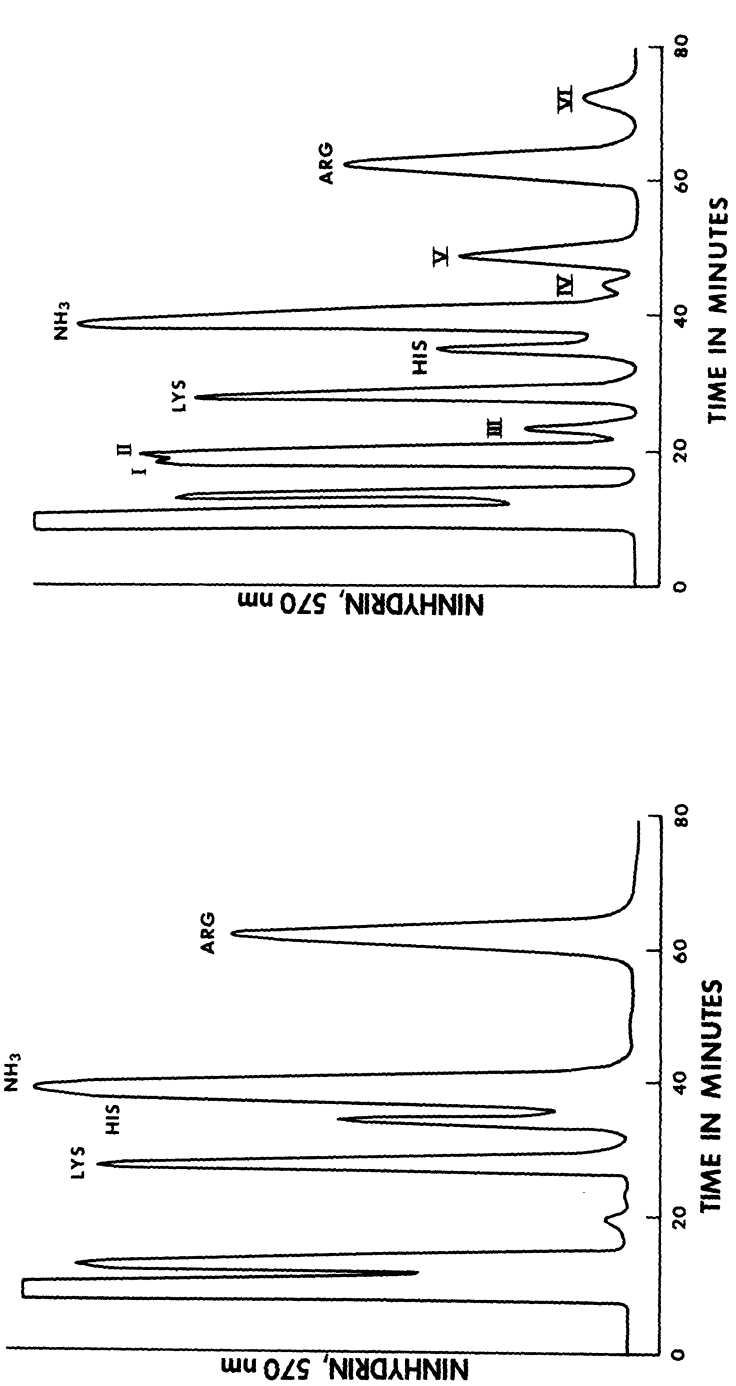


Fig. 3a. Short (basic) column amino acid chromatogram (Spackman, 1963) of a soy protein hydrolysate (untreated).

Fig. 3b. Short (basic) column amino acid chromatogram (Spackman, 1963) of alkali-treated soy protein hydrolysate. Note new peaks. Five times the usual sample size was applied to the column. See text.

Table 1

Elution Times of Dehydroalanine Derivatives of Amino[a] and Polyamino Acids[b] in Amino Acid Chromatography[c]

Amino Acid Derived From	Elution Time
Lysine	23
Polylysine	23
Ornithine	22
Polyornithine	22
Arginine	44
Polyarginine	22
Histidine	18.5; 23, 41
Polyhistidine	41
Tryptophan	36
Polytryptophan	33
Kynurenine	33
UNTREATED STANDARDS:	
Ornithine	28
Lysine	31
Histidine	35
NH_3	43
Arginine	65

[a] Copper complexes of the amino acid, additionally chelated with -alanine, were treated with a ten-fold excess of N-acetyldehydroalanine methyl ester, the copper removed with H_2S, and the mixture purified on a Dowex 50 X 8 column (4N HCl).

[b] Polyamino acids were treated with a ten-fold excess of dehydroalanine methyl ester and worked up as described in the Experimental Section.

[c] The following unknown peaks were observed on a chromatogram of hydrolyzed alkali-treated soy protein (elution times in minutes): 18 (double-peak), 22, 23, 43, 48, 73 (Cf. also, Fig. 3a).

Ornithine is eluted almost in the same position as lysine. In addition, the peak due ornithinoalanine is very close to that for lysinoalanine (they appear as a double peak). For this reason, it is quite possible that reported analyses for lysinoalanine may include measurable amounts of ornithinoalanine, which may be derived

from alkaline decomposition of arginine to ornithine. Current work is directed at unequivocal identification of the postulated amino acid derivatives as well as factors leading to their formation.

Although tryptophan side chains would not be expected to react with the double bond of dehydroalanine, certain oxidizing processing conditions could transform tryptophan to kynurenine (Finley and Friedman, 1973), the aromatic amino group of which could interact with the double bond of dehydroalanine. In addition, dehydroalanine residues liberate pyruvic acid under hydrolytic conditions (Morino and Snell, 1971). The pyruvic acid could, in principle, react with the α-carbon atoms of two tryptophan side chains by a mechanism analogous to that given for reaction of tryptophan with glyoxalic acid to give an analogous product (Friedman and Finley, 1975).

CONCLUDING REMARKS

Several new amino acids besides lysinoalanine appear to be formed when soy protein is treated with base at 65°C. These amino acids appear to be derived from histidine, arginine, and possibly tryptophan. Although these amino acid derivatives occur in smaller amounts than lysinoalanine, our results suggest that caution should be exercised in identifying peaks that appear before lysine chromatograms because several other amino acid derivatives from histidine, arginine, ornithine, and tryptophan also appear to elute near this position, so that they may be confused.

Although the nutritional and pharmacological consequences of these amino acid derivatives are not yet known, the observed non-correlation between lysinoalanine contents of several different proteins and their nephrotoxic effects in rat feeding tests (Gould and MacGregor, 1977), may very well result from contributions of other crosslinked amino acids to the effects. The extent of such contributions may vary from protein to protein. These and related aspects of this problem remain to be studied.

REFERENCES

Finley, J. W. and Friedman, M. (1973). New sweetening agents: N-formyl and N-acetylkynurenine. J. Agr. Food Chem., 33-34.

Finley, J. W., Snow, J. T., Johnston, P. H., and Friedman, M. (1977). Inhibitory effect of mercaptoamino acids on lysinoalanine formation during alkali-treatment of proteins. This volume.

Friedman, M. and Finley, J. W. (1975c). Evaluation of methods for tryptophan analysis in proteins. In "Protein Nutritional Quality of Foods and Feeds," Part I, M. Friedman (Ed.), Dekker, New York, 423-452.

Gould, D. G. and MacGregor, J. T. (1977). Nutritional and biological consequences of protein crosslinking: an overview. This volume.

Katz, S. H., Hediger, M. L., and Valleroy, L. A. (1974). Traditional maize processing techniques in the New World. Science, 184, 765-773.

Sternberg, M. and Kim, C. Y. (1977). Lysinoalanine formation in protein food ingredients. This volume.

Sternberg, M., Kim, C. Y., and Schwende, F. J. (1975). Lysinoalanine: presence in foods and food ingredients. Science, 190, 992-994.

Woodard, J. C., Short, D. D., Alvarez, M. R., and Reyniers, J. P. (1975). Biological effects of Nε-(DL-2-amino-2-carboxyethyl)-L-lysine, lysinoalanine. In "Protein Nutritional Quality of Foods and Feeds," Part 2, M. Friedman (Ed.), Marcel Dekker, Inc., New York, pp. 595-618.

9

α,β-Unsaturated and Related Amino Acids in Peptides and Proteins

Erhard Gross

National Institutes of Health

Bethesda, Maryland 20014

ABSTRACT

α,β-Unsaturated amino acids are potential precursors for the formation of crosslinkages in peptides and proteins. DEHYDROALANINE and DEHYDROBUTYRINE are constituents of NISIN (from Streptococcus lactis) and SUBTILIN (from Bacillus subtilis). Both peptides are crosslinked via sulfide bridges of no fewer than one residue of lanthionine and four residues of β-methyllanthionine presumably formed by the addition of the sulfhydryl group of cysteine residues across the double bond of dehydroalanine and dehydrobutyrine, respectively. CINNAMYCIN (from Streptomyces cinnamoneus) and DURAMYCIN (from Streptomyces cinnamoneus forma azacoluta) also display the crosslinking features of lanthionine and β-methyllanthionine. The reactive double bond of α,β-unsaturated amino acids is no longer seen in these two peptides. The presence of LYSINOALANINE in cinnamycin and duramycin establishes the imino bridge as a novel type of naturally occurring crosslinkage. The formation of the imino bridge is attributed to the addition of the ε-amino group of a lysine residue across the α,β-unsaturation of dehydroalanine, a reaction that takes place in nisin, nisin fragments, and subtilin under controlled alkaline conditions. The crypticity of α,β-unsaturated amino acids constitutes a continued impediment to their easy analytical detection. A more broadly based role for α,β-unsaturated amino acids in the physiological environment must not be ruled out at the present time.

INTRODUCTION

The double bond in α,β-unsaturated amino acids (Figure 1) is activated by the adjacent amide carbonyl group and thus the ready

subject of β-addition reactions by nucleophiles, such as mercaptans and amines. In peptides and proteins adequately nucleophilic agents of the type indicated are most commonly provided by the sulfhydryl group of cysteine and the ε-amino group of lysine. With the products of the addition reactions crosslinkages are introduced in the form of sulfide (Figure 2; X=S) and imino (Figure 2; X=NH) bridges.

The α,β-unsaturated amino acids DEHYDROALANINE (Figure 1; R=H) and DEHYDROBUTYRINE (Figure 1; R=CH_3) are well established constituents of the intensely crosslinked polypeptides NISIN (Gross and Morell, 1968) and SUBTILIN (Gross and Kiltz, 1973).In both cases no fewer than five crosslinkages are manifest in the presence of the thioether amino acids LANTHIONINE (Figure 3; R=H) and β-METHYL-LANTHIONINE (Figure 3; R=CH_3).

That α,β-unsaturated amino acids are vehicles of crosslinking in their own right, became evident when the molecular size of nisin had to be clarified (Gross and Morell, 1967). Employing dehydroalanine, this crosslinking potential has been successfully applied to the solid phase synthesis of biologically important peptide amides (Gross et al., 1973). In either case there is at stake the reversible reaction between α,β-unsaturated amino acids on the one hand and amides and keto acids on the other (Figure 4).

CINNAMYCIN and DURAMYCIN

Cinnamycin (Benedict et al., 1952) was isolated from Streptomyces cinnamoneus, duramycin (Shotwell et al., 1958) from Streptomyces cinnamoneus forma azacoluta. Early analytical investigations established the presence of lanthionine and β-methyllanthionine (Figure 3) in cinnamycin (Dvonch et al., 1954) as well as in duramycin (Shotwell et al., 1958). The extension of the studies on nisin (Gross and Morell, 1971) to subtilin (Gross et al., 1973) had shown that both peptides contain the α,β-unsaturated amino acids dehydroalanine and dehydrobutyrine in addition to the closely related residues of lanthionine and β-methyllanthionine. With the initial access to small samples of cinnamycin and duramycin, it was feasible to explore the possible presence of α,β-unsaturated amino acids in these lanthionine and β-methyllanthionine containing peptides. The results of this investigation were negative. The reactive α,β-unsaturation of dehydroalanine and dehydrobutyrine (Figure 1) is no longer seen in cinnamycin or duramycin. However, the amino acid analyses of both peptides revealed immediately the presence of two infrequently observed amino acids one of which had heretofore not been found to occur naturally. In this latter amino acid nature demonstrates another way of "masking" α,β-unsaturated amino acids and introducing crosslinkages. While lanthionine and β-methyllanthionine are postulated to be the products of the

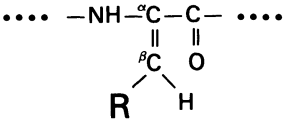


Fig. 1. The structure of α,β-unsaturated amino acids (R = H: dehydroalanine; R = CH_3: dehydrobutyrine).

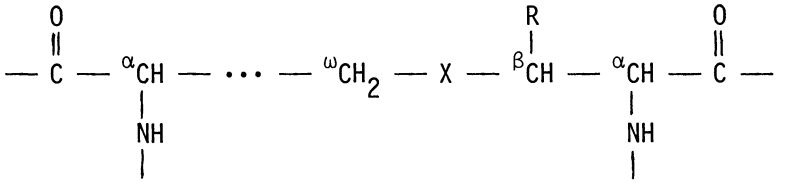


Fig. 2. Crosslinkages resulting from the β-addition of nucleophiles to α,β-unsaturated amino acids (R = substituent at the β-carbon atom of the α,β-unsaturated amino acid; X = S or NH).

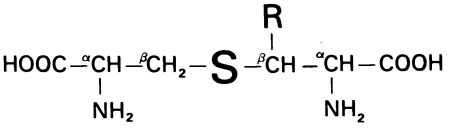


Fig. 3. The structure of the thioether amino acids lanthionine (R = H) and β-methyl-lanthionine (R = CH_3).

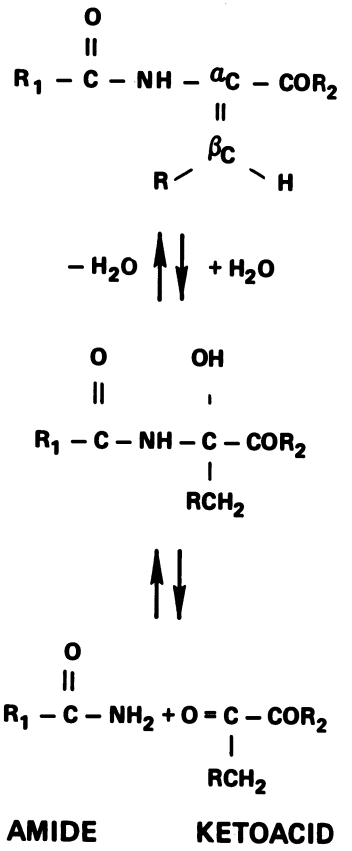


Fig. 4. The reversible reaction between α,β-unsaturated amino acids and amides and keto acids.

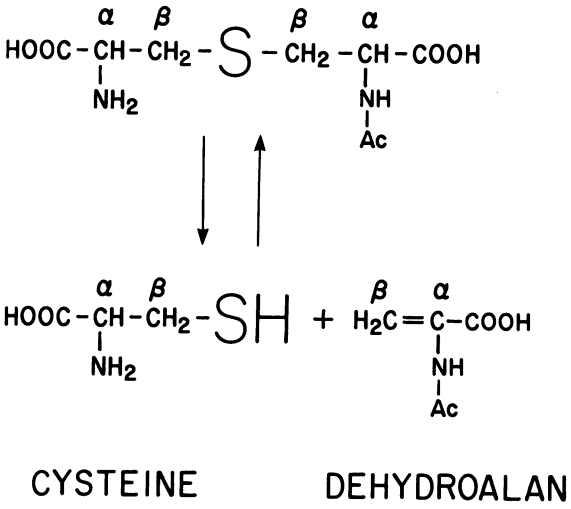


Fig. 5. The addition of cysteine to dehydroalanine with the formation of lanthionine. Ac = acyl or amino acyl; dehydroalanine with the free amino group would readily decompose in the presence of water to give ammonia and pyruvic acid.

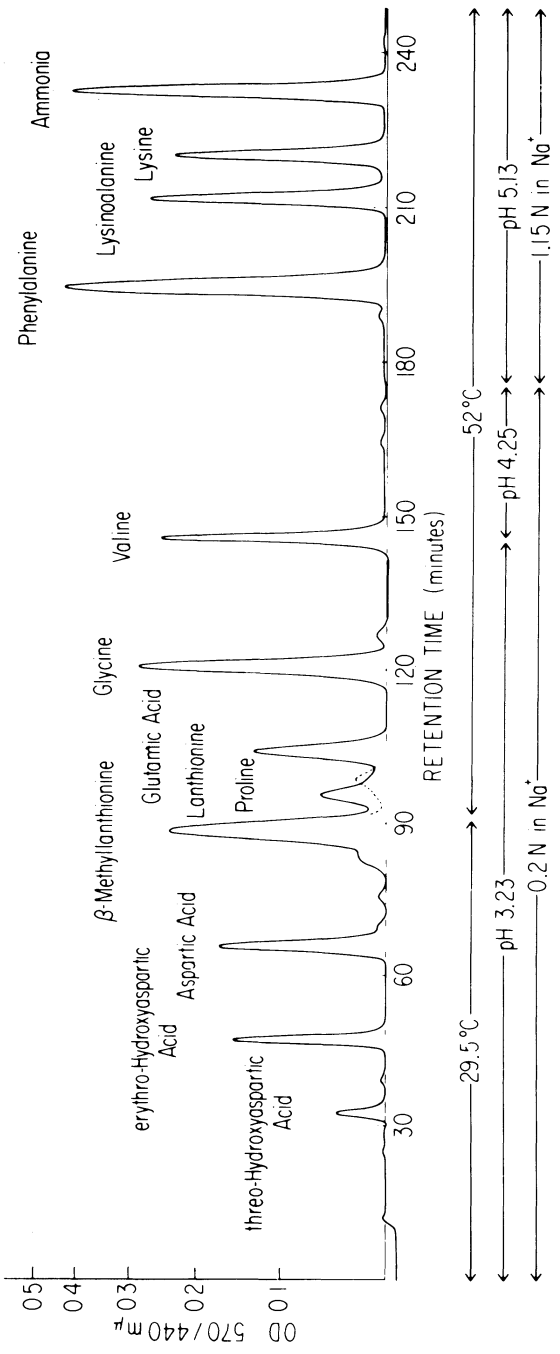


Fig. 6. Chromatogram of hydrolysate of duramycin. Elution conditions are given in the abscissa. Effluent volumes for threo-hydroxyaspartic acid, 32 ml; erythro-hydroxyaspartic acid, 46 ml; lysinoalanine, 212 ml.

addition of cysteine across the double bond of dehydroalanine (Figure 5) and dehydrobutyrine, respectively, for the newly discovered amino acid it soon became evident that the ε-amino group of a lysine residue had had to have been added to dehydroalanine.

A. Lysinoalanine. In the chromatogram (Figure 6) of the amino acid analysis of purified duramycin three peaks appeared in positions (effluent volumes 32, 46, and 212 ml) where in the hydrolysates of nisin and subtilin (cf. for instance, Gross et al., 1973) amino acids are not present. The peaks at effluent volumes 32 and 46 ml were found to correspond to the diastereoisomers of HYDROXYASPARTIC ACID (comparison with authentic samples; Calbiochem, San Diego, California, USA).

Hydroxyaspartic acid will not be discussed in greater detail in this context. May it, however, be mentioned that crosslinking potential is also intrinsic to this amino acid. Until aspects of the biosynthesis of hydroxyaspartic acid have been clarified, its formation from glycine and glyoxalic acid remains a possibility (vide supra, the crosslinking features claimed for α,β-unsaturated amino acids as the consequence of the reaction between amides and keto acids, there followed by the loss of water).

The amino acid eluting at the effluent volume of 212 ml is LYSINOALANINE, previously only found once in the hydrolysate of a naturally occurring peptide, namely that of cinnamycin (cf. the amino acid composition given in Table I). The identity of lysinoalanine in cinnamycin had been established (a) by comparison with an authentic sample synthesized by Okuda and Zahn (1965) from $^{\alpha}$N-acetyl-L-lysine and ethyl-α-acetaminoacrylate (acetyl dehydroalanine ethyl ester); (b) by oxidizing lysinoalanine from cinnamycin hydrolysates with periodate for which reaction it was determined that lysine, ammonia and formaldehyde had been formed at ratios of 1:1:1.

Cinnamycin and duramycin do not only have in common the presence of the novel crosslinking feature seen in lysinoalanine. With one exception all other amino acids in their compositions are also identical. The amino acid exchange observed is between LYSINE (in duramycin) and ARGININE (in cinnamycin).

Until the detection of lysinoalanine was made in cinnamycin and duramycin, the amino acid was known to be formed in proteins with a history of alkaline treatment. BOHAK (1964) isolated and characterized lysinoalanine from bovine pancreatic ribonuclease A that had been exposed to 0.2N NaOH at 40°C for two hours. While Bohak (1964) observed lysinoalanine formation also for alkali-treated lysozyme, papain, chymotrypsin, bovine plasma albumin, and phosvitin, it did not take place in pepsin (containing one lysine

Table I. The Amino Acid Composition of Cinnamycin

Amino Acid	moles/mole	Residues/mole
erythro-HYDROXYASPARTIC ACID	0.88	1
ASPARTIC ACID	1.01	1
β-METHYLLANTHIONINE	1.65	2
LANTHIONINE	1.06	1
PROLINE	1.05	1
GLUTAMIC ACID	1.03	1
GLYCINE	2.05	2
VALINE	1.00	1
PHENYLALANINE	2.88	3
LYSINOALANINE	1.03	1
AMMONIA	3.04	(3)
ARGININE	0.93	1
Total number of amino acids:		**15**

residue only) and pepsinogen. A few other early examples of lysinoalanine formation in alkali-exposed proteins are: S-dinitrophenylated reduced bovine pancreatic ribonuclease (Patchornik and Sokolovsky, 1964); wool (Ziegler, 1964); bovine serum albumin (Zahn and Lumper, 1968).

Since the time of these early encounters with lysinoalanine there has been a steady increase in the interest in this amino acid. Apart from its recently established natural occurrence (vide supra), lysinoalanine is the practically ever present constitutent of proteins that were exposed to heat and/or processed under alkaline conditions with the intention of addition to food products for human consumption. This, quite naturally, brought to the fore concern about possible adverse physiological effects that might be associated with the presence of lysinoalanine in the daily dietary intake of man. The studies that have been conducted to establish, for instance, the digestability, nutritive value, and toxic properties of proteins with lysinoalanine shall not be the subject of further discussion here. They will be dealt with more competently by other contributors to this volume.

It is important, however, to emphasize that lysinoalanine is a substance fairly ubiquitous when it comes to man's - not only present day - culinary preferences. The spectrum ranges from egg white (boiled for three minutes) to the commercial sample of a modern whipping agent with lysinoalanine contents of 140 and 50,000 micrograms per gram of protein, respectively (Sternberg et al., 1975). Did man adapt to, induce, and develop a system of enzymes that knows how to metabolize lysinoalanine appropriately?

In a way the situation is reminiscent of nisin (vide infra) one of peptides with α,β-unsaturated amino acids to which are attributed its - at times - not harmless physiological effects. One belief has it that man indeed underwent a process of adaptation over time by continuously consuming dairy products. Nisin is produced by Streptococcus lactis. One aspect not to be ignored is the route of administration. None of the physiological effects, known to be triggered by nisin, is seen subsequent to oral application.

A discussion of lysinoalanine must not be closed without paying attention to ORNITHINOALANINE. Ornithine is not a constitutent of proteins and rarely found to occur naturally. If at all, it is seen in peptides of microbial origin. However, alkaline conditions set the stage for the conversion of arginine to ornithine and for alkali-treated sericin (Ziegler et al., 1967) the observation was made that crosslinkages are present on the account of ornithinoalanine formation.

B. Structural Elucidation. The heavily crosslinked state of cinnamycin and duramycin - three sulfide and one imino bridge - does not bode well for structural studies. Both molecules contain one residue each of either arginine (cinnamycin; cf. Table I) or lysine (duramycin; vide supra), yet trypsin does not cleave these lysyl and arginyl peptide bonds, not even under the forced conditions that were successful in the case of subtilin (Kiltz and Gross, 1973). Likewise, the three phenylalanyl peptide bonds in either of the two peptides resist cleavage by chymotrypsin.

THERMOLYSIN is the only protease that, thus far, provided fragments useful to some extent for structural studies on cinnamycin and duramycin. The application of thermolysin to the cleavage of peptide bonds in subtilin (Kiltz and Gross, 1973) had been more rewarding in that it differentiated at room temperature and 60°C between the aminoacyl bonds of leucine and phenylalanine residues on the one hand and that of a valine residue on the other. The conclusion may be justified that cinnamycin and duramycin are more compact molecules than nisin or subtilin.

Advantage was taken in the case of nisin (Gross and Morell, 1970) of the applicability of the cyanogen bromide reaction (Gross, 1967). The fragments secured proved useful in structural studies and two of them display the biological properties of the parent molecule.

For cinnamycin and duramycin there are a few options for the direct application of fragmentation techniques of the nonenzymatic order. One that was applied successfully is the treatment with dilute acid.

Aspartic acid, a constituent of cinnamycin and duramycin, is known to be released from peptides and proteins upon exposure to weakly acidic conditions at elevated temperatures (Schultz, 1967). Expecting that hydroxyaspartic acid will also be susceptible to this reaction, duramycin was treated for up to 54 hours at 110°C with 0.03 N hydrochloric acid. Aspartic and hydroxyaspartic acid were released in integral numbers together with glycine (Figure 7). The liberation of one residue of glycine speaks in favor of its placement between aspartic and hydroxyaspartic acid.

The compound represented by the peak at effluent volume 39 ml (Figure 7) is still the subject of conclusive identification. It is likely to be derived from hydroxyaspartic acid. [cf. its absence in the total hydrolysate of duramycin (Figure 6)]. Dehydroaspartic acid is a strong suspect, possibly formed in an α,β-elimination reaction in the course of which water is lost from dehydroxyaspartic acid (vide supra, the reaction proposed for the formation of hydroxyaspartic acid from glycine and glyoxalic acid).

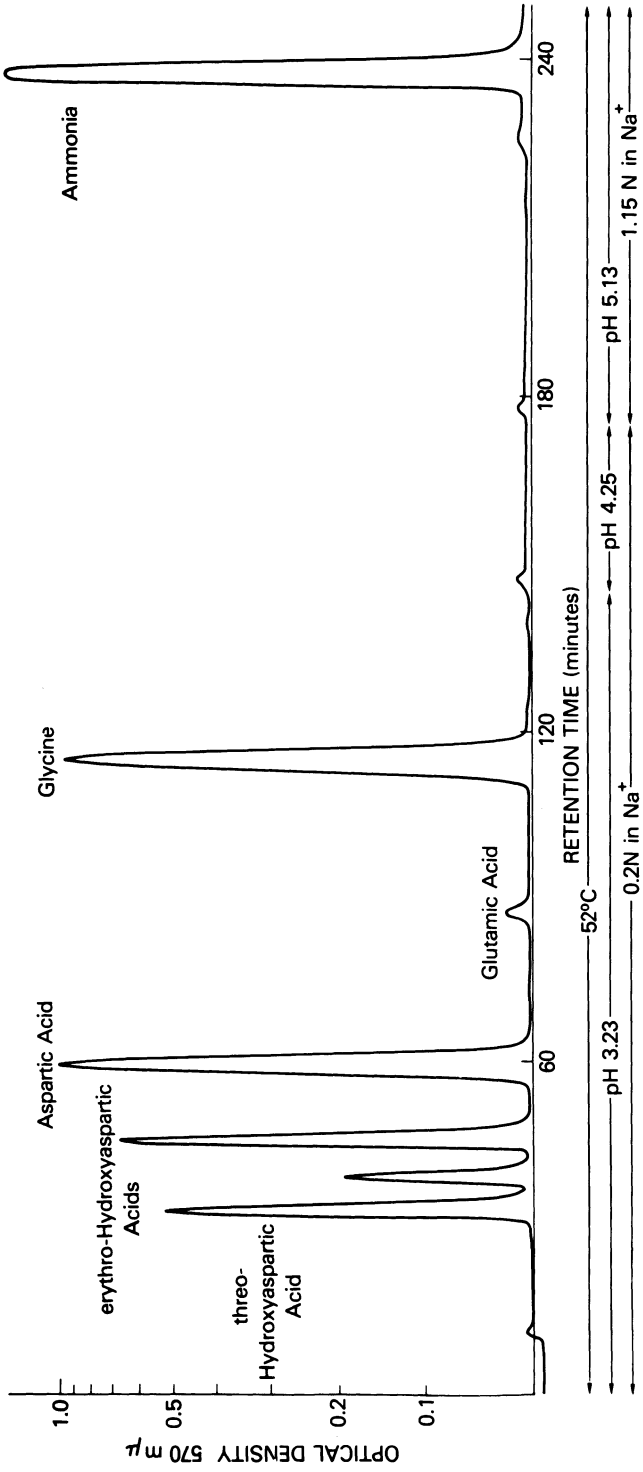


Fig. 7. Amino acids released from duramycin upon treatment with 0.03 N hydrochloric acid at 110°C for 54 hours. Hydroxyaspartic acid (threo- plus erythro-form), 0.965 μmoles; aspartic acid, 0.932 μmoles; glutamic acid, 0.019 μmoles; glycine, 0.999 μmoles. The identity of the ninhydrin-positive compound at the effluent volume of 39 ml remains to be established (cf. text!).

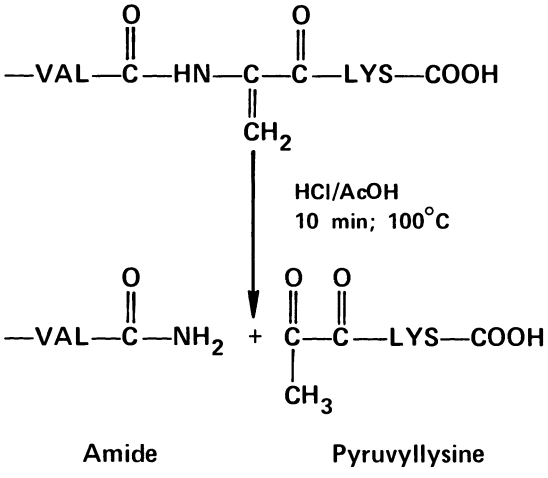


Fig. 8. The conversion of α,β-unsaturated amino acids to amide and keto acid. Dehydroalanine in the penultimate position of nisin; formation of -valyl amide and pyruvyllysine.

It must not be disturbing that 0.019 μmoles of glutamic acid are released (Figure 7) from duramycin upon treatment with 0.03 N hydrocloric acid. This is merely the result of a 1.6-type neighboring group interaction which proceeds more slowly than those of the 1.5-type in aspartic and hydroxyaspartic acid. For the practical purpose of securing additional fragments of cinnamycin and duramycin, it may become desirable to enhance the rate of release of glutamic acid.

The studies discussed for duramycin provided partial sequences only. Further information is derived from stepwise degradations done on (a) the parent molecules, (b) performic acid oxidized duramycin to which bisulfite was added, (c) desulfurized duramycin. The data combined entered into this working hypothesis for the primary structure of duramycin: H_2N-ALA(1)-LYS-GLU-ALA(4)-ALA(5)-ABA(6; aminobutyric acid)-PHE-GLY-PRO-PHE-ALA(11)-PHE-VAL-ALA(14)-HO-ASP-GLY-ASP-ABA(18)-LYS(19)-COOH. Crosslinking bonds are tentatively assigned in this way: sulfide bridges, lanthionine between residues 4 and 14, β-methyllanthionine between residues 1 and 6, and 11 and 18. The imino bridge of lysinoalanine links residues 5 and 19. The placements given for the crosslinking features impart upon duramycin the structure of a heterodetic pentacyclic peptide. Against the background information on hand and with only one amino acid exchange known for the two peptides, it stands to reason that the structure of cinnamycin differs from that of duramycin only to the extent that a residue of arginine takes the place of one of lysine.

NISIN and SUBTILIN.

A. Structure. It was the interest in the chemistry of thioether amino acids that started the early investigation of nisin. The cyanogen bromide reaction (Gross, 1966) was to be extended to lanthionine and β-methyllanthionine. It was the fortunate pursuit of a minor "contaminant" in commercial preparations of nisin and the isolation and structural determination of PYRUVYLLYSINE (Figure 8) that led to the detection of the α,β-unsaturated amino acids in this peptide. Pyruvyllysine had been formed after the addition of water to the double bond of a residue of DEHYDROALANINE (Gross and Morell, 1967) in the penultimate position of nisin. The breakdown products were to be an amide and a ketoacyl amino acid (pyruvyllysine, Figure 8). Mercaptan addition reactions on purified nisin and nisin fragments from cyanogen bromide cleavage eventually established the presence of two residues of dehydroalanine and one residue of DEHYDROBUTYRINE (Gross and Morell, 1968).

The structural elucidation of nisin required the development of new analytical methods too numerous to account for all of them here. The assignment of the sulfide bridges of lanthionine and

β-methyllanthionine had to be faced for the first time. Since next to lysinoalanine, lanthionines are observed in alkali-treated proteins, it may be of interest to briefly delineate chemical steps for the characterization of their sulfide bridges: oxidation with performic acid gives the sulfone; in the presence of bisulfite and at elevated temperature β-elimination takes place and bisulfite is added to the unsaturations generated; sulfinic acid groups remaining are newly exposed to performic acid and oxidized to sulfonic acids (Gross and Morell, 1970). At this level of the highest state of oxidation of sulfur, it is safe to proceed with subsequent steps of characterization, such as stepwise degradation. It should not be overlooked that, under the conditions specified, all sulfur functions in a protein will be oxidized. The products from the oxidation of cysteine or cystine will be the same as those from the oxidation of lanthionine followed by bisulfite addition and renewed oxidation: CYSTEIC ACID.

NISIN (Figure 9) is a heterodetic pentacyclic peptide (Gross and Morell, 1971). The five crosslinking bonds are the sulfide bridges of one residue of lanthionine and four residues of β-methyllanthionine. Five of the amino acids that joined in the crosslinkages show the infrequently seen D-configuration at their α-carbon atoms. These five amino acids occupy positions 3, 8, 13, 23, and 25 (Figure 9). They are considered to have been α,β-unsaturated amino acids - DEHYDROALANINE and DEHYDROBUTYRINE - in a precursor state to which the sulfhydryl groups of cysteine residues were added in stereospecific manner. The question about the origin of the dehydroalanine and dehydrobutyrine residues is best answered in the overall concept proposed for the biosynthesis of nisin. It is assumed that nisin is derived from a ribosomally synthesized precursor of higher molecular weight. This PRONISIN undergoes postsynthetic modification consisting of β-elimination reactions on suitably substituted, likely β-hydroxy amino acids (serine or threonine) followed by the stereospecific addition reactions of cysteine sulfhydryl groups. Finally the modified precursor molecule is fragmented by the action of proteolytic enzymes. There are no answers as yet to the questions about the remaining three intact α,β-unsaturated amino acids and their distribution.

With the knowledge of the presence of lanthionine and β-methyllanthionine residues in subtilin (Alderton, 1953), the peptide was investigated for the possible occurrence of α,β-unsaturated amino acids. Application of the reaction conditions that released pyruvyllysine from nisin (vide supra) liberated the ketoacyl amino acid also from subtilin (Gross et al., 1969), thus establishing immediately the carboxyl terminal sequence dehydroalanyllysine to be identical with the two terminating residues of nisin. An additional residue of dehydroalanine and one of dehydrobutyrine were recognized subsequent to mercaptan addition

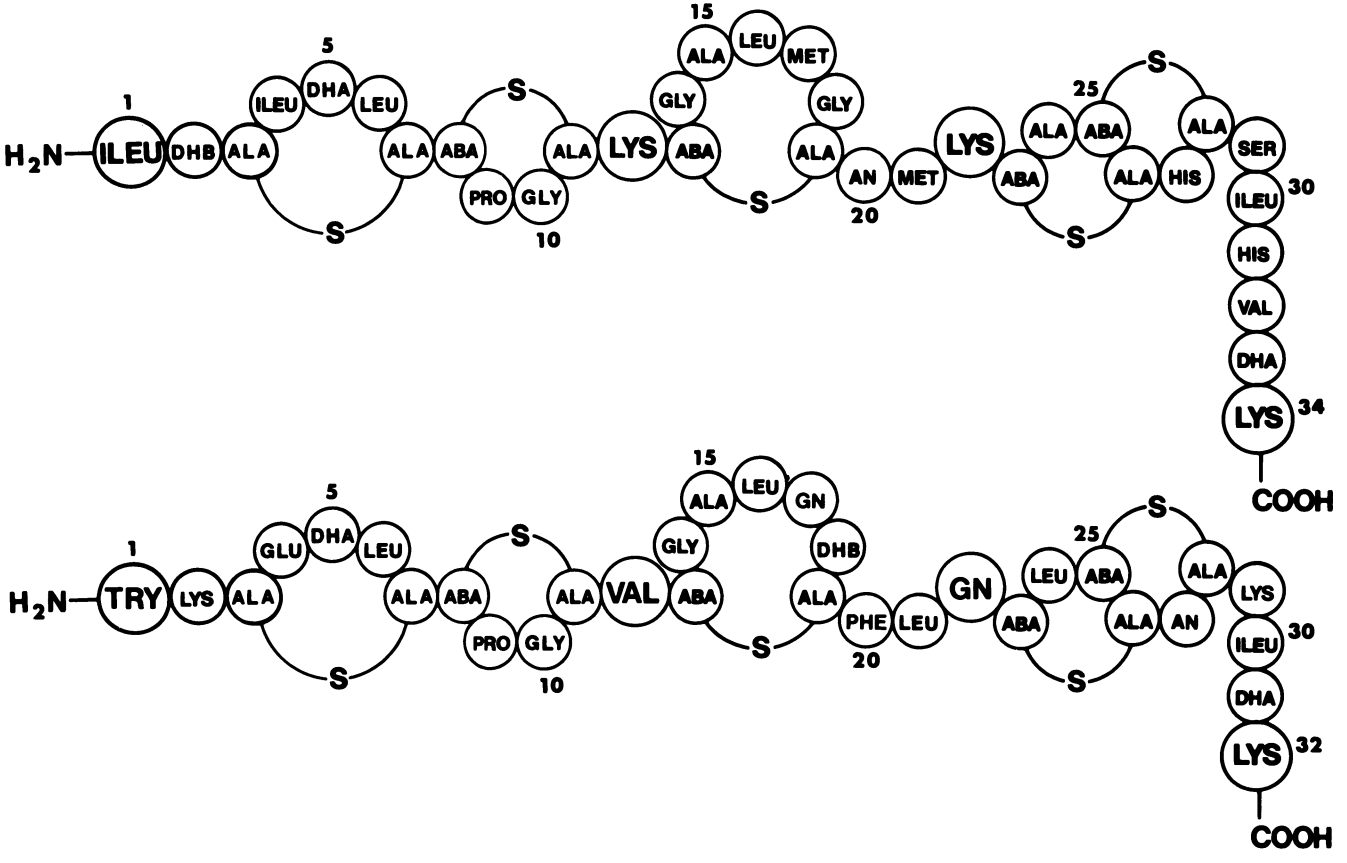


Fig. 9. The structure of nisin (top) and subtilin (bottom). ABA = aminobutyric acid; DHA = dehydroalanine; DHB = dehydrobutyrine (β-methyldehydroalanine); ALA-S-ALA = lanthionine; ALA-S-ABA = β-methyllanthionine.

to subtilin or subtilin fragments (Gross and Kiltz, 1973) from tryptic and thermolytic fragmentation (Kiltz and Gross, 1973). Much of the remaining structural work benefited from and made use of the methods developed for nisin.

The close relationship between the structures of nisin and subtilin is evident from Figure 9. Dehydroalanine residues occupy identical positions. Dehydrobutyrine in subtilin has moved into an endocyclic position (ring C). All amino acids participating in sulfide bridges are found in positions identical with those in nisin, consequently all rings are of same size as those in nisin. Amino acid exchanges, obviously, occurred in a number of places. The phylogenetic questions to be asked are numerous. One of them inquires about the reasons for the preservation of structure in two microorganisms - a spore-forming bacillus and a streptococcus - that are no longer so closely related.

Much remains to be learned about the function of NISIN, SUBTILIN, CINNAMYCIN, and DURAMYCIN in their native biological environment. It is speculative to say that they are membrane constituents, possibly participating in ion transport and/or being essential components of defense mechanisms in microbes fighting for survival.

B. Biology. For nisin, removed from its native biological environment, concepts have been developed for the mode of biological activity it displays in various ways. One of such hypotheses is based on no other structural feature but that seen in α,β-unsaturated amino acids. Assume that these residues react in the fashion, so amply demonstrated before, with essential sulfhydryl groups of proteins or enzymes, for instance, in biological membranes, then an element is introduced that is detrimental to physiological processes. It was for chemical reasoning that this hypothesis was first probed on tissue growing in utero. If alkaline yeast extracts - in which α,β-unsaturated amino acids may have been generated - are capable of inducing fetal resorption in rodents, why should nisin and its fragments not do the same? The acclaimed active principle is present in them to begin with.

When nisin and its cyanogen bromide derived so-called H_2N-terminal fragments were given intravenously or intraperitoneally to pregnant rats and rabbits, fetal resorption was induced at various stages of gestation. Exploring the effect, seen on neonatal tissue, on neoplasms, inhibitory effects on the growth of tumor tissue were observed. Nisin and certain of its fragments may be considered membrane-active peptides inasmuch as they cause the lysis of cells (erythrocytes) and subcellular constituents (lysosomes).

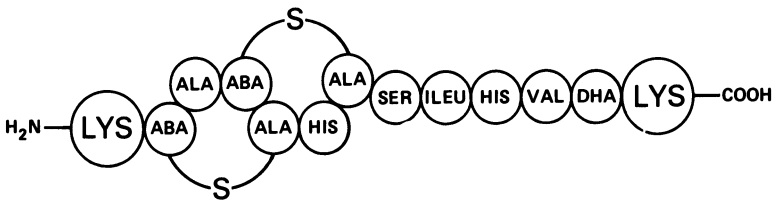


Fig. 10. The COOH-terminal fragment of nisin. ABA = aminobutyric acid; DHA = dehydroalanine; ABA-S-ALA = β-methyllanthionine.

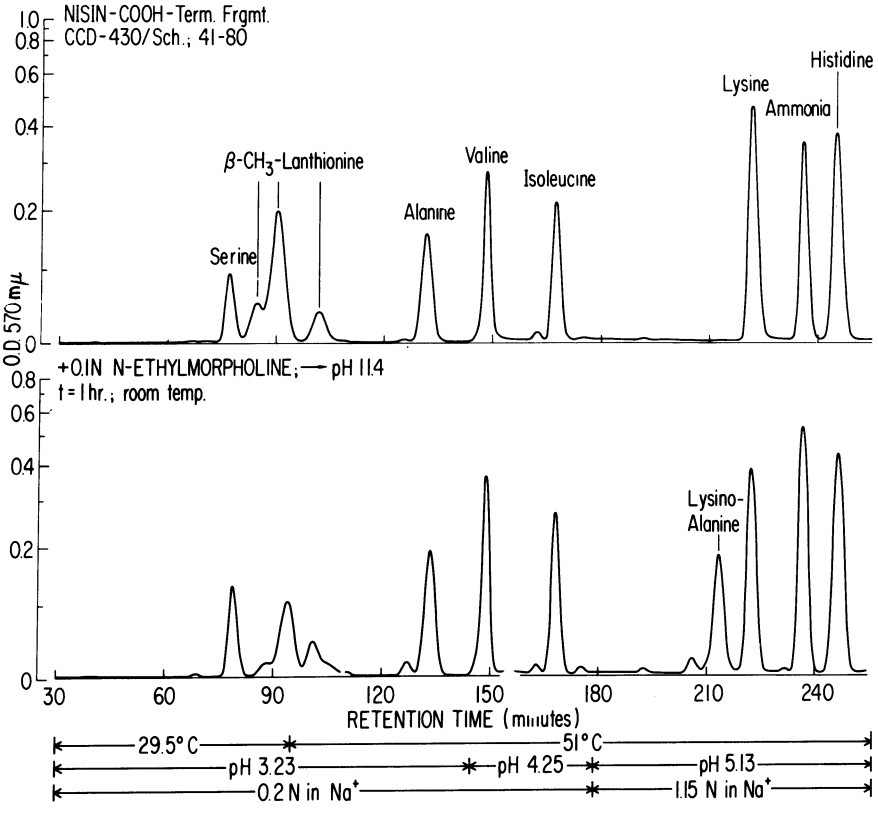


Fig. 11. Formation of lysinoalanine in the carboxyl-terminal fragment of nisin. Under the pH conditions selected, β-methyllanthionine is already subject to partial β-elimination (cf. decrease in peak area for β-methyllanthionine comparing the amino acid analysis of base-exposed (lower chromatogram) versus untreated (upper chromatogram) peptide.

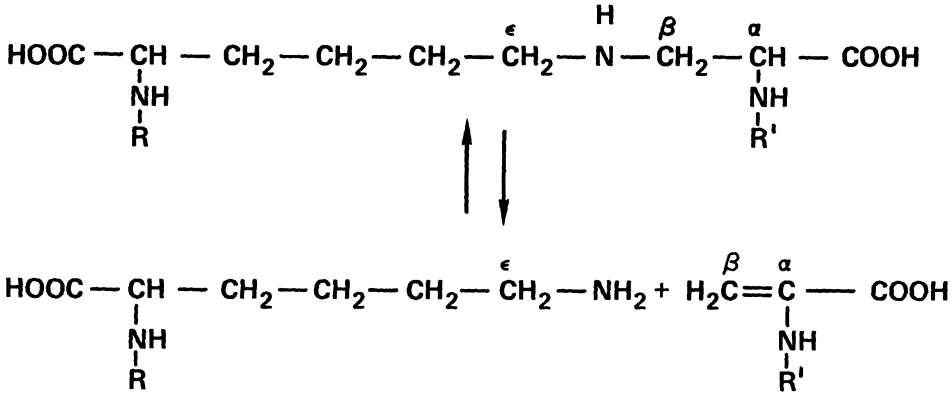


Fig. 12. The addition of the ε-amino group of lysine across the double bond of dehydroalanine.

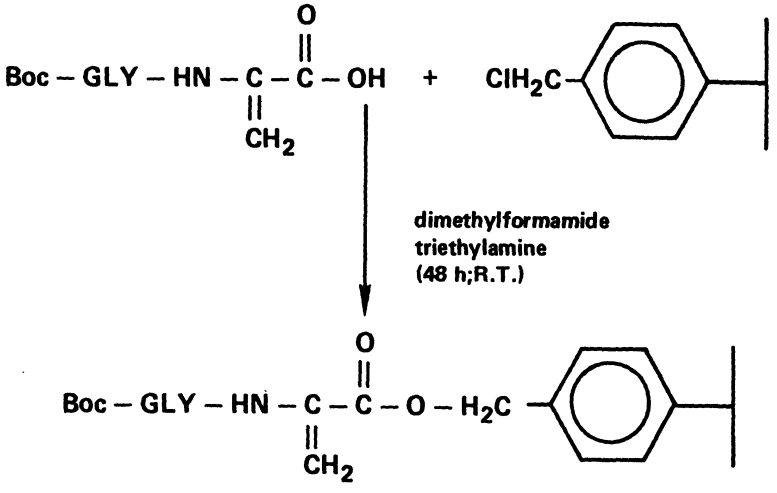


Fig. 13. The synthesis of dehydroalanine resin.

C. Formation of Lysinoalanine in Nisin and Nisin Fragments. The prerequisites met by the presence of dehydroalanine and lysine, nisin and its cyanogen bromide derived fragments were exposed to alkaline conditions for possible lysinoalanine formation. May the example of the COOH-terminal fragment (Figure 10) serve to demonstrate that in pH 11.4 0.1 N N-ethyl morpholine buffer at room temperature within 1 hour lysinoalanine is formed to a considerable extent (cf. the chromatogram of the amino acid analyses in Figure 11). The outcome of this experiment constitutes proof for the addition of the ε-amino group of lysine residues across the double bond of dehydroalanine (Figure 12).

CROSSLINKING OF PEPTIDES TO SOLID SUPPORTS

Dehydroalanine Resin. The acid catalyzed breakdown of α,β-unsaturated amino acids with the formation of amide and keto acid (Figure 4) suggested the application of this reaction to the synthesis of peptide amides on solid supports. "Crosslinking" a protected amino acyl group via DEHYDROALANINE to a resin the peptide may be elongated step by step to its desired chain length before it is removed under acid catalyzed conditions with the formation of a carboxyl-terminal amide while the pyruvic acid remains covalently attached to the solid support.

In general specifically prepared and protected aminoacyl dehydroalanine is attached to the resin as illustrated in Figure 13. The example of the synthesis of a tripeptide amide, the thyropin releasing factor (TRF) - pyroglutamylhistidylproline amide (E. Gross et al., 1973) - is illustrated in Figure 14. The product met all the activity criteria applied to this class of compounds.

CONCLUSION

α,β-UNSATURATED AMINO ACIDS are now recognized as key constituents of peptides and proteins. Firmly established is their natural occurrence in a number of peptides, among them nisin and subtilin. The presence of α,β-unsaturated amino acids is not restricted to low molecular weight entities of microbial origin. Dehydroalanine has been detected in L-phenylalanine ammonia lyase of potato tubers (Hanson and Havier, 1970) and in histidine ammonia lyase of Pseudomonas putida (Givot et al., 1969; Wickner, R. B., 1969). A first report is also on hand suggesting dehydroalanine as a constituent in a mammalian protein, namely the enzyme histidine ammonia lyase in rat liver (Givot and Abeles, 1970).

It is of considerable interest that in Pseudomonas putida an enzyme has been found, namely urocanase (George and Phillips,1970), that employs β-ketobutyrate as prosthetic group. It would not be surprising to see this enzyme related to DEHYDROBUTYRINE (vide supra, the interactions between keto acids and amides and α,β-unsaturated amino acids).

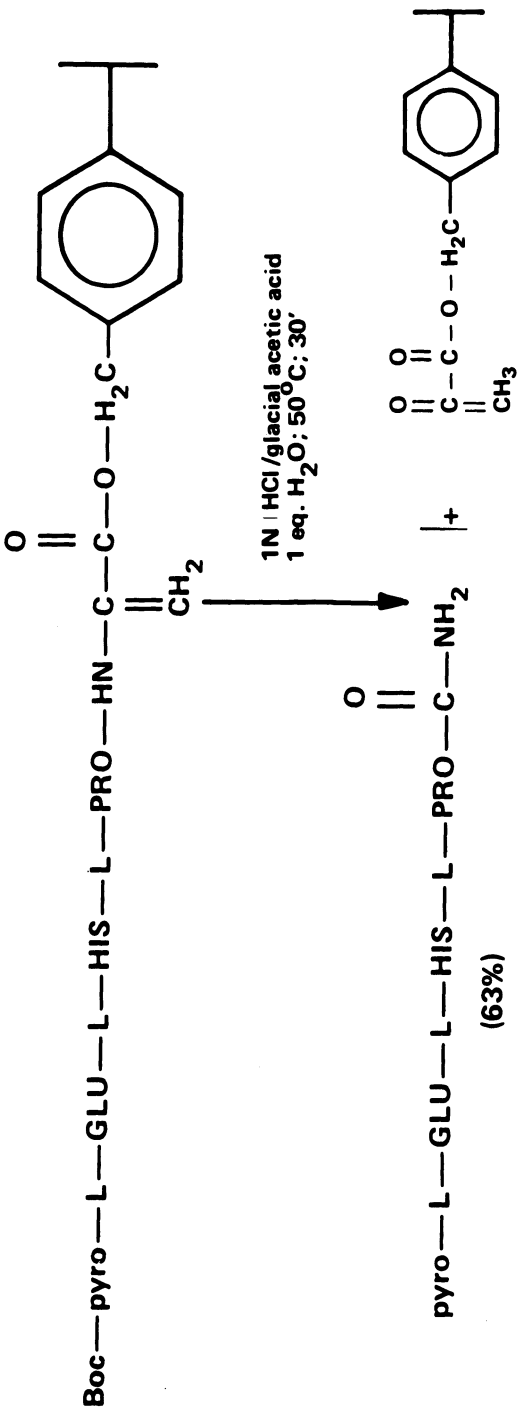


Fig. 14. The synthesis of the thyrotropin releasing factor (TRF) on the dehydroalanine resin.

It must be borne in mind that α,β-unsaturated amino acids are dynamic chemical entities that are in principle always ready to enter into addition reactions. With water as participant in the reaction the addition product is not stable and decomposition follows to give amide and keto acid. The reversibility of this reaction, the addition of the amide group to α-keto acids (Figure 4), further broadens the scope and multiplicity of interactions in which we must be prepared to find α,β-unsaturated amino acids. - What are the observations made good for? They intend to point to the likelihood of a much more frequent involvement of α,β-unsaturated amino acids in pathways in the physiological environment than is presently appreciated.

Under the necessary but rather severe conditions of alkaline treatment there is the formation of LYSINOALANINE in peptides and in proteins to provide food supplements for man. Again, an α,β-unsaturated amino acid - DEHYDROALANINE - is the key intermediate for the formation of the imino bridge in the crosslinking amino acid that, as of recent, is also accounted for as constituent in natural products. Why do streptomyces strains take advantage of the crosslinking potential vested in dehydroalanine and lysine? Advantageous it to be in the natural habitat of the microorganisms, so we assume.

The utility of crosslinking peptides via α,β-unsaturated amino acids to solid support lay before us once the dynamics of their chemistry were recognized. Many hormone releasing factors are peptide amides. To their synthesis the DEHYDROALANINE RESINS have been applied with considerable success. Peptide synthesis is likely to benefit from other applications of the chemistry of α,β-unsaturated amino acids.

Last, but not least, the time had come that it stood to reason that the structural components of α,β-unsaturated amino acids might hold potential as agents of chemotherapy. Removed from their source of origin and offered to new physiological environment, peptides with α,β-unsaturated amino acids were still believed to accept nucleophiles of biological origin. Conclusive evidence and the elements of the final proof admittedly still lacking, the results are rated positive when it comes to an accounting of the tests conducted to prove the working hypothesis. The action of peptides with α,β-unsaturated amino acids on NEONATAL and NEOPLASTIC TISSUE is promising and worthy of further pursuit. In the biological environment such molecules continue to constitute agents of desirable CROSSLINKING.

ACKNOWLEDGEMENTS

The studies discussed were only possible with the enthusiastic support of able coworkers. Mr. John L. Morell did the pioneering

studies on nisin that were useful for the structural determination of subtilin by Drs. Hans Hermann Kiltz and Eike Nebelin. Hans Hermann Kiltz began the investigations that showed the presence of lysinoalanine in cinnamycin. Structural work on cinnamycin was continued by Dr. Hao-Chia Chen and is now in the hands of Mr. Charles C. Chapin. Miss Judith H. Brown is responsible for the structure of duramycin. Dr. Shigeru Nanno and Mr. Charles C. Chapin investigated the formation of lysinoalanine in peptides with lysine and dehydroalanine, and Dr. Kosaku Noda was successful with the development of the dehydroalanine resin method of peptide amide synthesis.

REFERENCES

Alderton, G. (1953). A new sulfur-containing amino acid from subtilin. J. Am. Chem. Soc. 75, 2391-2392.

Benedict, R. G., Dvonch, W., Shotwell, O. L., Pridham, T. G., and Lindenfelser, L. A., (1952). Cinnamycin, an Antibiotic from Streptomyces cinnamoneus Nov. Sp., Antibiotics and Chemotherapy 2,591-594.

Bohak, Z. (1964). $^{\epsilon}$N-(DL-2-Amino-2-carboxyethyl)-L-lysine, a new amino acid formed on alkaline treatment of proteins. J. Biol. Chem. 239, 2878-2887.

Dvonch, W., Shotwell, O. L., Benedict, R. G., Pridham, T. G., and Lindenfelser, L. A., (1954). Further studies on cinnamycin, polypeptide antibiotic. Antibiotics and Chemotherapy 4, 1135-1142.

George, D. J. and Phillips, A. T. (1970). Identification of α-ketobutyrate as the prosthetic group of urocanase from Pseudomonas putida. J. Biol. Chem. 245, 528-537.

Givot, I. L., Smith, T. A., and Abeles, R. H. (1969). Studies on the mechanism of action and the structure of the electrophilic center of histidine ammonia lyase. J. Biol. Chem. 244, 6341-6353.

Givot, I. L. and Abeles, R. H. (1970). Mammalian histidine ammonia lyase. In vivo inactivation and presence of an electrophilic center at the active site. J. Biol. Chem. 245, 3271-3273.

Gross, E. (1966). The cyanogen bromide reaction. Methods in Enzymology. Enzyme Structure. Ed. C. H. W. Hirs, Academic Press, Inc., New York, New York. 11, 238-255.

Gross, E. and Morell, J. L. (1967). The presence of dehydroalanine in the antibiotic nisin and its relationship to activity. J. Am. Chem. Soc. 89, 2791-2792.

Gross, E. and Morell, J. L. (1968). The number and nature of α,β-unsaturated amino acids in nisin. FEBS Letters 2, 61-64.

Gross, E., Morell, J. L., and Craig, L. C. (1969). Dehydroalanyl-lysine: Identical COOH-terminal structures in the peptide antibiotics nisin and subtilin. Proc. Nat. Acad. Sci. 62, 952-956.

Gross, E. and Morell, J. L. (1970). NISIN. The assignment of sulfide bridges of β-methyllanthionine to a novel bicyclic structure of identical ring size. J. Am. Chem. Soc. 92, 2919-2920.

Gross, E. and Morell, J. L. (1971). The structure of nisin. J. Am. Chem. Soc. 93, 4634-4635.

Gross, E. and Kiltz, H. H. (1973). The number and nature of α,β-unsaturated amino acids in subtilin. Biochem. Biophys. Res. Commun. 50, 559-565.

Gross, E., Kiltz, H. H., and Craig, L. C. (1973). Subtilin, II. Die Aminosäurezusammensetzung des Subtilins. Hoppe-Seyler's Z. Physiol. Chem. 354, 799-801.

Gross, E., Kiltz, H. H., and Nebelin, E. (1973). Subtilin, VI. Die Struktur des Subtilins. Hoppe Seyler's Z. Physiol. Chem. 354, 810-812.

Gross, E., Noda, K., and Nisula, B. (1973). Solid phase synthesis of peptides with carboxyl-terminal amides - thyrotropin releasing factor (TRF). Angew. Chem. Internat. Edit. 12, 664-665.

Hanson, K. R. and Havier, E. A. (1970). L-Phenylalanine ammonia lyase, IV. Evidence that the prosthetic group contains a dehydroalanyl residue and mechanism of action. Arch. Biochim. Biophys. 141, 1 (1970).

Kiltz, H. H. and Gross, E. (1973). Subtilin, III. Enzymatische Fragmentierung mit Trypsin und Thermolysin. Hoppe-Seyler's Z. Physiol. Chem. 354, 802-804.

Okuda, T. and Zahn, H. (1965). Synthese von $^{\varepsilon}$N-(2-amino-2-carboxy-äthyl)-L-Lysin, einer neuen Aminosäure aus alkalibehandelter Wolle. Chem. Ber. 98, 1164-1167.

Patchornik, A. and Sokolovsky, M. (1964). Chemical interactions between lysine and dehydroalanine in modified bovine pancreatic ribonuclease. J. Am. Chem. Soc. 86, 1860-1861.

Schultz, J. (1967). Cleavage at aspartic acid. Methods in Enzymology. Enzyme Structure. Ed. C. H. W. Hirs, Academic Press, Inc., New York, New York. 11, 255-263.

Shotwell, O. L., Stodola, F. H., Michael, W. R., Lindenfelser, L. A., Dworschack, R. G., and Pridham, T. G., (1958). Antibiotics against plant disease. III. Duramycin. A new antibiotic from Streptomyces cinnamoneus forma azacoluta. J. Am. Chem. Soc., 80, 3912-3915.

Sternberg, M., Kim, C. Y., and Schwende, F. J. (1975). Lysinoalanine: Presence in foods and food ingredients. Science 190, 992-994.

Wickner, R. B. (1969). Dehydroalanine in histidine ammonia lyase. J. Biol. Chem. 244, 6550-6552.

Zahn, H. and Lumper, L. (1968). Die Bildung von Lanthionin und Lysinoalanin - Querbrücken bei der alkalischen Denaturierung von Rinderserumalbumin. Hoppe-Seyler's Z. Physiol. Chem. 349, 77-84.

Ziegler, K. (1964). New cross-links in alkali-treated wool. J. Biol. Chem. 239, PC2713-PC2714.

Ziegler, K., Melchert, I., and Lübken, C. (1967). $^{\omega}$N-(2-amino-2-carboxyethyl)-ornithine, a new amino acid from alkali-treated proteins. Nature 214, 404-405.

10

BEHAVIOR OF O-GLYCOSYL AND O-PHOSPHORYL PROTEINS IN ALKALINE SOLUTION

John R. Whitaker and Robert E. Feeney

Department of Food Science and Technology

University of California, Davis, California 95616

I. ABSTRACT

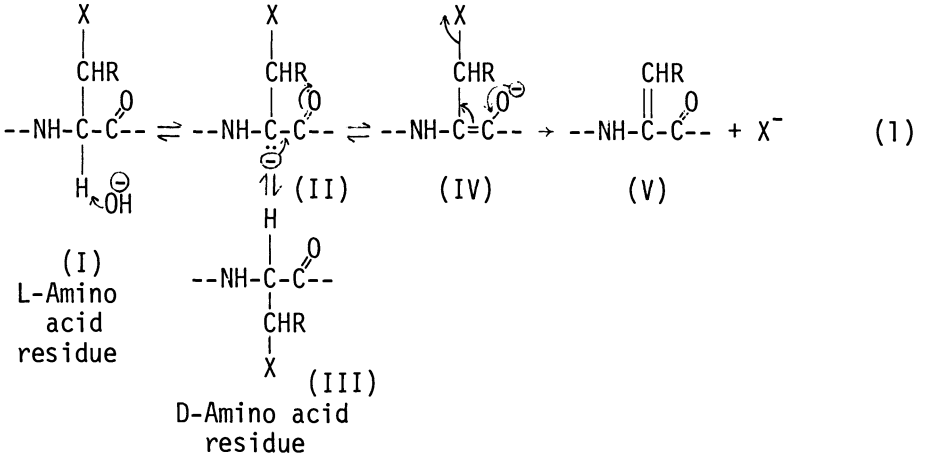
O-Glycosyl and O-phosphoryl groups, as well as disulfide bonds, are rapidly removed from proteins in alkaline solution primarily via β-elimination. The reaction is initiated by abstraction of the α-hydrogen of an amino acid residue by hydroxide ion. The carbanion undergoes rearrangement expelling the glyco- or phosphoryl group resulting in formation of a dehydroalanyl (from serine) or β-methyl-dehydroalanyl (from threonine) residue. The unsaturated derivatives are reactive with internal protein nucleophilic groups and with external nucleophiles. These addition reactions, some leading to crosslinking, result in changed properties of the protein. Several factors may affect rates of β-elimination and addition. For these studies, we used two unique proteins: phosvitin, a well-characterized protein with 120 O-phosphoryl groups and no cystine or O-glycosyl groups; and a glycopeptide related to the antifreeze protein from Antarctic fish, a well-characterized protein consisting of $(Ala\text{-}Ala\text{-}Thr)_n$ in which all of the threonyl residues are glycosylated. At the dilute concentrations of proteins used, rates of β-elimination and addition were independent of protein concentration but directly dependent upon the hydroxide ion concentration. With phosvitin, rates of β-elimination and addition were quite dependent on ionic strength and the rate of β-elimination was increased 20-fold in the presence of Ca^{2+}. Activation energies for both β-elimination and addition were near 20 kcal/mole. Implications of these reactions for protein chemistry and protein processing will be discussed.

II. INTRODUCTION

Sodium hydroxide, on the GRAS list of chemicals, is used in the food industry for peeling, solubilizing, neutralizing, detoxifying, and texturing foods. Although it has been known for a long time that alkaline conditions can cause chemical reactions of proteins in foods, careful and detailed investigations of the nature of these reactions have only been initiated recently. This increased interest is due to the greater use of sodium hydroxide particularly in the treatment of high protein foods for texturing purposes, to greater concern with maintaining the nutritional qualities of food and the concern for possible formation of toxic constituents.

In alkali solution, proteins are known to undergo the following types of reactions: (1) hydrolysis of some peptide bonds, (2) hydrolysis of the guanido group of arginine (Ziegler et al., 1967), (3) some destruction of the amino acids cystine, cysteine, serine, arginine, threonine (Pickering and Li, 1964; Geschwind and Li, 1964; Ziegler et al., 1967; Mellet, 1968; Blackburn, 1968; DeGroot and Slump, 1969; Parisot and Derminot, 1970; Whiting, 1971; Gottschalk, 1972; Provansal et al., 1975), (4) racemization (Dakin, 1912; Levene and Bass, 1928; Pickering and Li, 1964; Tannenbaum et al., 1970), (5) elimination of certain side chain groupings to form double bonds (Mecham and Olcott, 1949; Asquith and Carthew, 1972; Miro and García-Domínquez, 1973) and (6) formation of new amino acids (see below).

With the exception of hydrolysis (reactions 1 and 2), the other reactions are initiated through a common intermediate, a carbanion, which is produced by extraction of the α-hydrogen from the amino acid residue as shown in Equation 1. In Equation 1, X = H, O-glycosyl, O-phosphoryl, -S-CH_2-R, aliphatic or aromatic residue; R = H or CH_3. A hydrogen can add back to the carbanion to give either



the D or L amino residue (racemization) or with certain electron withdrawing side chains as those of cystine, cysteine, phosphoryl- and glycosylserine and phosphoryl- and glycosylthreonine there is rearrangement of the carbanion leading to expulsion of the substituting group to give an olefinic side chain (Equation 1). Formation of the enolic anion intermediate as postulated by Isbell (1944, 1971) appears to be an essential step in β-elimination since compounds in which the carboxyl or amino group is free do not undergo β-elimination (Derevitskaya et al., 1967; Wakabayashi and Pigman, 1974).

The olefinic residue [dehydroalanine (2-aminopropenoic acid) from O-substituted serine, cystine or cysteine; β-methyldehydroalanine (2-amino-2-butenoic acid) from O-substituted threonine (Plantner and Carlson, 1975)] is quite reactive with nucleophilic compounds. Reaction of dehydroalanine residues with nucleophilic groups of the side chains of the amino acid residues, lysine, ornithine, cysteine, asparagine, glutamine, histidine or tryptophan lead to the formation of cross-linkages in proteins (Fig. 1) and the production of new amino acid residues in the protein. Products of addition reactions between dehydroalanine and the ε-amino group of lysine (Patchornik and Sokolovsky, 1964; Bohak, 1964; Ziegler, 1964; Corfield et al., 1967; Robson and Zaidi, 1967; Whiting, 1971), the δ-amino group of ornithine (Ziegler et al., 1967) and the sulfhydryl group of cysteine (Corfield et al., 1967; Asquith and García-Domínquez, 1968; Nashef et al., 1976) are well characterized. Reaction of dehydroalanine residues with external nucleophiles such as ammonia and sulfite lead to formation of β-aminoalanine (Asquith et al., 1969) and 2-amino-3-sulfonylpropionic acid (Simpson et al., 1972) on hydrolysis of the protein. Analogous reactions between β-methyldehydroalanine and internal and external nucleophiles are expected to occur but have been less studied because O-substituted threonine residues occur much less frequently in most proteins than do O-substituted serine residues.

Certain proteins contain naturally occurring cross-linkages as a consequence of post translational *in vivo* enzymatic modifications of the proteins. These are described in detail in other papers of this monograph. In particular, lysyl oxidase catalyzed oxidation of the side chains of lysine and hydroxylysine lead to formation of several new amino acids in collagen and elastin (Gallop et al., 1972; Tanzer, 1973) while the action of glutamyltransaminase leads to the formation of ε-N(γ-glutamyl)lysine residues in fibrin (Pisano et al., 1971). ε-N-(γ-Glutamyl)lysine residues are also found in proteins heated in the dry or semi-dry state (Bjarnason and Carpenter, 1970).

PHOSVITIN AND ANTIFREEZE GLYCOPROTEINS IN ALKALINE SOLUTIONS

While the above chemical modifications of proteins are known to occur in alkaline solutions, very little is known about the rates at

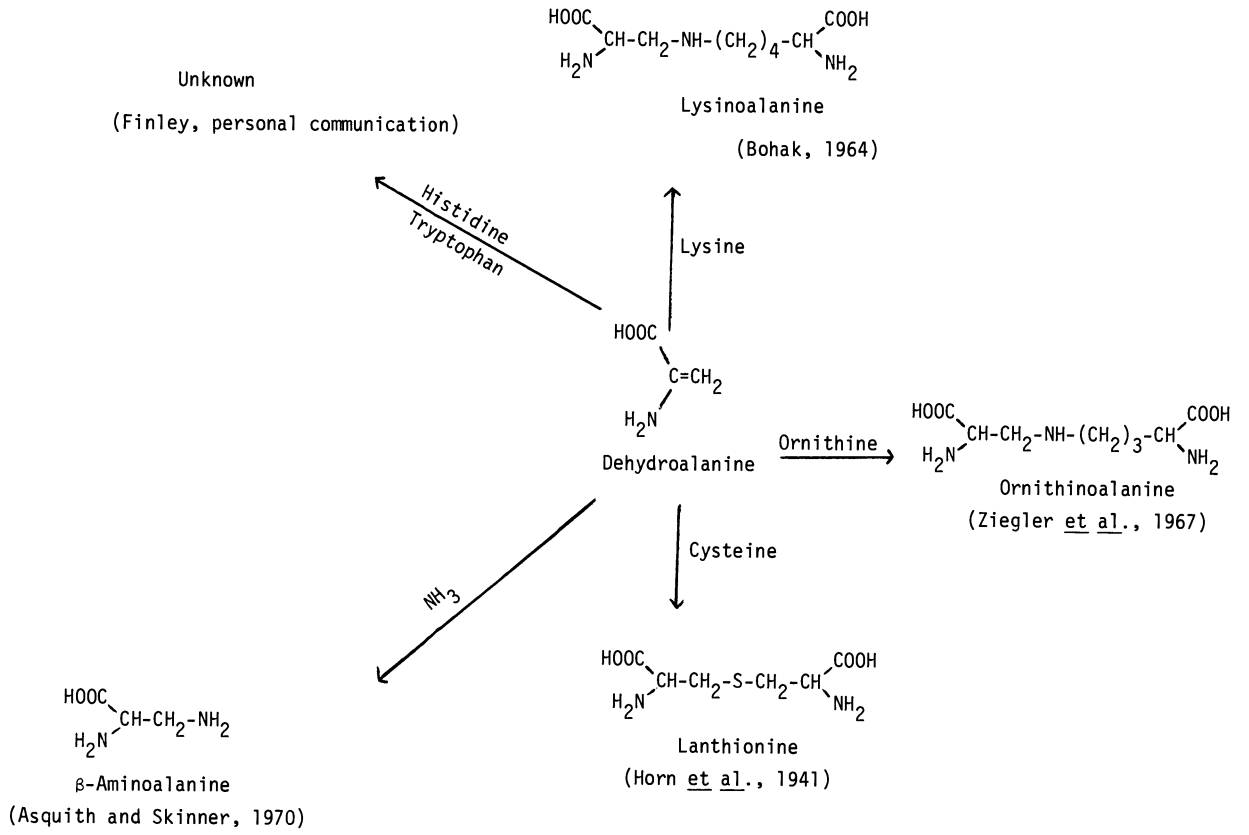


Fig. 1. New amino acids which may be formed through reaction of a dehydroalanine residue with internal or external nucleophiles in alkali treated proteins (adapted from Nashef et al., 1976).

which these reactions occur under various experimental conditions. Our laboratories have undertaken a systematic study of these reactions in model systems involving carefully selected proteins or small substrates. From the information gained from such systems, we then plan to examine the rates of these reactions under various conditions in more complex food systems. In the present paper, we summarize some of our results on the behavior of phosphoproteins (Sen et al., 1976) and glycoproteins (Lee et al., 1976) in alkaline solutions.

Phosvitin was chosen as a model for the behavior of phosphoproteins in alkaline solution because it is easily purified from egg yolk, much of its primary structure is available (Belitz, 1965), and there is already some data available on its behavior in alkaline solutions (Taborsky, 1974). It has a MW of 35,500 daltons, contains 10% phosphate (w/w) as 119 phosphoserine and one phosphothreonine residues, has no disulfide bonds and the single chain of carbohydrate is base stable, being attached to the amide group of an asparagine residue. At lower ionic strengths and pHs above 3 the fully phosphorylated protein is a random coil, thus eliminating possible interference by a denaturating step in determining the initial rates of β-elimination. There appears to be at least eight sequences of six consecutive phosphoserine residues followed by a basic amino acid and one cluster, near the carbohydrate moiety, of eight consecutive phosphoserine residues followed by an arginine residue (Belitz, 1965). Thus, nearly, half the phosphoserine residues are located in these nine clusters.

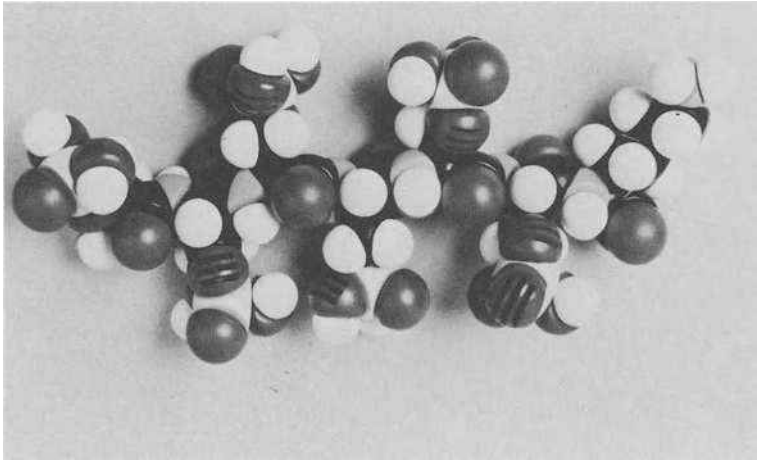


Fig. 2. Corey-Pauling-Koltun (CPR) space filling model of a cluster of six phosphoserine residues terminating with a lysine residue in phosvitin.

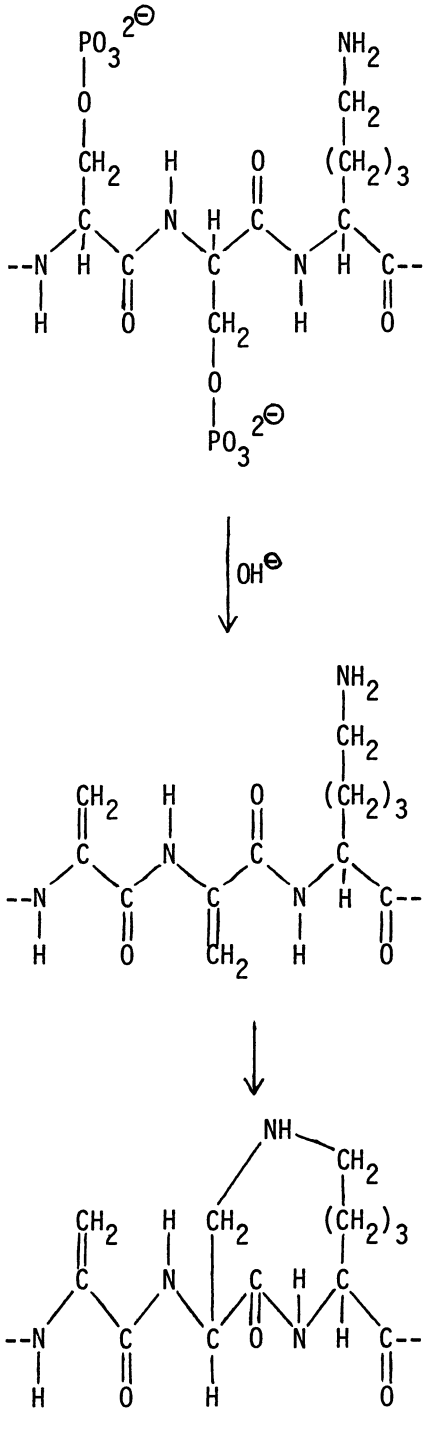


Fig. 3. β-Elimination of phosphoserine residues in phosvitin to produce dehydroalanine residues followed by internal addition of the ε-amino group of an adjacent lysine residue in alkaline solution.

A Corey-Pauling-Koltun (CPK) space filling model of one of these clusters is shown in Figure 2. Each consecutive phosphoserine residue is purposely displaced by 180° to minimize electrostatic repulsion of the phosphate groups in alkaline solution. β-Elimination of the phosphate group and internal addition of the ε-amino group of a lysine residue are shown in Figure 3.

One of the antifreeze glycoproteins (Feeney, 1974) was used in our investigation of the behavior of glycoproteins in alkaline solution (Lee et al., 1976). The larger antifreeze glycoproteins consist of repeating tripeptide units of Ala-Ala-Thr in which the hydroxyl group of each threonine is O-linked to a D-galactosyl-D(1→3)N-acetylgalactosamine. There are two additional alanine residues at the C-terminal end. A CPK model of four of the repeating glycotripeptide units in a random coil form is shown in Figure 4.

Our studies on β-elimination (Lee et al., 1976) were done with the smallest member of the antifreeze glycoproteins, termed AFGP-8. AFGP-8 consists of only four of the glycopeptide units plus the two C-terminal alanines and has a proline substituted for an alanine following two of the threonines. Its MW is approximately 2700 daltons. The reaction involving β-elimination of the carbohydrate moiety in alkaline solution is shown in Figure 5. There are no internal nucleophiles to add to the β-methyldehydroalanine residue formed.

Changes in Amino Acid Composition. The β-elimination reactions of phosvitin and AFGP-8 can be followed by determining the decrease in serine and threonine contents of acid hydrolyzates of the two pro-

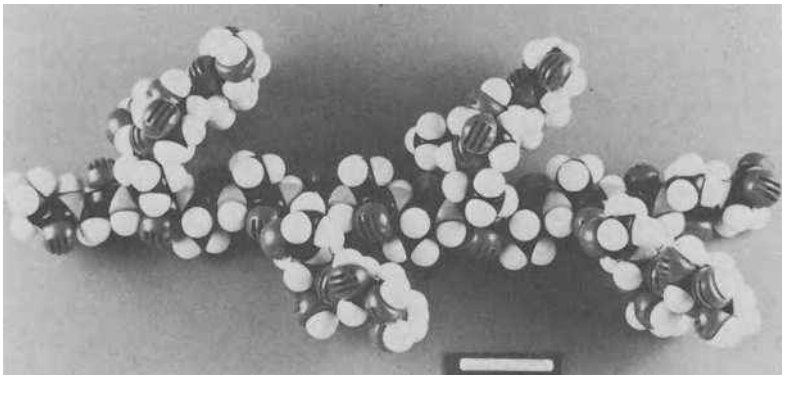


Fig. 4. Corey-Pauling-Koltun (CPK) space filling model of antifreeze glycoprotein shown in a random coil. The side chains represent the galactosyl (1→3) N-acetylgalactosamine residues attached to the four threonine residues (from Feeney, 1974).

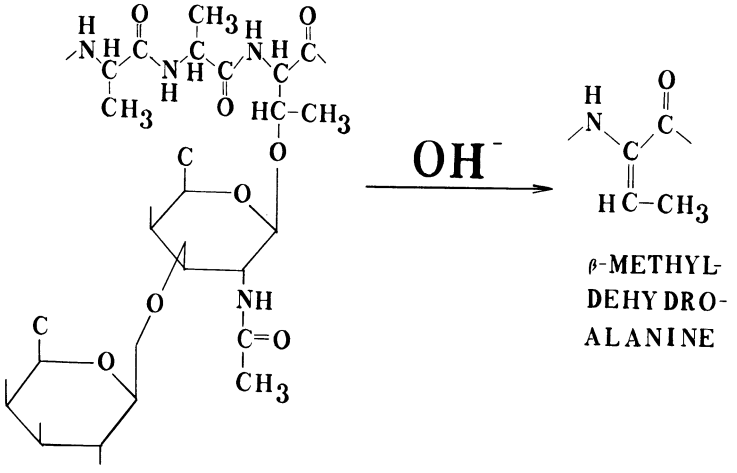


Fig. 5. β-Elimination of the carbohydrate moiety of antifreeze glycoprotein in alkaline solution to produce β-methyldehydroalanine residues (from Ahmed et al., 1973).

teins, respectively, by amino acid analysis since dehydroalanine is converted to pyruvic acid and NH_3 and β-methyldehydroalanine to α-ketobutyric acid and NH_3 duing acid hydrolysis. Results obtained by this method are shown in Tables 1 and 2. In 0.123 N NaOH at 60°C for 80 min, there was a loss of 79 moles of serine and 18 moles of lysine and the formation of 18 moles of lysinoalanine per mole of phosvitin (Table 1). The additional 61 moles of serine lost were largely as dehydroalanine in the unhydrolyzed protein (as determined by absorbance at 241 nm) although the possible formation of other addition compounds has not been ruled out. A detectable loss of arginine occurred only after 80 minutes in the alkaline solution.

The rate of loss of threonine in AFGP-8 during treatment at 37°C in 0.5 N NaOH is shown in Table 2. The rate of threonine loss is much more rapid early in the reaction with half of the threonine being lost in 120 min but requiring an additional 1320 min to cause loss of one half of the remaining threonine residues. It is known that O-glycosylserine and O-glycosylthreonine residues do not undergo β-elimination when either the amino or carboxyl group of the hydroxyamino acid residue is free in the compound (Derevitskaya et al., 1967; Wakabayashi and Pigman, 1974). Even a free amino (Derevitskaya et al., 1967) or a free carboxyl group (Ozeki and Yosizawa, 1971)

TABLE 1

Change in Amino Acid Composition of Phosvitin in Alkaline Solution[a]

Time (min)	Serine[b]	Lysine	Dehydroalanine[c]	Lysinoalanine	Serine + Dehydroalanine + Lysinoalanine
	(moles/mole phosvitin)				
0	115	23.1	0	0	115
20	90.2	18.9	9.9	4.20	104
40	70.1	12.9	23.5	8.83	102
80	36.4	5.33	45.1	17.9	99

[a] From Sen et al., 1976. The reactions were performed in KCl-NaOH buffer at 0.123 N NaOH, 60°C, 1 X 10^{-5} M phosvitin and ionic strength of 0.170.

[b] Corrected for loss during acid hydrolysis of protein. The results represent average of duplicate amino acid determinations after 24, 48, and 72 hr hydrolysis.

[c] Determined by increase in absorbance at 241 nm and calculated using ε 4.20 X 10^{3} M^{-1} cm^{-1}.

TABLE 2

Changes in Composition of AFGP-8 in Alkaline Solution[a]

Time (min)	Threonine[b] (moles/mole AFGP-8)	Galactosamine[c] (moles/mole AFGP-8)
0	4.0	4.0
15	3.36	3.12
60	3.04	2.80
120	2.16	1.88
240	1.64	1.68
420	1.56	1.28
600	1.40	1.08
1440	0.96	0.48
2880	0.56	0.32

[a]From Lee et al., 1976. In 0.5 N NaOH at 37°C with 2.22 X 10^{-3} M AFGP-8.

[b]Corrected for loss during acid hydrolysis of protein at 110°C for 22 hr in 6 N HCl.

[c]Determined by Technicon AutoAnalyzer following hydrolysis of protein in 3 N HCl at 95°C for 15 hr.

one amino acid removed from the hydroxyamino acid residue prevents β-elimination. In AFGP-8, none of the threonines are terminal but there are two followed by alanine and two by proline residues.

The effect of alkali on AFGP-8 can also be followed by the rate of loss of galactosamine (Table 2). The rate of loss of galactosamine is higher than that for threonine loss. Differences in rates of loss of threonine and galactosamine were found at other temperatures and hydroxide ion concentrations. In contrast to our results, Plantner and Carlson (1975) reported that the loss of β-hydroxy amino acids in alkaline solutions was always 10-20% greater than the loss of N-acetylgalactosamine. The more rapid loss of galactosamine from AFGP-8 might be a result of β-elimination involving the 1→3 glycosidic linkage of the D-galactosyl-D-(1→3) N-acetylgalactosamine residue (Pigman and Moschera, 1973). The possible addition of water to the β-methyldehydroalanine residue to form a threonine residue has not been excluded.

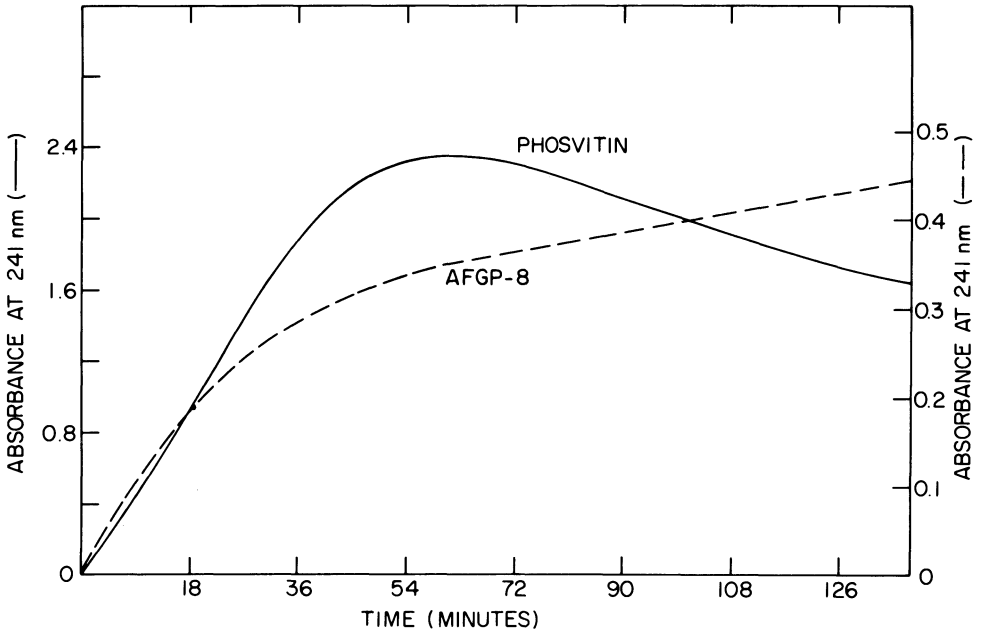


Fig. 6. Absorbance changes at 241 nm for phosvitin and AFGP-8 in alkaline solution. For phosvitin, the reaction was in 0.187 N NaOH at 60°C at 1.09 X 10^{-5} M phosvitin and ionic strength of 0.170 (Sen et al., 1976). For AFGP-8, the reaction was at 0.1 mg/ml of AFGP-8 in 0.5 N NaOH at 37°C (Lee et al., 1976).

Absorbance Changes. The β-elimination reactions can also be monitored by the increase in absorbance at 241 nm (Fig. 6) as shown originally by Mecham and Olcott (1949). With phosvitin in alkaline solution the increase in absorbance at 241 nm is followed eventually by a decrease in absorbance as a result of the rate of addition of nucleophilic groups to dehydroalanine becoming faster than the β-elimination reaction. With AFGP-8 there is no decrease in absorbance since the protein contains no nucleophilic groups (except the N-terminal α-amino of alanine). In the case of AFGP-8, the absorbance change is most likely complicated by contribution from chromogens (Mayo and Carlson, 1970; Lee et al., 1976) produced from the dehydration and cyclization of the hexosamine residues. Other complications concern the lack of agreement of the reported molar extinction coefficient for the dehydroalanine residue which ranges from 4200 to 6050 M^{-1} cm^{-1} (Carter and Greenstein, 1946a,b; Price and Greenstein, 1947; Lee et al., 1976). We have used 4200 M^{-1} cm^{-1} (Carter and Greenstein, 1946a,b) in the results reported here.

TABLE 3

Effect of Hydroxide Ion Concentration on Initial Rate of β-Elimination of Phosvitin and AFGP-8

Protein	$[OH^-]$ ($\underline{M} \times 10^2$)	Initial rate[a] (min^{-1})	Initial rate/$[OH^-]$ ($\underline{M}^{-1}$ min^{-1})
AFGP-8[b]	0.001	4.13×10^{-5}	4.13[c]
	0.01	6.14×10^{-5}	0.614
	0.1	5.70×10^{-4}	0.570
	1.0	2.26×10^{-3}	0.226
	50	2.04×10^{-1}	0.408
			ave = 0.454 (0.647 at 60°C)[d]
Phosvitin[e]	1.74	6.44×10^{-4}	0.0370
	5.41	2.78×10^{-3}	0.0514
	12.3	7.65×10^{-3}	0.0622
	18.7	12.1×10^{-3}	0.0647
			ave = 0.0538

[a]Calculated as [dehydroalanine]/[protein]min where protein concentration is expressed in terms of phosphoserine concentration in case of phosvitin and glycothreonine concentration for AFGP-8.

[b]Performed at 50.0°C in 0.2 $\underline{M}$ phosphate-NaOH buffers and 3.70×10^{-5} $\underline{M}$ AFGP-8 (Lee et al., 1976).

[c]Left out of average.

[d]Calculated using E_a = 9.60 kcal/mol.

[e]Performed at 60.0°C in KCl-NaOH buffers with 1.0×10^{-6} to 1.1×10^{-5} $\underline{M}$ phosvitin. Rates corrected to ionic strength of 0.170 (Sen et al., 1976).

Effect of Hydroxide Ion Concentration. The initial rate of β-elimination of phosvitin and AFGP-8 was found to be directly dependent on the hydroxide ion concentration of the reaction (Table 3). The initial rate of β-elimination of the glycothreonine residue of AFGP-8 was 12 times more rapid at 60°C than was β-elimination of the phosphoserine residue of phosvitin. Generally, β-elimination from glycoserine residues occurs at about the same rate as from glycothreonine residues (Tanaka et al., 1964) although faster (Wakabayashi and Pigman, 1974) and slower (Plantner and Carlson, 1975) relative rates for glycoserine in relation to glycothreonine residues have

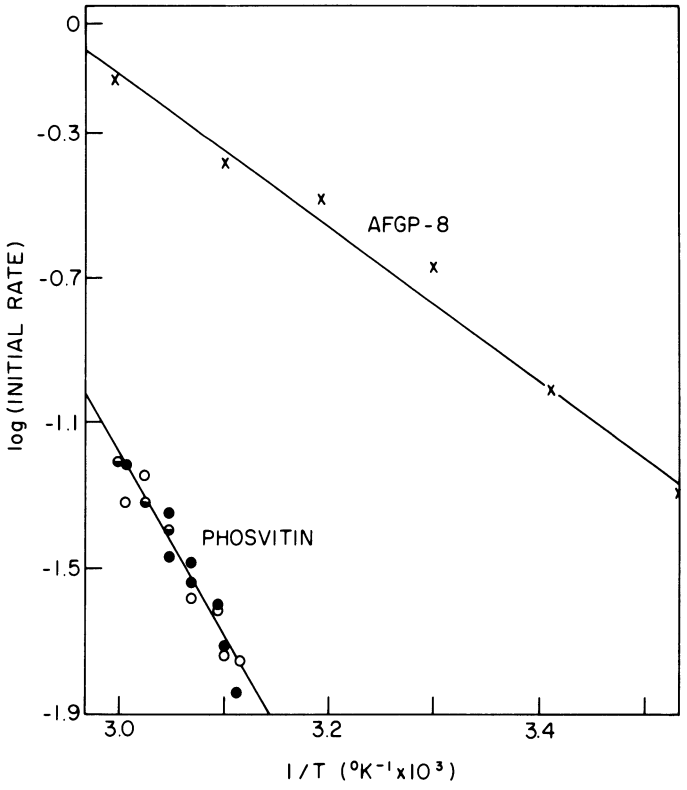


Fig. 7. Effect of temperature on initial rate of β-elimination of phosvitin and AFGP-8 in alkaline solution. For phosvitin, the reactions were performed in KCl-NaOH buffers at several different concentrations of phosvitin (Sen et al., 1976). All reactions were corrected to an ionic strength of 0.170. 0, hydroxide ion concentration constant at 0.123 N at all temperatures. 0, hydroxide ion concentration was different at different temperatures. Initial rates are expressed as [dehydroalanine]/([phosphoserine]$_0$ [OH^-] min). For AFGP-8, the reactions were performed at 6 mg/ml protein in 0.5 N NaOH (Lee et al., 1976). The initial rates are expressed as [threonine loss]/([glycothreonine]$_0$ [OH^-] min).

been reported. The reason for the marked increase in initial rate of β-elimination of AFGP-8 at pH 9 is not known, although the value given was readily reproducible.

Effect of Temperature. The initial rate of β-elimination of phosphoserine residues in phosvitin was more affected by a change in temperature than was β-elimination of glycothreonine residues in

TABLE 4

Thermodynamic Activation Parameters for the β-Elimination of O-Phosphoserine, O-Glycothreonine and Cystine Groups

Protein	E_a (kcal/mol)	$\Delta H^{\neq}$ (kcal/mol)	$\Delta F^{\neq a}$ (kcal/mol)	$\Delta S^{\neq a}$ (cal/mol/deg)
Phosvitin (phosphoserine)[b]	20.2	19.5	24.1	-13.8
AFGP-8 (O-glycothreonine)[c]	9.60	8.94	22.4	-39.9
Lysozyme (cystine)[d]	23.8	23.1	20.2	8.71
GAX ovomucoid (cystine)[e]	14.2	13.5	20.3	-20.4

[a]At 60.0°C.

[b]Corrected to 0.170 ionic strength. Experiments performed at variable and constant ionic strength and hydroxide ion concentrations with 1.0-11 X 10^{-6} M phosvitin (Sen et al., 1976).

[c]In 0.5 N NaOH and 2.22 X 10^{-3} M AFGP-8 (Lee et al., 1976).

[d]Reaction conditions were 1 X 10^{-5} M lysozyme in 0.1 N NaOH (Nashef et al., 1976).

[e]GAX ovomucoid = golden pheasant ovomucoid. Reaction conditions were 1 X 10^{-5} M ovomucoid in 0.1 N NaOH (Nashef et al., 1976).

AFGP-8 (Fig. 7). E_a values were 20.2 and 9.60 kcal/mol for β-elimination of the phosphoserine and glycothreonine residues, respectively. As shown in Table 4, $\Delta F^{\neq}$ for the reactions at 60°C are not greatly different since the initial rates differ by only some 12 fold (Table 3). The markedly different values of E_a (and $\Delta H^{\neq}$) are compensated for by markedly different $\Delta S^{\neq}$ values. The much higher $\Delta H^{\neq}$ for β-elimination of phosphoserine residue must represent the increased work necessary to bring the hydroxide ion into proximity of the α-hydrogen (Equation 1) in the presence of the negative charge on the phosphate group of the phosphoserine residue. There is no charge to work against in the glycothreonine residue. On the other hand, the much larger negative $\Delta S^{\neq}$ value for the glycothreonine indicates a requirement for a much larger change in order of the system, possibly the removal of several water molecules from the vicinity of the α-

TABLE 5

Effect of Calcium Ion Concentration on Initial Rate of β-Elimination of Phosvitin[a]

$CaCl_2$ ($M \times 10^4$)	Initial rate of β-elimination[b] (M^{-1} $min^{-1} \times 10^2$)
0	4.74
2.24	10.0
3.36	24.8
4.48	36.1
5.60	43.2
6.72	40.3
8.96	53.8
11.2	93.4

[a]At 60°C, 0.123 N NaOH, 5.54×10^{-6} M phosvitin (Sen et al., 1976).
[b]Initial rates expressed as [dehydroalanine]/(phosphoserine]$_0$[OH^-] min).

hydrogen. (We have assumed that the rate determining step is extraction of the α-hydrogen which seems reasonable). Even with β-elimination from the same grouping, cystine (Table 4 for lysozyme and golden pheasant ovomucoid), the environment around the group was found to greatly affect $\Delta H^{\neq}$ and $\Delta S^{\neq}$. It is of interest that golden pheasant ovomucoid contains about 24% carbohydrate amide linked to asparagine while lysozyme does not contain any carbohydrate.

Effect of $CaCl_2$. Because of the effect of the negative charge of the phosphate group of phosphoserine on the rate of β-elimination any suppression of this charge should increase the rate. It was found that an increase in ionic strength increased the rate of β-elimination. Addition of $CaCl_2$ also markedly affected the rate (Table 5). At 1.12×10^{-3} M $CaCl_2$ the rate was 20 times more rapid than in the absence of $CaCl_2$. There was essentially no change in $\Delta H^{\neq}$ (19.5 and 20.1 kcal/mol in the absence and presence of $CaCl_2$) but $\Delta S^{\neq}$ increased from -13.8 cal/mol/degree in the absence of $CaCl_2$ to -7.21 kcal/mol/degree in the presence of 7.47×10^{-4} M $CaCl_2$ (Sen et al., 1976).

Addition to Dehydroalanine and β-Methyldehydroalanine Residues. As discussed earlier, the olefinic compounds formed by β-elimination

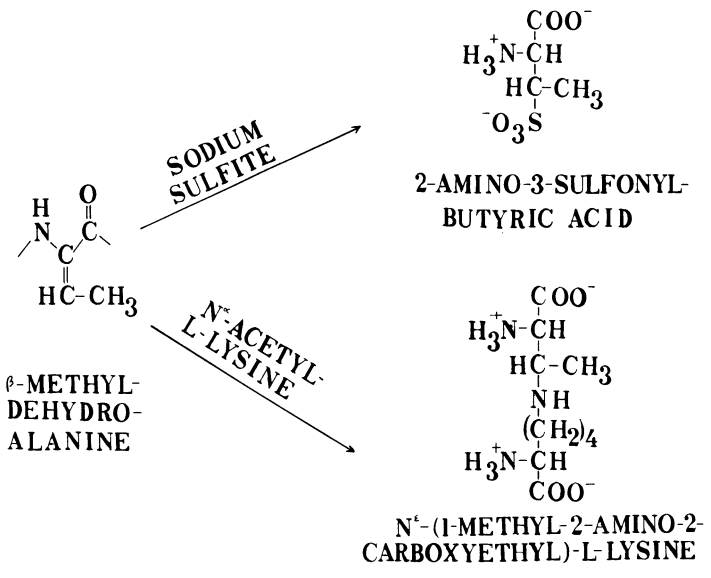


Fig. 8. Addition of sulfite and α-N-acetyl-L-lysine to β-methyldehydroalanine produced from β-elimination of AFGP-8 in sodium hydroxide followed by acid hydrolysis of protein (Lee et al., 1976).

TABLE 6

Sulfite Addition to β-Eliminated AFGP-8 at Different pH's[a]

pH	Sulfite addition (%)
9.7	55
10.7	70
11.7	67
12.5	69

[a]From Lee et al., 1976. AFGP-8 was treated for 24 hr at 37°C in 0.5 N NaOH to give an 80% decrease of threonine. Three mg of the treated AFGP-8 were incubated with 0.1 M Na_2SO_3, adjusted to the indicated pH with NaOH, for 24 hr at 37°C. Percent addition was calculated according to the actual available β-methyldehydroalanine, not total threonine content. Cysteic acid was used as standard on the amino acid analyzer.

are quite reactive with nucleophiles leading to formation of new products. The ε-amino group of lysine residues of phosvitin readily added to the dehydroalanine residues produced to give lysinoalanine (Table 1). In 0.123 N NaOH at 60°C, in 80 min 18 moles lysinoalanine per mole phosvitin were formed.

Externally added nucleophiles also react with the olefinic compounds. As shown in Figure 8 and Table 6, sulfite adds readily to the β-methyldehydroalanine residues of AFGP-8 to give 2-amino-3-sulfonylbutyric acid. Lee et al. (1976) did not achieve quantitative conversion (Table 6) although Simpson et al. (1972) have reported such to be achievable.

N-α-Acetyl-L-lysine has also been shown to add to β-methyldehydroalanine of AFGP-8 to give two products identified as the D and L-isomers of ε-N-(1-methyl-2-amino-2-carboxyethyl)-L-lysine (Lee et al., 1976).

Significance and Application of β-Elimination Reactions. The occurrence of β-elimination reactions and the possible ensuing reactions are important to the food scientist, nutritionist, toxicologist and food manufacturer. The possibility that alkali treatment can cause both the loss of essential nutrients (e.g. lysine) and the formation of compounds of questionable healthfulness (e.g. lysinoalanine) (DeGroot et al., 1976; Feeney, 1977; Gross, 1977) requires that the chemistry of these reactions be well understood and the reactions be controlled in food processing. In contrast to the wide spread existence of disulfides in both animal and plant proteins, significant amounts of O-phosphoryl and O-glycosyl groups are found mainly in animal products. This is probably the reason why the β-elimination from disulfides has received the most extensive attention, particularly by food scientists interested in new markets for plant proteins. Animal proteins, however, are frequently present in processed foods and the rapidity of β-elimination of O-phosphoryl and O-glycosyl groups must also be considered by the food processor.

In other areas, the β-elimination reaction has proved to be a useful one, rather than one to be avoided as with foods. It is a procedure commonly used by carbohydrate chemists to distinguish between O-glycosyl linkages of carbohydrates to serine and threonine residues in proteins from amide linkages of carbohydrates to asparagine residues in proteins (Downs and Pigman, 1976). The O-glycosyl groups β-eliminate on treatment with alkali while the amide-linked sugars are not removed.

Applications of β-eliminations of O-glycosyl groups have also been useful in studying structure-function relationships of glycoproteins. The β-elimination of the disaccharides from the active antifreeze glycoproteins was used to show the essentiality of the

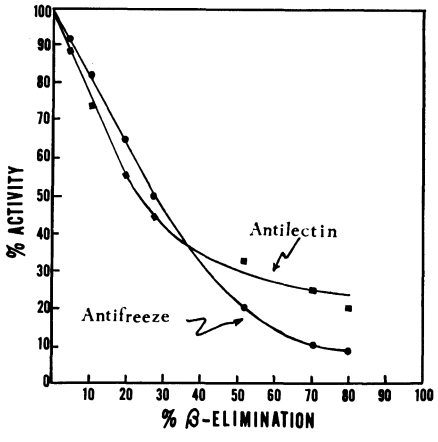


Fig. 9. Inactivation of antifreeze and antilectin activities of antifreeze glycoprotein by β-elimination of carbohydrate. β-Elimination was done with glycoprotein concentrations of 0.1 mg per ml in 0.1 N NaOH at 20°C (Ahmed et al., 1973).

carbohydrate side chains for function as an antifreeze agent as well as for a lectin-inhibiting activity. The similarities of the losses of the two different activities also indicated that the carbohydrate has a major role in these two different functions (Fig. 9).

ACKNOWLEDGMENTS

The authors appreciate advice from Honson Lee, Aws Nashef, and L. C. Sen in preparation of the manuscript, assistance from Linda Geiger, Chris Howland and Clara Robison in typing and editing the manuscript and financial support from grant FD 00568 from the Food and Drug Administration.

REFERENCES

Ahmed, A. I., Osuga, D. T. and Feeney, R. E. (1973). Antifreeze glycoprotein from an Antarctic fish. J. Biol. Chem., 248, 8524.

Asquith, R. S., Booth, A. K. and Skinner, J. D. (1969). The formation of basic amino acids on treatment of proteins with alkali. Biochim. Biophys. Acta, 181, 164.

Asquith, R. S. and Carthew, P. (1972). An investigation of the mechanism of alkaline degradation of cystine in intact protein. Biochim. Biophys. Acta, 278, 8.

Asquith, R. S. and García-Domínguez, J. J. (1968). New amino acids in alkali-treated wool. J. Soc. Dyers Colour., 84, 155.

Asquith, R. S. and Skinner, J. D. (1970). Modification of keratin and related proteins by alkali. Textilveredlung, 5, 406.

Belitz, H. -D. (1965). Egg yolk proteins and their fractionated products. IV. Amino acid sequences in phosvitin. Lebensm.-Untersuch. Forsch., 127, 341.

Bjarnason, J. and Carpenter, K. J. (1970). Mechanisms of heat damage in proteins. 2. Chemical changes in pure proteins. Brit. J. Nutr., 24, 313.

Blackburn, S. (1968). "Amino Acid Determination, Method and Techniques." Marcel Dekker, New York, N.Y.

Bohak, Z. (1964). N^{ϵ}-(DL-2-Amino-2-carboxyethyl)-L-lysine, a new amino acid formed on alkaline treatment of proteins. J. Biol. Chem., 239, 2878.

Carter, C. E. and Greenstein, J. P. (1946a). A spectrophotometric method for the determination of dehydropeptidase activity. J. Biol. Chem., 165, 725.

Carter, C. E. and Greenstein, J. P. (1946b). Spectrophotometric determination of dehydropeptidase activity in normal and neoplastic tissues. J. Nat. Cancer Inst., 7, 51.

Corfield, M. C., Wood, C., Robson, A., Williams, M. J. and Woodhouse, J. M. (1967). Formation of lysinoalanine during the treatment of wool with alkali. Biochem. J., 103, 15c.

Dakin, H. D. (1912). The racemization of proteins and their derivatives resulting from tautomeric change. J. Biol. Chem., 13, 357.

DeGroot, A. P. and Slump, P. (1969). Effects of severe alkali treatment of proteins on amino acid composition and nutritive value. J. Nutr., 98, 45.

DeGroot, A. P., Slump, P., Van Beek, L., and Feron, V. J. (1976). Severe alkali treatment of proteins. In "Proteins for Humans: Evaluation and Factors Affecting Nutritional Value." C. E. Bodwell, ed., Avi, Westport, Conn.

Derevitskaya, V. A., Vafina, M. G. and Kochetkov, N. K. (1967). Synthesis and properties of some serine glycosides. Carbohyd. Res., 3, 377.

Downs, F. and Pigman, W. (1976). Determination of O-glycosidic linkages to L-serine and L-threonine residues of glycoproteins. Meth. Carbohydr. Chem., 7, 200.

Feeney, R. E. (1974). A biological antifreeze. Am. Scientist, 62, 712.

Feeney, R. E. (1977). Chemical modification of food proteins. In "Improvement of Food Proteins Through Chemical and Enzymatic Modifications," R. E. Feeney and J. R. Whitaker, eds., Advances in Chemistry Series, Am. Chem. Soc., Washington, D. C.

Gallop, P. M., Blumenfeld, O. O. and Seifter, S. (1972). Structure and metabolism of connective tissue proteins. Ann. Rev. Biochem., 41, 617.

Geschwind, I. I. and Li, C. H. (1964). Effects of alkali-heat treatment on β-melanocyte-stimulating hormone. Arch. Biochem. Biophys., 106, 200.

Gottschalk, A. (1972). "Glycoproteins," Elsevier Publ. Co., New York, N.Y.

Gross, E. (1977). The chemistry and biology of amino acids in food proteins: lysinoalanine. In "Improvement of Food Proteins Through Chemical and Enzymatic Modifications," R. E. Feeney and J. R. Whitaker, eds., Advances in Chemistry Series, Am. Chem. Soc., Washington, D. C.

Horn, M. J., Jones, D. B. and Ringel, S. J. (1941). Isolation of a new sulfur-containing amino acid (lanthionine) from sodium carbonated-treated wool. J. Biol. Chem., 138, 141.

Isbell, H. S. (1944). Interpretation of some reactions in the carbohydrate field in terms of consecutive electron displacement. J. Res. Nat. Bur. Stand., Sect. A, 32, 45.

Isbell, H. S., Linek, K. and Hepner, K. E. Jr. (1971). Transformations of sugars in alkaline solutions: II. Primary rates of enolization. Carbohydr. Res., 19, 319.

Lee, H. S., Osuga, D. T., Nashef, A. S., Ahmed, A. I., Whitaker, J. R. and Feeney, R. E. Effect of alkali on glycoproteins: β-Elimination and nucleophilic addition reactions of substituted threonyl residues. Submitted for publication (1976).

Levene, P. A. and Bass, L. W. (1928). Studies on racemization. VII. The action of alkali on casein. J. Biol. Chem., 78, 145.

Mayo, J. W. and Carlson, D. M. (1970). Effect of alkali and sodium borohydride at alkaline pH on N-acetylchondrosine: reduction vs cleavage. Carbohyd. Res., 15, 300.

Mecham, D. K. and Olcott, H. S. (1949). Phosvitin, the principal phosphoprotein of egg yolk. J. Am. Chem. Soc., 71, 3670.

Mellet, P. (1968). Influence of alkali treatment on native and denatured proteins. Text. Res. J., 38, 977.

Miro, P. and García-Domínquez, J. J. (1973). Action of ammonium and sodium hydroxides on keratin fibers in relation to their morphological structure. J. Soc. Dyers Colour., 89, 137.

Nashef, A. S., Osuga, D., Lee, H. S., Ahmed, A. I., Whitaker, J. R. and Feeney, R. E. Effects of alkali on proteins. Disulfides and their products. Submitted for publication (1976).

Ozeki, T. and Yosizawa, Z. (1971). Glycopeptides isolated from bovine submaxillary mucin. Arch. Biochem. Biophys., 142, 177.

Parisot, A. and Derminot, J. (1970). Formation of amino acids in wool treated with 0.1 N sodium hydroxide at various temperatures. Bull. Inst. Text. Fr., 24, 603.

Patchornik, A. and Sokolovsky, M. (1964). Nonenzymatic cleavages of peptide chains at the cysteine and serine residues through their conversion into dehydroalanine. I. Hydrolytic and oxidative cleavage of dehydroalanine residues. J. Am. Chem. Soc., 86, 1206.

Pickering, B. T. and Li, C. H. (1964). Adrenocorticotropins XXIX. The action of sodium hydroxide on adrenocorticotropin. Arch. Biochem. Biophys., 104, 119.

Pigman, W. and Moschera, J. (1973). Uses and misuses of bases in studies of glycoproteins. In "Carbohydrates in Solution," Advan. Chem. Ser., 117, 220.

Pisano, J. J., Finlayson, J. S., Peyton, M. P. and Nagai, Y. (1971). ε-(γ-Glutamyl)lysine in fibrin: Lack of crosslink formation in factor XIII deficiency. Proc. Nat. Acad. Sci. USA, 68, 770.

Plantner, J. J. and Carlson, D. M. (1975). Studies of mucin-type glycoproteins. Olefinic amino acids, products of the β-elimination reaction. Anal. Biochem., 68, 153.

Price, V. E. and Greenstein, J. P. (1947). Dehydropeptidase activity in certain animal and plant tissues. J. Biol. Chem., 171, 477.

Provansal, M. M. P., Cuq, J. -L. A. and Cheftel, J. -C. (1975). Chemical and nutritional modifications of sunflower proteins due to alkaline processing. Formation of amino acid cross-links and isomerization of lysine residues. J. Agric. Food Chem., 23, 938.

Robson, A. and Zaidi, Z. H. (1967). The formation of lysinoalanine during the treatment of silk fibroin with alkali. J. Text. Inst. Trans., 58, 267.

Sen, L. C., Gonzalez Flores, E., Whitaker, J. R. and Feeney, R. E. Reactions of phosphoproteins in alkaline solutions. Submitted for publication (1976).

Simpson, D. L., Hranisavljevic, J. and Davidson, E. A. (1972). β-Elimination and sulfite addition as a means of localization and identification of substituted seryl and threonyl residues in proteins and proteoglycans. Biochemistry, 11, 1849.

Taborsky, G. (1974). Phosphoproteins. Adv. Protein Chem., 28, 1.

Tanaka, K., Bertolini, M. and Pigman, W. (1964). Serine and threonine glycosidic linkages in bovine submaxillary mucin. Biochem. Biophys. Res. Comm., 16, 404.

Tannenbaum, S. R., Ahern, M. and Bates, R. P. (1970). Solubilization of fish protein concentrate. Food Technol. (Chicago), 24, 604.

Tanzer, M. L. (1973). Cross-linking of collagen. Science, 180, 561.

Wakabayashi, K. and Pigman, W. (1974). Synthesis of some glycodipeptides containing hydroxyamino acids, and their stabilities to acids and bases. Carbohyd. Res., 35, 3.

Whiting, A. H. (1971). Isolation of lysinoalanine from the protein-polysaccharide complex of cartilage after alkali treatment. Biochim. Biophys. Acta, 243, 332.

Ziegler, K. (1964). New cross-links in alkali-treated wool. J. Biol. Chem., 239, 2713.

Ziegler, K., Melchert, I. and Lürken, C. (1967). N_{δ}-(2-amino-2-carboxyethyl)-ornithine, a new amino-acid from alkali-treated proteins. Nature (London), 214, 404.

11

^{35}S-SULFIDE INCORPORATION DURING ALKALINE TREATMENT OF KERATIN AND ITS RELATION TO LANTHIONINE FORMATION

S. H. Feairheller, M. M. Taylor, and D. G. Bailey

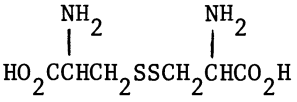
Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, Pennsylvania 19118

ABSTRACT

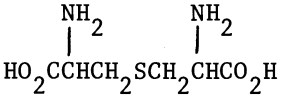
Cattle hair exposed to solutions of ^{35}S-sulfide ions at pH 12.5, hydrolyzed with acid, and analyzed for amino acids with simultaneous measurement of the radioactivity of the eluate from the analytical column showed 87 percent of the radioactivity incorporated in the hair in three amino acids: cysteic acid, lanthionine, and cystine. This and other evidence presented lend further support to the intermediacy of dehydroalanyl residues formed by a β-elimination reaction in the conversion of cystinyl residues to lanthionyl residues in proteins. The presence of radioactively labelled cystine in the hydrolyzate indicates that the dehydroalanyl residues are also capable of reforming cystinyl residues under these same conditions through a series of reversible reactions.

INTRODUCTION

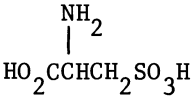
Conditions and reagents used to remove hair or wool from animal hides and skins in the manufacture of leather cause the same reactions of the sulfur-containing amino acids as those described in the papers both preceding and following this one in the Symposium. These alkaline solutions (pH 12.5) of sulfide ions convert cystinyl residues in the keratin to lanthionyl and/or lysinoalanyl residues, depending on the quantities of sulfide ions present. If sufficient sulfide ions are present most of the hair or wool is completely dissolved. If limited amounts are present



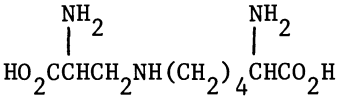
Cystine



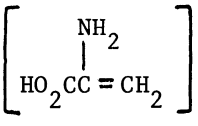
Lanthionine



Cysteic Acid



Lysinoalanine



Dehydroalanine

the hair or wool remains intact but its attachment to the hide or skin is considerably weakened, substantial conversion of the cystinyl residues to lanthionyl and lysinoalanyl residues takes place, and the hair or wool protein becomes more resistant to further attack by the reagents.

In early investigations, the formation of the lanthionyl residues was considered responsible for the increased resistance of the treated hair (Merrill, 1956; Windus and Showell, 1968). Subsequent observation that lysinoalanyl residues were also formed led to consideration of their involvement, the proposal that a β-elimination reaction is the initial step in these conversions,

and that dehydroalanyl residues are intermediates in the reactions (Feairheller et al., 1972). Further supporting evidence was obtained when the addition of mercaptans and secondary amines to these reaction mixtures resulted in the chromatographic detection of S-substituted cysteines and N-substituted β-aminoalanines (the products expected from the addition of these added compounds to the dehydroalanyl residues) in hydrolyzates of the hair (Feairheller et al., 1976). These authors also presented evidence suggesting the reversibility of these reactions or, at least, the interconversions of the sulfur-containing amino acids. This evidence and the results of our further investigations in this area are described in this report.

EXPERIMENTAL

Experiment Using $Na_2^{35}S$*

Five mg of thoroughly washed and solvent degreased cattle hair, 8 mg of $Ca(OH)_2$, and 0.9 ml of water were placed in a glass hydrolysis tube and to this was added 0.1 ml of a solution of 0.5 mg of $Na_2{}^{35}S$ (specific activity 9.2 mCi/mM)**. The resulting mixture was allowed to stand for three days. The solution was then removed from the residual hair and the hair was washed ten times with distilled water using a pipette and without removing the hair from the tube. The hair was washed once with ten percent hydrochloric acid solution and finally 2.5 ml of 6N hydrochloric acid solution were added. The contents of the tube were frozen in a dry ice-acetone bath, the tube was sealed under vacuum and the sealed tube and contents were then heated at 105°C for 18 hr. The tube was opened and the contents were evaporated to dryness under a stream of nitrogen. The residue was taken up in exactly 1 ml of 0.1N hydrochloric acid solution for analysis. The solution was analyzed for amino acid composition on a single column system (Piez and Morris, 1960) using a continuous gradient elution buffer. A stream splitting device was used to divert 25% of the eluate from the analytical column through the normal analyzer detection system. The remaining 75% simultaneously flowed through the detector of a liquid scintillation system. The overall system

---

*Experimental conditions for all other reactions discussed can be found in Feairheller et al., 1972; and Feairheller et al., 1976.

**Caution must be exercised in the use of sulfides because of the toxic nature of the gas (H_2S) generated on acidification. All radioisotopes must be handled in accordance with prescribed AEC guidelines for safe use.

was precalibrated with known samples of radioactively labelled amino acids. Excellent correlation was obtained between the elution times of the amino acids and the peaks of radioactivity. Fractions of the effluent from the scintillation counter containing the radioactivity were mixed with solutions of the appropriate amino acid and rechromatographed to further substantiate the correlation.

RESULTS AND DISCUSSION

Previous work (Feairheller et al., 1972; Feairheller et al., 1976) indicated that the addition of sulfide ions to these reaction mixtures of alkali-treated hair keratin had a threefold effect which was contradictory to the sole involvement of lanthionine in this increase in the stability of the hair protein. Our present study confirmed this effect (Table 1). First, the addition of sulfide ions caused a decrease in the resistance of the hair to further dissolution. This effect is well known to leather chemists (sodium sulfide is usually added to "unhairing" baths prior to addition of the alkali). Second, the amount of lanthionine formed increased in the presence of added sulfide ions. In some cases the amount actually doubled. Finally, the amount of lysinoalanine formed decreased to a trace or less in the presence of added sulfide ions. The amount of cystine lost from the hair keratin as a result of these treatments did not vary greatly, regardless of whether sulfide ions were present or not. Clearly, all of these treatments render the hair considerably more resistant to further dissolution; however, there is a significant two- or threefold difference between those samples exposed to sulfide ions and those not exposed.

We determined what the involvement of the sulfide ion was in these reactions by using ^{35}S-sulfide in the reaction mixtures, isolating the exposed hair, hydrolyzing it in preparation for amino acid analysis, chromatographically analyzing the hydrolyzate, and measuring the amino acid content and radioactivity simultaneously using a stream-splitting device on the analyzer (Table 2).

The lanthionine contained a substantial amount of ^{35}S, indicating that the reactions by which it was formed permitted the incorporation of sulfide ion or some other form of sulfur derivable from sulfide ion under these conditions. Its specific activity was about 45% that of the added sulfide. The oxidized product, cysteic acid, had by far the highest specific activity, approaching (94%) that of the added sulfide and indicating that the reactions leading to its formation involve exclusive incorporation of sulfur in some form from solution, rather than from sulfur originally present in the hair keratin. This is especially significant since cysteic acid is a final product in the reaction sequence.

TABLE 1

Crosslinking Amino Acid Content of Alkali-Treated Hair Keratin and Resistance to Further Degradation

Alkali Treatment			Amino Acids (As % of Original Cystine)				Solubility[e] (As % of Sample Exposed)
Reagent(s)[a]	Time (Days)	pH	$(Cys)_2$	Lan[b]	Lya[c]	Total	
Control	-	-	100	0	0	100	81
$Ca(OH)_2$	1	12.4	37	30	4	71	6
$Ca(OH)_2$	5	12.4	26	38	8	72	5
$Ca(OH)_2 + Na_2S$	3	12.4	25	59	0	84	10
$Ca(OH)_2 + Na_2S$[d]	3	12.4	23	55	0	78	18
NaOH	1	12.6	51	25	4	76	6
$NaOH + Na_2S$	1	12.0	47	38	0	85	11

[a]Refer to Feairheller et al. (1972) for experimental details. [b]Lanthionine. [c]Lysinoalanine. [d]Double amount of Na_2S. [e]Hair recovered from alkali treatments weighed and shaken vigorously in a solution which was 2% by weight of sodium hydroxide and 1.5% by weight of sodium sulfide at 40°C for one hr. Remaining hair was recovered, washed, dried and weighed.

Table 2

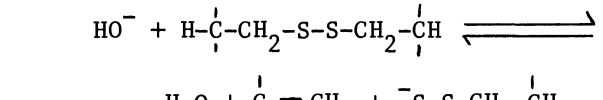
Distribution of Sulfur-Containing Amino Acids and Radioactivity

Amino Acid	Quantity	Specific[a] Activity
	(mM)	(mCi/mM)
Cystine	.32	3.66
Lanthionine	.56	4.18
Cysteic Acid	.18	8.67

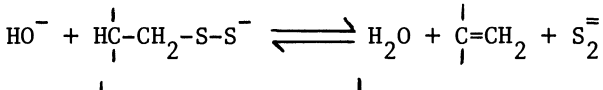
[a]Specific activity of added $Na_2^{35}S$:9.2mCi/mM.

While not as heavily labelled as the other two amino acids, the cystine itself contained a significant amount of ^{35}S, about 40% of the specific activity of the added sulfide. The sequence of reactions involved in its decomposition permits its reformation from some intermediate which has acquired sulfur from solution. Its reformation could take place by a series of reversible reactions or a separate sequence.

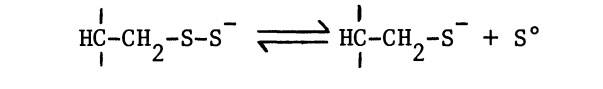
A group of reactions, some of which can be used to explain these results, is set forth in equations 1-8. The evidence is overwhelming (Danehy, 1971) that the β-elimination reaction resulting in the formation of dehydroalanyl residue, equation 1 is the initial reaction. This residue can take part in a number of



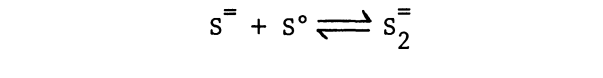
$$HO^- + H\text{-}\underset{|}{\overset{|}{C}}\text{-}CH_2\text{-}S\text{-}S\text{-}CH_2\text{-}\underset{|}{\overset{|}{C}}H \rightleftharpoons H_2O + \underset{|}{\overset{|}{C}}=CH_2 + {}^-S\text{-}S\text{-}CH_2\text{-}\underset{|}{\overset{|}{C}}H \quad (1)$$



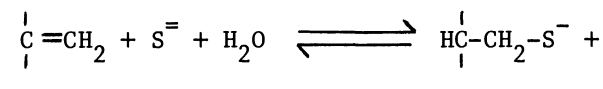
$$HO^- + H\underset{|}{\overset{|}{C}}\text{-}CH_2\text{-}S\text{-}S^- \rightleftharpoons H_2O + \underset{|}{\overset{|}{C}}=CH_2 + S_2^= \quad (2)$$



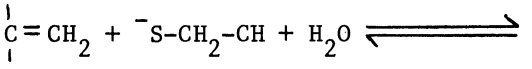
$$H\underset{|}{\overset{|}{C}}\text{-}CH_2\text{-}S\text{-}S^- \rightleftharpoons H\underset{|}{\overset{|}{C}}\text{-}CH_2\text{-}S^- + S^\circ \quad (3)$$

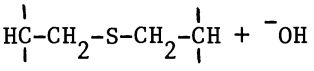


$$S^= + S^\circ \rightleftharpoons S_2^= \quad (4)$$

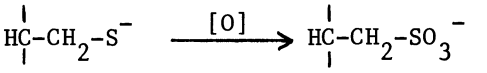


$$\underset{|}{\overset{|}{C}}=CH_2 + S^= + H_2O \rightleftharpoons H\underset{|}{\overset{|}{C}}\text{-}CH_2\text{-}S^- + {}^-OH \quad (5)$$

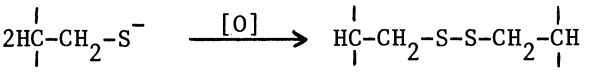




(6)



(7)



(8)

addition reactions, including the reformation of cystine by the reverse of this reaction by which it is formed. In the intact hair keratin, the reversibility of the reaction would be enhanced by the close proximity of the two residues. Other reactions of the dehydroalanyl residues are illustrated in equations 5 and 6. An additional reaction, not shown, is the formation of lysinoalanyl residues by reaction with nearby lysinyl residues.

Equations 2 and 3 illustrate alternative modes of decomposition of the second product of the reaction in equation 1. Neither equation 2 nor 3 is known to have any precedent. However, if they do occur and are reversible, they would account for the incorporation of either ionic or elemental sulfur from solution. These forms of sulfur are in equilibrium by way of the reaction shown in equation 4.

The addition of sulfide ion to a dehydroalanyl residue, equation 5, is to be expected under these conditions, as is the addition of the cysteinyl residue anion, equation 6. This sequence would obviously lead to the incorporation of the added sulfide ion into lanthionine, and its presence might be expected to compete very well with lysinyl residues in the addition reactions and thus hinder or limit the formation of lysinoalanyl residues. Only a finite number of the lysinyl residues, which are fixed in the protein, are available, while the sulfide ions are present in excess and free in solution. Nothing is known about the reversibility of equation 5 at this time. Evidence for the reversibility of the reaction in equation 6 is given later. The β-elimination reaction of a lanthionyl residue, the reverse of equation 6, would indicate that it is not the stable crosslink it is commonly thought to be (Danehy, 1971). The reversal of the reaction in equation 5 is unlikely, since it results in the accumulation of a second negative charge on an atom already containing one full negative charge. At pH 12.5, the sulfur would not be protonated and this elimination reaction is unlikely. A similar argument could be used against the forward reaction in equation 2, but in this case the negative charge is spread over two sulfur atoms.

Excess sulfide ions would strongly favor the forward reaction of equation 5 as opposed to the reverse reaction, regardless of the other factors involved, and could successfully compete with the cysteinyl residue anions in the addition reactions thus preventing the formation of lanthionyl residues, equation 6. However, as dictated by their mode of formation, the dehydroalanyl residues and cysteinyl residue anions are in close proximity to each other in the intact protein and are thus able to overcome the excess sulfide ions. Therefore the reaction in equation 6 takes place. This was not the case with the lysinoalanine formation where the sulfide ions successfully competed with the lysinyl residues and prevented the formation of lysinoalanyl residues.

These six reactions, in appropriate combination, along with the oxidation of the cysteinyl residues to cysteic acid residues, equation 7, would account for the ^{35}S incorporation found.

An alternative route to the formation of cystine that would also account for the incorporation of ^{35}S from sulfide ions in solution is the oxidative reaction, equation 8. Oxidation is taking place as indicated by the formation of cysteic acid. The reactions in equation 1, 5, 6, 7, and 8 could then account for the different products, but not the ^{35}S incorporation into lanthionine. This sequence of reactions would have resulted in the formation of lanthionine with a specific activity approaching that of the added sulfide, and this was not found. The reaction in equation 3 is almost certainly involved, and the lanthionine is formed from cysteinyl residue anions produced by this reaction as well as by the addition of sulfide to a dehydroalanyl residue, equation 5.

Therefore, the reactions most likely involved are those shown in equations 1, 3, 4, 5, 6, and 7, with the reactions shown in equations 1, 3, and 4 being completely reversible under the conditions that we used. The reactions shown in equations 2 and 8 are not as likely and are not necessary to explain the results. It is also unlikely that the reaction in equation 5 is reversible.

Finally, evidence presented by Feairheller et al. (1976) bears on the reversibility of the reaction in equation 6. Feairheller et al. (1972) had shown that the conversion of cystinyl residues to lanthionyl residues in proteins was effected by sodium hydroxide solutions with a pH of about 12.5 as well as by saturated solutions of calcium hydroxide, commonly used in "unhairing" reactions at the same pH. However, Danehy (1971) concluded that cystine, the free amino acid, can not be converted to lanthionine under these same conditions. This appears to be substantiated on theoretical grounds. In the free amino acid, the amino group and negatively charged carboxylate anion attached to the α-carbon atom generate a high electron density and prevent the abstraction of the α-hydrogen atom by base. Exposure of cystine to solutions of

lithium, sodium, or potassium hydroxide at a pH of about 12.5 caused no reaction over a period of several days. However, exposure of cystine to a solution of either calcium or strontium hyroxide at the same pH resulted in reaction of the cystine and formation of lanthionine. Feairheller et al. (1976) suggested that these divalent metal ions are capable of forming complexes of sufficient stability with the cystine, involving the carboxylate anions and α-amino groups, to reduce the electron density about the α-carbon atom and thus permit the β-elimination reaction to take place. Magnesium and barium hydroxides were not effective. The lanthionine accounted for one-third of the cystine in these reaction mixtures after 48 hr but then it started to disappear. The cystine was completely consumed within 14 days and the lanthionine within 28 days. Other products detected were alanine, cysteic acid, ammonia, hydrogen sulfide, and 2-methylthiazolidine-2,4-dicarboxylic acid (Dann et al., 1957).

We next exposed lanthionine to the same conditions with calcium hydroxide and, as was indicated by the results of the previous experiments, it decomposed but did so initially at a much slower rate. After two days exposure, 86% of the lanthionine was recoverable. However, cystine was also present to the extent of 5% of the amount of the starting lanthionine or 36% of the lanthionine which had reacted. After seven days' exposure the amount of lanthionine recoverable had dropped to 20% of its original value and no cystine was detectable. The different metallic hydroxides had the same effect with lanthionine as they had with cystine. Calcium and strontium hydroxides caused the decomposition of lanthionine, while lithium, sodium, potassium, magnesium, and barium hydroxides did not.

There is no reason to suppose that a mechanism other than the β-elimination reaction is operative here and, if this is the case, it must be concluded that lanthionyl residues in proteins are capable of the same reactions. The reaction shown in equation 6 is therefore reversible, and one or both products of the reverse reaction are capable of being converted to cystine.

CONCLUSIONS

The involvement of the β-elimination reaction and the intermediacy of dehydroalanyl residues in reactions of hair keratin are further substantiated by the results of this study. The vulnerability of lanthionine to the same conditions adds still further support and indicates that this residue in proteins is not a completely inert crosslink. Its reaction in proteins through the elimination type mechanism is implied. The lanthionine was found to be more inert to these conditions than the cystine and did increase the

stability of the treated hair but, not unexpectedly, the lysinoalanine appears to represent the most inert of those crosslinks known to be present. It is also reasonable, in view of the reaction sequence discussed, that added sulfide ions should compete with lysinyl residues and favor formation of lanthionyl residues at the expense of lysinoalanyl residues.

REFERENCES

Danehy, J. P. (1971). The alkaline decomposition of organic disulfides and related nucleophilic displacements of sulfur from sulfur. Int. J. Sulfur Chem. B., 6, 103.

Dann, J. R., Oliver, G. L., and Gates, J. W. (1957). The reaction of cystine and lanthionine with aqueous calcium hydroxide. The identification of 2-methylthiazolidene-2,4-dicarboxylic acid. J. Am. Chem. Soc., 79, 1644.

Feairheller, S. H., Taylor, M. M., Filachione, E. M., and Windus, W. (1972). New amino acids formed in hair during unhairing. J. Am. Leather Chem. Assoc., 67, 98.

Feairheller, S. H., Taylor, M. M., Bailey, D. G., and Windus, W. (1976). Further evidence in support of the elimination reactions as the mechanism of alkaline unhairing. J. Am. Leather Chem. Assoc., in press.

Merrill, H. B. (1956). "Chemistry and Technology of Leather." Vol. II, Chapter 8 (O'Flaherty, F., Roddy, W. T., and Lollard, R. M., eds.), Reinhold Publishing Corp., New York, NY.

Piez, K. A., and Morris, L. (1960). A modified procedure for the automatic analysis of amino acids. Anal. Biochem., 1, 187.

Windus, W., and Showell, J. S. (1968). An interpretation of the mechanism of unhairing as a nucleophilic displacement. J. Am. Leather Chem. Assoc., 63, 258.

12

LYSINOALANINE FORMATION IN WOOL AFTER TREATMENTS WITH SOME PHOSPHATE SALTS

C. Touloupis and A. Vassiliadis

Department of Industrial Chemistry, University of Athens, I3A Navarinou Street, Athens (I44), Greece

I. ABSTRACT

Treatment of wool with solutions of sodium tripolyphosphate, potassium tetrapyrophosphate, and sodium phosphate results in the formation of lysinoalanine, the amount of which increases from the first to the last reagent. As this may be due to the different pH, solutions of the three salts of the same pH were also tried. This series of experiments suggests that although the hydroxyl ions contribute to the formation of lysinoalanine, the governing factor is the kind of the anions concerned, as the sodium phosphate acts in a more rigorous way than the two other salts, in spite of the smaller concentration and the faster reduction of the pH of the solution during treatment.

II. INTRODUCTION

Lysinoalanine N^{ε}-(DL-2-amino-2-carboxyethyl)-L-lysine, abbreviated in the following as LAL, has been isolated for the first time in I964 after alkaline treatment of S-dinitrophenyl-ribonuclease by Patchornic and Sokolovsky (I964), who suggested that its formation was due to the reaction of the ε-aminogroup of lysine with dehydroalanine.

At the same time it was found (Bohak, I964) that LAL was formed from other proteins, also after alkaline treatments, and its structure was established by independent synthesis from N^{α}-carbobenzyloxy-L-lysine and N-phenylacetyldehydroalanine.

In the same year Ziegler (I964,I965) reported the formation

of LAL in wool after treatments with a number of alkaline reagents and suggested that dehydroalanine is formed from either cystine or serine.

The possibility of dehydroalanine formation from cystine and serine has been investigated by several workers (Mellet and Swanpoel, I965; Sotiriou-Provata and Vassiliadis, I966; Robson and Zaidi,I967; Mellet, I968; Williams and Mellet, I969; Ebert, I974) and it seems that both hypotheses are probable,because dehydroalanine can be formed from both aminoacids by a β-elimination reaction.

In the present work the formation of LAL in wool is studied after treatments with sodium tripolyphosphate, potassium tetrapyrophosphate or sodium phosphate, as well as with sodium carbonate, which has been studied extensively, for comparison reasons.

III. EXPERIMENTAL

Wool. Australian merino 64's has been used throughout this work.
Reagents. All reagents used were of analytical grade.
Determination of LAL. Miro and Garcia-Dominguez's (I967) method was used, but LAL was estimated directly on the strips by scanning at 520mμ with a Densicord-Photovolt Model 542 scanner.

IV. RESULTS AND DISCUSSION

A. Sodium carbonate. This reagent, which has been studied extensively in the past, was included in our experiments as a basis of comparison with the other reagents. From the results of this series, shown in Tables I and 2, it is clear that the amount of LAL formed increases with temperature and concentration of the solution. The latter may indicate that the reaction is pH dependent, as the pH of the solution increases with the concentration.

It is also evident that the LAL formed represents the maximum that could be expected, as calculated from the lysine content of wool given in the literature.

Reduction of the concentration of the solution, in order to achieve a pH of 9.4, reduces the amount of LAL formed to a considerable extent. And although this may be due to the lower pH, it seems more probable, when the next series of experiments is also taken into account, that this is the result of the absorption of carbonates by wool, which reduces the already low concentration of the reagent.

B. Phosphate salts. In this series of experiments wool was treated

Table I

Effect of concentration and temperature on the amount of lysinoalanine (LAL) formed in wool after treatment for one hour in solutions of sodium carbonate (liquor:wool = 60)

Concentration of the solution mmoles/l	pH of the solution	Temperature °C	LAL formed µmoles/g wool
I0	I0.00	50	6
20	I0.25	50	I0
30	I0.45	50	I7
50	I0.85	50	34
I0	I0.00	75	43
?0	I0.25	75	70
30	I0.45	75	93
40	I0.65	75	I09
50	I0.85	75	II8
I0	I0.00	I00	77
20	I0.25	I00	I38
30	I0.45	I00	209
50	I0.85	I00	267

Table 2

Effect of time on the amount of lysinoalanine (LAL) formed in wool after treatments in a solution of sodium carbonate (I mmole/l) at pH 9.4 (liquor:wool = 60)

Time of treatment h	pH of the solution after treatment	LAL formed µmoles/g wool
0.5	9.I5	4
I.0	9.I0	6
I.5	9.05	8
2.0	8.90	I0
2.5	8.80	I5

Table 3

Effect of concentration and temperature on the amount of lysinoalanine (LAL) formed in wool after treatments for one hour in solutions of sodium tripolyphosphate (liquor:wool = 60)

Concentration of the solution mmoles/l	pH of the solution	Temperature ^{o}C	LAL formed μmoles/g wool
10	9.10	50	0
20	9.25	50	0
30	9.45	50	0
50	9.55	50	0
10	9.10	75	3
20	9.25	75	5.5
30	9.45	75	6.5
40	9.50	75	7
50	9.55	75	7.5
10	9.10	100	3
20	9.25	100	5.5
30	9.45	100	6.5
50	9.55	100	7.5

Table 4

Effect of concentration and temperature on the amount of lysinoalanine (LAL) formed in wool after treatments for one hour in solutions of potassium tetrapyrophosphate (liquor:wool = 60)

Conventration of the solution mmoles/g	pH of the solution	Temperature ^{o}C	LAL formed µmoles/g wool
I0	9.8	50	0
20	I0.I	50	0
30	I0.7	50	3
50	I0.95	50	5
I0	9.8	75	7
20	I0.I	75	I0
30	I0.7	75	I2
40	I0.85	75	I2.5
50	I0.95	75	I3
I0	9.8	I00	7
20	I0.I	I00	I5
30	I0.7	I00	20
50	I0.95	I00	25

Table 5

Effect of concentration and temperature on the amount of lysinoalanine (LAL) formed in wool after treatments for one hour in solutions of sodium phosphate (liquor:wool = 60)

Concentration of the solution mmoles/g	pH of the solution	Temperature °C	LAL formed µmoles/g wool
10	10.45	50	4.5
20	11.2	50	7
30	11.3	50	8.5
50	11.5	50	17
10	10.45	75	9.5
20	11.2	75	20
30	11.3	75	29.5
40	11.45	75	37
50	11.5	75	42
10	10.45	100	17
20	11.2	100	30
30	11.3	100	60
50	11.5	100	104

Table 6

Effect of time on the amount of lysinoalanine (LAL) formed in wool after treatments in a solution of sodium tripolyphosphate 25mmoles/l at pH 9.4 (liquor:wool = 60)

Time of treatment h	pH of the solution after treatment	LAL formed µ moles/g wool
0.5	9.1	2
1.0	9.0	6.5
1.5	8.9	10.5
2.0	8.85	17
2.5	8.75	21.5

Table 7

Effect of time on the amount of lysinoalanine (LAL) formed in wool after treatments in a solution of potassium tetrapyrophosphate (3 mmoles/l) at pH 9.4 (liquor:wool = 60)

Time of treatment h	pH of the solution after treatment	LAL formed µmoles/g wool
0.5	9.2	2
I.0	9.05	6.5
I.5	8.95	I3
2.0	8.9	2I.5
2.5	8.8	30

Table 8

Effect of time on the amount of lysinoalanine (LAL) formed in wool after treatments in a solution of sodium phosphate (I.2 mmoles/l) at pH 9.4 (liquor:wool = 60)

Time of treatment h	pH of the solution after treatment	LAL formed µmoles/g wool
0.5	8.95	I3
I.0	8.8	2I.5
I.5	8.75	32
2.0	8.6	38.5
2.5	8.4	53.5

with solutions of sodium tripolyphosphate, potassium tetrapyrophosphate and sodium phosphate. The LAL formed is given in Tables 3 to 8. From the first three Tables, 3 to 5, it is evident that the influence of the reagent increases from tripolyphosphate to phosphate. Again, it could be claimed that this is due to the pH of the solutions. From Tables 6 to 8 however, a distinct difference between sodium phosphate and the other reagents is observed, although the pH is the same. Moreover, the concentration of the sodium phosphate is lower, and because of this the fall of the pH during treatment is much higher.

It is clear, therefore, that the type of the anion must contribute to the phenomenon. In favour of this view is also the fact that although the sodium phosphate solutions have a much higher pH than those of sodium carbonate (Tables 1 and 5), the LAL formed is lower, which would not be expected for a pH-dependent reaction.

Comparison of Tables 2 and 8 leads to the same couclusion, for again the LAL formed is higher in the case of phosphate, although the pH of its solution is at any time lower than of the carbonate solution.

C. The mechanism of the reaction: The accepted mechanism for the formation of LAL is by reaction of the ε-aminogroup of lysine with dehydroalanine, the latter being formed either from serine or cystine by a,β-elimination reaction. The suggested mechanisms by hydrolysis of cystine (Schöberl and Wiesner, I933, I940) or from cystine (Nicolet, I93I) can be considered as β-eliminations too.

The results of this work suggest, as it has already been reported (Touloupis and Vassiliadis, I975), that although β-elimination is surely involved, a substitution mechanism, through the anions of the reagents studied, is also possible.

REFERENCES

Bohak, Z. (I964). N^{ε}(DL-2-Amino-2-carboxyethyl)-L-Lysine, a new amino acid formed on alkaline treatment of proteins. J.Biol.Chem., 239, 2878-87.

Ebert, Ch. (I974). Chemical modification of keratins by methylation of amino acid side groups. Colloid Polym. Sci., 252, I00-I6.

Mellet, P. and Swanepoel, O. (I965). The modification of native and denatured keratin by alkali. Naturwissenschaften, 52, 495-6.

Mellet, P. (I968). Influence of alkali treatment on native and denatured proteins. Text. Res. J., 38, 977-83.

Miro, P. and Garcia-Dominguez, J. (I967). Simple method for lysinoalanine determination. Bull. Inst. Text. Fr., 2I,9I-I00.

Nicolet, B.H. (I93I). The mechanism of sulfur lability in cysteine and its derivatives. I. Some thio ethers readily split by alkali. J. Am. Chem. Soc. 53, 3066-72.

Patchornik, A. and Sokolovsky, M. (I964). Chemical interaction between lysine and dehydroalanine in modified bovine pancreatic ribonuclease. J. Am. Chem. Soc., 86, I860-6I.

Robson, A. and Zaidi, Z.H. (I967). The formation of lysinoalanine during the treatment of silk fibroin with alkali. J. Text. Inst., Trans., 58, 267-9.

Schöberl, A. and Wiesner, M. (I933). Model experiments on the oxidative degradation of biologically important organic sulfur compounds. Liebigs Ann. Chem., 507, III-27.

Schöberl, A. and Wiesner, M. (I940). Biochem. Z., 306, 269.

Sotiriou-Provata, M. and Vassiliadis, A. (I969). Treatments of wool in organic solvents and their effect on the ureabisulfite test. Text. Res. J., 36, I03I-36.

Touloupis, C. and Vassiliadis, A. (I975). On the mechanism of lysinoalanine formation in wool. C. R. Acad. Sci. Paris., 280, C 65-68.

Vassiliadis, A. (I963). Lanthionine formation in wool during treatments with sodium thiosulfate. Text. Res. J., 33, 376-8I.

Williams, M.J. and Mellet, P. (I969). Influence of alkali treatment on native and denatured proteins. Text. Res. J., 39, 789-90.

Ziegler, K. (I964). New cross-links in alkali-treated wool. J. Biol. Chem., 239, PC 27I3-I4.

Ziegler, K. (I965). The influence of alkali treatment on wool. Third Int. Wool Text. Res. Conference, Paris, 2, 403-I4.

13

THE FORMATION AND CLEAVAGE OF LYSINOALANINE CROSSLINKS

G. Ebert and Ch. Ebert

Institute of Polymers, University of Marburg/L
Lahnberge, Geb. H 3550 Marburg/L (West Germany)

ABSTRACT

Treatment of lysinoalanine (LAL) with methyl iodide at pH-values above 8 leads to cleavage to dehydroalanine (DHA) and Nε-di- or/and trimethyllysine, as was shown by ultraviolet spectra and amino acid analysis. This reaction is due to methylation of the crosslinking NH group of LAL to the quaternary ammonium compound; nucleophilic β-elimination follows. By this reaction, the ε-amino groups of polylysine and its copolymers can be methylated to form di- and/or trimethyl-lysine residues. Chemically modified polypeptides containing Nε-dimethyllysine can be made in this way.

INTRODUCTION

Some years ago we found that treating SH-containing proteins like keratins with CH_3I for blocking the thiol groups--especially after partial or total reduction of the cystine -S-S-bonds--forms peptide-bonded DHA through a Hoffmann elimination reaction of the dimethylsulfonium intermediate(1,2,3):

$$B^- + H\overset{\diagdown}{\underset{\diagup}{C}}\text{-}CH_2\text{-}\overset{+}{S}(CH_3)_2 \rightarrow BH + \overset{\diagdown}{\underset{\diagup}{C}}\text{-}CH_2\text{-}\overset{+}{S}(CH_3)_2 \rightarrow \overset{\diagdown}{\underset{\diagup}{C}} = CH_2 + S(CH_3)_2 \qquad (1)$$

The base B^- may be not only OH^- but also $H_2PO_4^-$, HPO_4^{-2}, etc. Therefore this process can take place even at pH 6. This α,β-unsaturated amino acid is formed in alkaline media, too; its formation and reactions have been investigated by several authors.

The double bond of the DHA residue reacts, according to Schöberl (4), with neighboring unreacted SH groups to form lanthionine. The ε-amino group of lysine also reacts with DHA, giving LAL (5,6). These two reactions,occuring in alkaline solutions of proteins with unmethylated free SH groups, are expected to take place also after the β-elimination of dimethylsulfonium cysteine. Therefore, the lysine content of the protein should be decreased by methylation with CH_3I, whereas the content of LAL should be increased.

RESULTS

Amino acid analyses of keratin fibers, untreated and after repeated reduction with thioglycolate and subsequent treatment with CH_3I, are shown in Table 1.

Table 1

The contents of lysine, lysinoalanine, and Nε-methylated lysines of keratin fibers after repeated reduction and methylation(7)

	Cys (Mol%)	Lys	Lal	Nε-mono meth lys	Nε-dimeth lys Nε-trimeth lys
untreated	10.8	3.50	—	—	—
1 x red. and meth.	3.5	2.72	0.78	≈ 0.05	0.10
2 x red. and meth.	2.8	1.89	0.69	0.13	0.85
6 x red. and meth.	0.8	1.52	0.37	0.25	1.45

The initial results seem surprising in that the content of LAL passes a maximum depending on the degree of repeated methylation, because it is expected to increase in parallel to the decreases of lysine and cysteine. However, the amino acid chromatogram obtained by the method for physiological liquids showed an additional peak between ammonia and lysine (Fig. 1) that became bigger with repeated methylation and the corresponding decrease of LAL.

This peak was attributed to Nε-methylated lysines. Paik and Kim (8) have developed a method for separating the Nε-methyllysines. By their method, indeed, a rather small peak of Nε-monomethyl-lysine and a bigger one corresponding to Nε-di- and trimethyl-lysine were observed (Fig. 2). This was confirmed by testing the pure Nε-methylated lysines. However, the dimethyl-lysine could not be separated from the trimethylated product (9).

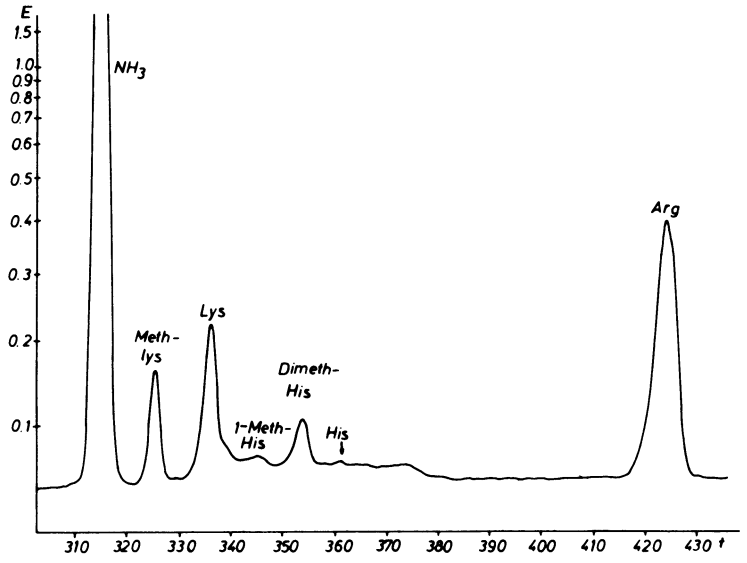


Figure 1. Amino acid chromatogram of keratin fibers after 6 x repeated reduction and methylation (physiological run).

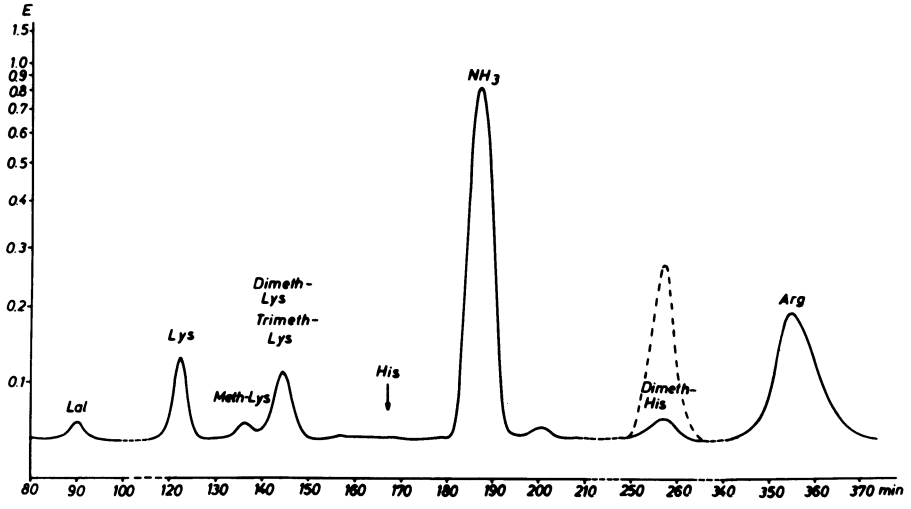
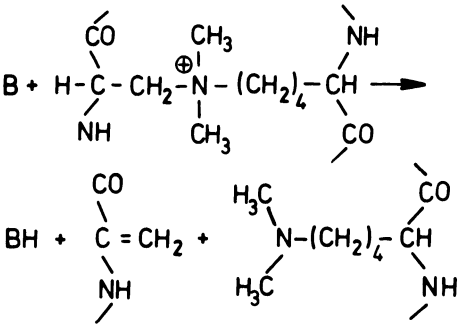


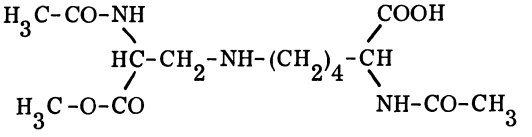
Figure 2. Amino acid chromatogram of keratin fibers after 6 x repeated reduction and methylation (0.8% cysteine). Method of Paik and Kim (8).

According to the amino acid analysis, the content of the Nε-methylated lysines, especially of di- and/or trimethyl-lysine, increases remarkably with repeated methylation as shown in the two right-hand columns of Table 1. Because with repeated methylation, the content of LAL decreases after passing a maximum (Table 1), whereas the lysine content decreases continuously, the Nε-methylated lysines appear unlikely to be formed by direct methylation. Direct methylation is also unlikely because at pH values of 8 or 9 the ε-amino group of lysine is protonated (pK-value 10.0). Therefore we conclude that the crosslinking NH-group of LAL is methylated to the quaternary ammonium compound, which then undergoes a nucleophilic β-elimination process like that of the cysteine-dimethylsulfonium ion:



(2)

To prove this cleavage mechanism of lysinoalanine by methylation, Nε-(2-acetamino-2-methoxy-carbonylethyl)-N-α-acetyllysine (I) was used as a model substance. It was treated with CH_3I on 0.01M phosphate buffer solution at constant temperature and pH. Because protons are consumed by the β-elimination reaction, the pH value had to be kept constant by using a pH-stat. As expected, the hydrolysate of the reaction product showed that besides LAL, the main product was di- or/and trimethyl-lysine (Fig. 3).



(I)

Nε-monomethyl-lysine was not observed after this reaction. Therefore the small amount of this compound present in the hydrolysates of methylated keratins (Fig. 2) may possibly be formed by a direct methylation of the amino side chain of lysine. The rather intense ammonia peak is very likely due to decomposition of free DHA by hydrolysis to pyruvic acid an ammonia. According to Gross and Morell (10) DHA absorbs in the ultraviolet region between 225 and

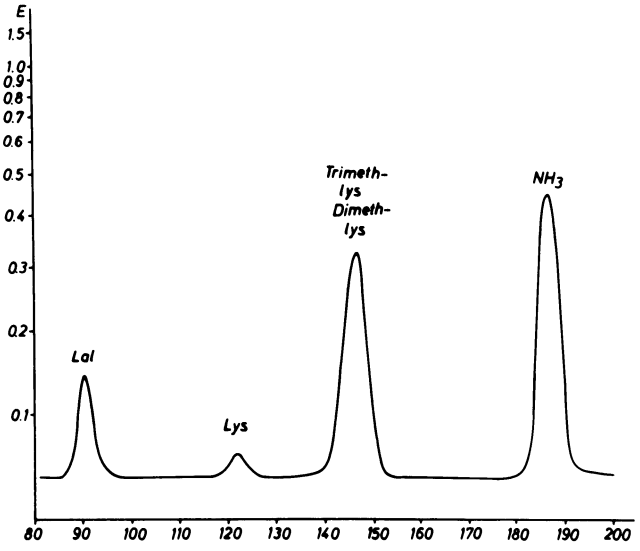
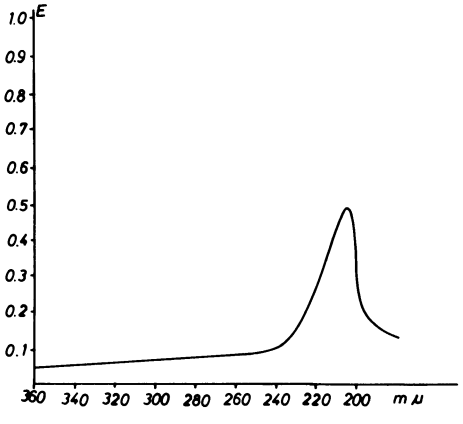


Figure 3. Amino acid chromatogram of methyl iodide treated (I).



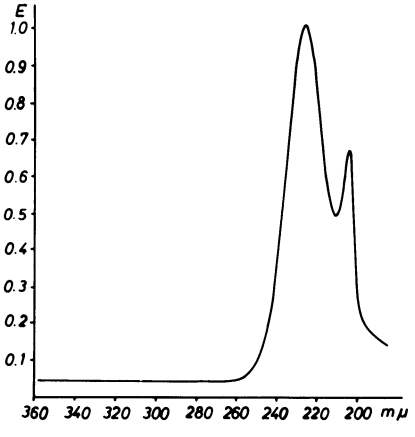


Figure 4. Absorption spectra
a) of Nε-(2-acetamino-2-methoxycarbonylethyl)-N-α-acetyllysine (I) in aqueous solution at pH 8.
b) after treatment of (I) with methyliodide.

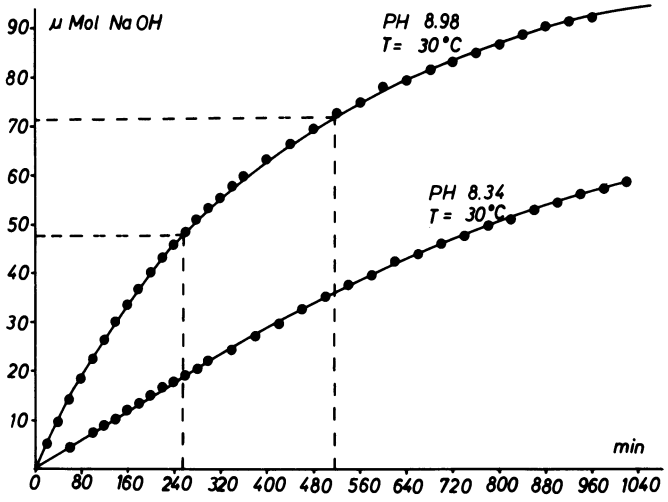


Figure 5a. Kinetic study of the cleavage of Nε-(2-acetamino-2-methoxycarbonylethyl)-N-α-acetyllysine by methylation at 30°C; pH 8.34 and 8.98.

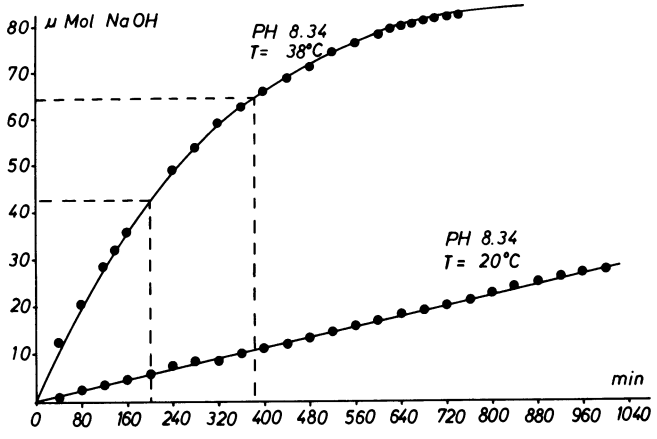


Figure 5b. Kinetic study of the same reaction as in Figure 5a; pH 8.38, T 20°C and 38°C.

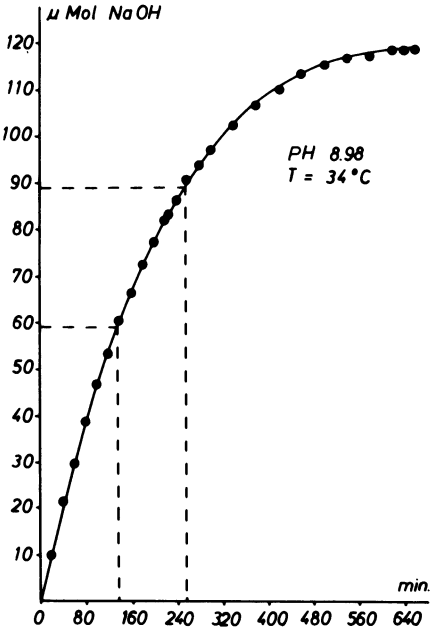


Figure 5c. Kinetic study as in Figure 5a; pH 8.98, T 34°C.

250 nm. Therefore one should expect that the solution obtained by methylation of (I) should have an absorption peak at these wavelengths due to N-acetylaminodehydroalanine methyl ester. This is indeed the case, as one can see from Figure 4b. These results show that the proposed cleavage mechanism of lysinoalanine is valid.

This reaction depends rather strongly on temperature and pH, as shown by kinetic measurments (Fig. 5c-c).

This cleavage reaction of LAL allows one to prepare polymers or copolymers of dimethyl-/trimethyl-lysine and lysine in the following way: Polylysine is treated with N-acetyldehydroalanine to form the corresponding polylysinoalanine derivative. Subsequent methylation forms the di- or/and trimethyl-lysine polymer. This reaction, which is now being investigated by Young-H Kim (12), allows study of the conformation stability of side-chain methylated lysines. These amino acids seem to play an important role during cell mitosis.

ACKNOWLEDGMENT

The authors are very much obliged to the Deutsche Forschungsgemeinschaft for financial support.

REFERENCES

1. Ebert, G. (1967). Kolloid Z. Z. Polym., 216/217, 306.
2. Ebert, Ch. (1967). Diplomarbeit, Frankfurt.
3. Ebert, G., Ebert, Ch. and Wendorff, J. (1970). Kolloid Z. Z. Polym., 237, 229.
4. Schöberl, A. (1942). Biochem. Z., 313, 314.
5. Bohak, Z. (1964). J. Biol. Chem., 239, 2878.
6. Ziegler, K. (1968). Communic. de la IIIème Congrès International de la Recherche Textile Laniere, Paris, France, II, 403.
7. Ebert, Ch. (1971). Thesis, Marburg.
8. Paik, W. K. and Kim, S. (1967). Biochem. et Biophys. Res. Commun., 27, 479.
9. Hempel, K., Lange, H. W. and Birkofer, L. (1969). Hoppe-Seylers Z. Physiol. Chem., 350, 966.
10. Gross, E. and Morell, J. L. (1968). FEBS Letters, 2, 61.
11. Gross, E., Morell, J. L. and Craig, L. C. (1969). Proc. Natl. Acad. Sci. U. S., 62, 952.
12. Ebert, G. and Kim, Young-Ha. To be published.

14

ON THE SPECIFIC CLEAVAGE OF CYSTEINE CONTAINING PEPTIDES AND PROTEINS

Ch. Ebert, G. Ebert, and G. Rossmeissl

Institute of Polymers, University of Marburg/L.

Lahnberge, Geb.H 3550 Marburg/L. (West Germany)

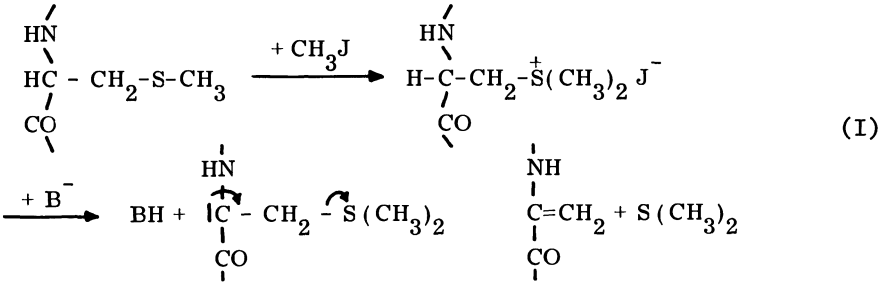
ABSTRACT

Addition of cysteine to the double bonds of polydehydroalanine and copolymers of dehydroalanine (DHA) and methylcysteine, alanine, leucine or γ- methyl-L-glutamate is accompanied by increased solubility and decreased molecular weight. This result is due to a peptide bond cleavage caused by formation of a thiazolidine as a consequence of nucleophilic attack by the sulfur atom on the preceding C=O group and subsequent splitting of the peptide bond by nucleophilic attack of an OH^- ion. This mechanism is predominant in alkaline media; in acid another mechanism is favoured; carbonyl-oxygen attacks the carbonyl-C-atom of the cysteine residue, forming a second ring system. Addition of one water molecule then yields two peptide fragments, one of them a terminal cysteine residue. Both mechanisms could be confirmed in the case of γ-L-glutamyl-dehydroalanyl-glycine by adding cysteine. Furthermore, it could be shown that SH-glutathione is decomposed at elevated temperatures according to the two mechanisms mentioned. This SH-induced pepetide bond cleavage can be used for selective peptide chain splitting of cysteine-containing polypeptides and proteins under relatively mild conditions.

INTRODUCTION

We have tried to prepare polylanthionine and copolymers of this amino acid by adding cysteine and H_2S to the double bond of polydehydroalanine. For this we used poly-S-methylcysteine and--because of the very poor solubility of this copolymer--copolymers with

γ-methyl-L-glutamate or L-alanine, made by polymerization of the respective N-carboxy-anhydrides (1-3). By treatment with methyl iodide, the S-methylcysteine was methylated to dimethylcysteine-sulfonium ion, which undergoes nucleophilic β-elimination (Hoffmann elimination) to form peptide-bonded dehydroalanine (4-6).



(I)

By adding cysteine, one should obtain single chain molecules of polanthionine or its copolymers, by adding H_2S, however, a multi-chain crosslinked poly-lanthionine, etc.

RESULTS

The degree of polymerization of the polymers used was about 100. Because of the insolubility of the polymers in the solvents used for methylation, the reactions took place in heterogeneous systems. However, during addition of cysteine to the DHA, the polymers became soluble and the molecular weight decreased. This behaviour can be explained as due to cleavage of the polypeptide chain. The case of a S-methylcysteine/γ-methylglutamate - 1:1 copolymer is given as an example. Figure 1 shows the amino acid chromatogram of the hydrolysed polymer.

This copolymer was treated with methyl iodide according to the reaction scheme (I) for transferring the S-methyl-residues into DHA. Thereafter, cysteine was added to get single-chain, peptide-bonded lanthionine. The polymer became soluble; this solution was subjected to amino acid analysis without hydrolysis (Fig. 2). Besides cysteine and cysteic acid, glutamic acid and lanthionine were also present. Some other peaks probably may be attributed to small peptides formed by cleaving the peptide chain. After hydrolysis these minor peaks were absent (Fig. 3). The S-methylcysteine (SMC) peak became very small (Cf. Fig. 1). About 95% of the SMC has been converted to DHA by methylation and elimination.

We have explained the cleavage mechanism in the following way (2,3). If the mobility of the peptide chain is high enough, the sulfur atom of cysteine is able to attack the carbon atom of the preceding carbonyl group, forming a five-membered ring. By the subsequent attack of OH^- --cysteine addition was carried at pH 8.5--

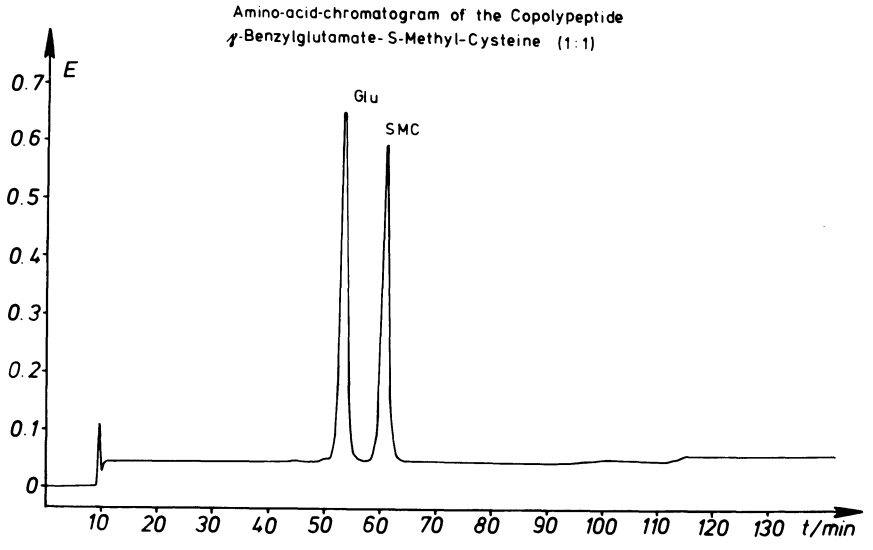


Figure 1. Amino acid chromatogram of Poly(γ-methylglutamate-S-methylcysteine) 1:1.

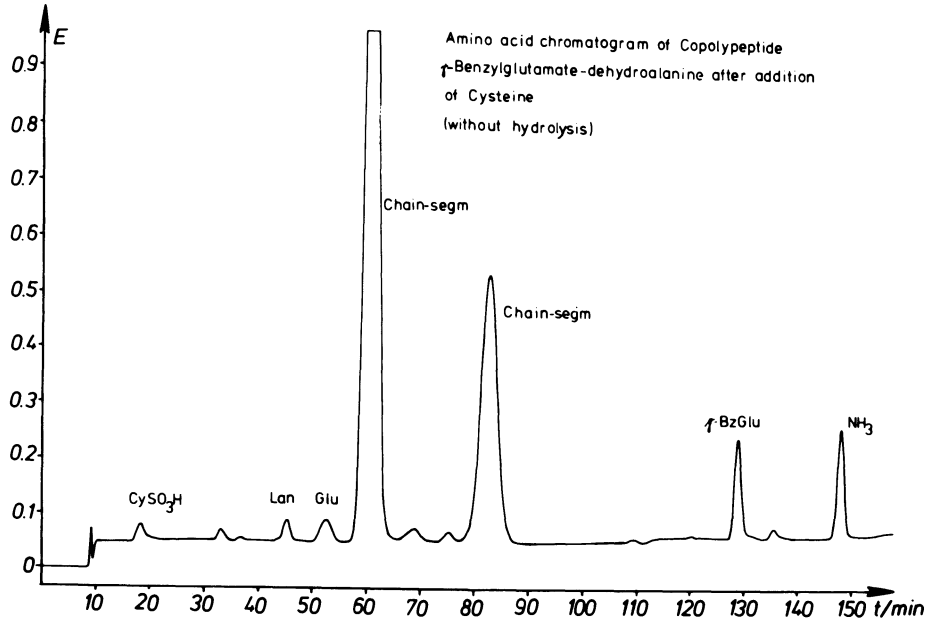


Figure 2. Amino acid chromatogram of the solution of Poly(γ-methylglutamate-dehydroalanine) after treatment with cysteine (unhydrolyzed sample).

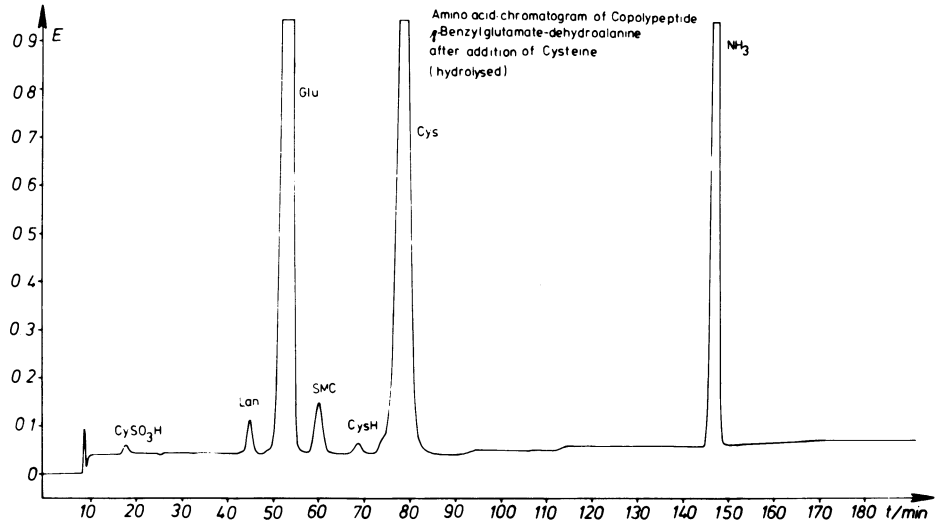
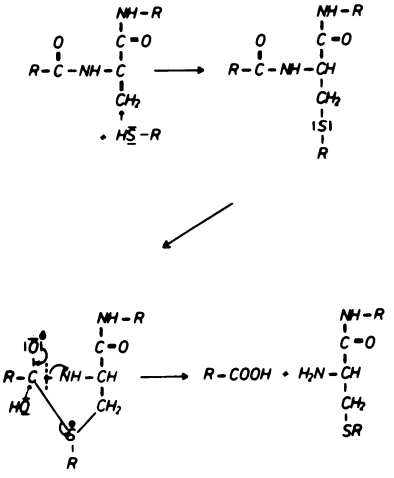


Figure 3. Amino acid chromatogram of the soluble fraction as in Figure 2, but after hydrolysis.

this peptide bond in the ring is cleaved as shown in the following reaction scheme.



To prove this mechanism, we have used SH-glutathione as a model substance not only because it is a small molecule with a well known sequence, but also because its single SH group per molecule allows one to draw definite conclusions with respect to the reaction mechanism. Because glutathione is soluble, the reaction can be performed in a homogeneous system. Furthermore, the SH-glutathione

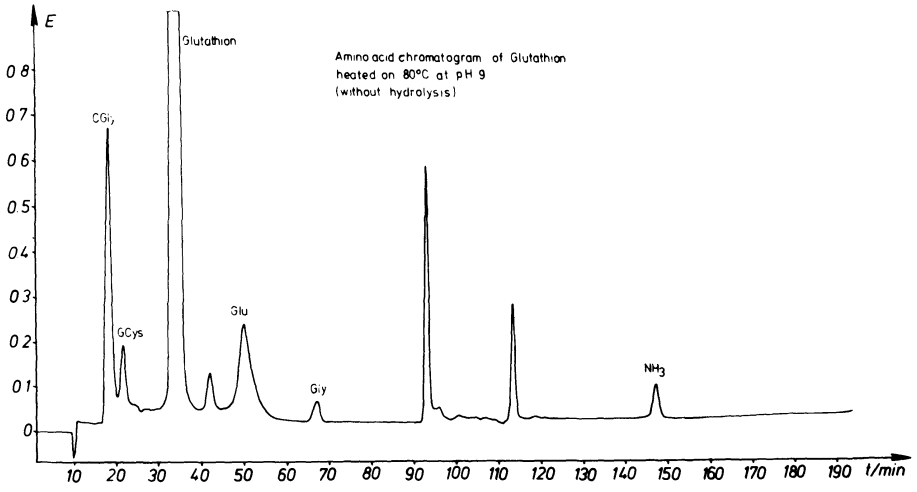
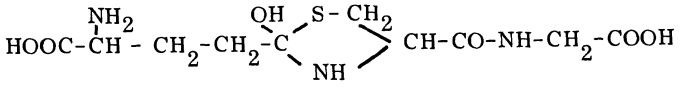


Figure 4. Amino acid chromatogram of a solution of SH-glutathione stored at pH 9.0 and 80°C.

is relatively stable toward oxidation because it is stabilized by forming a thiazolidine ring:



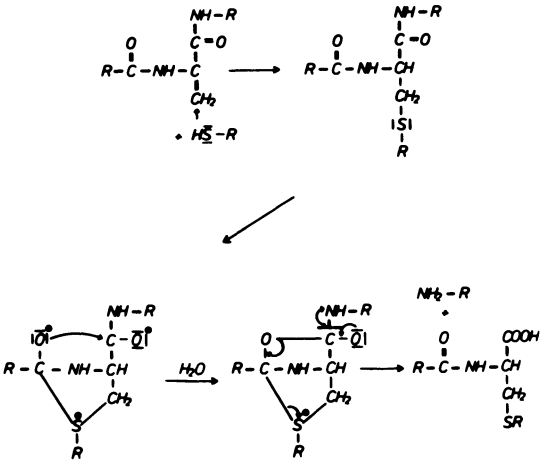
Two kinds of experiments on SH-glutathione were carried out.
1) The cysteine residue was changed into the DHA residue as mentioned above (I); cysteine was then added to the double bond.
2) SH-glutathione was dissolved in buffer solutions of various pH values and stored at an elevated temperature (80°C) to see if cleavage also occurs under these conditions.

In both cases amino acid analysis of the reaction product without hydrolysis showed the presence of free amino acids. This means that the peptide bond is cleaved under both conditions. The reaction products obtained in acid and in alkaline media are different. In an alkaline medium the free acid is mainly glutamic acid. In an acid solution, however, glycine is the main product. For example, in Figure 4, glutamic acid and a small amount of glycine are to be seen after 34 min besides unreacted SH-glutathione. The dipeptides cysteinyl-glycine (18 min) and γ-glutamylcysteine (22 min) appear in front of the SH-glutathione.

From these results is follows that at acid pH the cleavage mechanism differs from that in alkaline solution. It very likely takes place in the following way:

glutathione
glutamyl-cysteine
glycine
glutamic acid
cysteinyl-glycine
P_H 5.0
P_H 9.0

Figure 5



As in alkaline media, a five-membered ring is formed by attack of a free electron-pair to the preceding carbonyl group. However, at acid pH-values, the electronegativity of this carbonyl-oxygen atom is high enough to form a second five membered ring with the carbonyl C of the following peptide group, which is cleaved subsequently.

Figure 5 summarizes the reaction products due to cleavage of the SH-glutathione-peptide-chain at different pH-values.

We are now studying the cysteine-induced peptide-bond cleavage with other polypeptides. Preliminary results with the A-chain of insulin show that in the denatured state, this polypeptide too is cleaved in these ways.

ACKNOWLEDGMENT

The authors are grateful to the Deutsche Forschungsgemeinschaft for financial assistance.

REFERENCES

1. Rossmeissl, G. (1973). Diplomarbeit, Marburg.
2. Ebert, Ch., Ebert, G. and Rossmeissl, G. (1976). Proc. Fifth Intern. Wool Textile Conf., Aachen, West Germany, III, 97.
3. Rossmeissl, G. (1976). Thesis, Marburg.
4. Ebert, Ch. (1967). Diplomarbeit, Frankfurt.
5. Ebert, G., Ebert, Ch. and Wendorff, J. (1970). Kolloid Z. Z. Polym., 237, 229.
6. Ebert, Ch. (1971). Thesis, Marburg.
7. Utzinger, G. E., Strait, L. A. and Tuck, L. D. (1963). Experientia, 19, 324.
   Utzinger, G. E. (1963). Experientia, 19, 327.

15

REACTIONS OF PROTEINS WITH DEHYDROALANINES

Mendel Friedman, John W. Finley and Lai-Sue Yeh

Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Berkeley, California 94710

ABSTRACT

Reactions of proteins with dehydroalanine or derivatives of dehydroalanine were studied as models for protein crosslinking. Treatment of casein, bovine serum albumin, lysozyme, wool or polylysine with acetamido- and phenylacetamido acrylic acid methyl esters at pH 9-10 converted varying amounts of lysine to lysinoalanine residues. However, complete transformation was not achieved. Incomplete reaction is attributed to partial hydrolysis of the esters to the less reactive acrylic acids under the reaction conditions. Similar studies were made of the reactivities of protein SH groups generated by reduction of disulfide bonds by tributylphosphine. The SH groups could be completely alkylated at pH 7.6 in aqueous propanol, as shown by nearly quantitative recovery of lanthionine. Such a procedure might therefore be used to estimate cystine contents of proteins.

INTRODUCTION

Dehydroalanine residues are reactive intermediates formed by base and heat-catalyzed elimination reactions of serine and cystine residues in proteins. Since dehydroalanine has a reactive, conjugated double bond system, it reacts with accessible protein functional groups. In particular, the sulfhydryl groups of cysteine and ε-amino groups of lysine residues react to form lanthionine, and lysinoalanine, respectively (Figures 1-3). The relative rates of these reactions govern the nature of products formed during alkali treatment of foods. In order to obtain insight into the control of these reactions, we are determining the conditions that influence the reactivity of dehydroalanine with

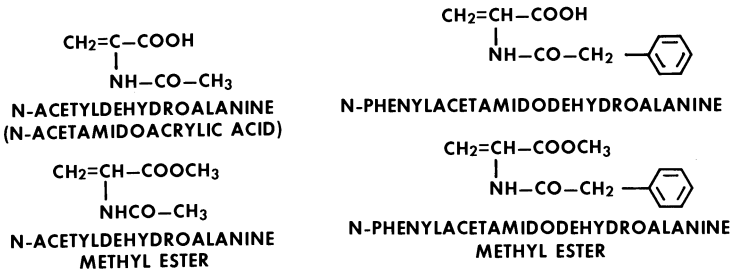


Fig. 1. Structures of dehydroalanine and derivatives.

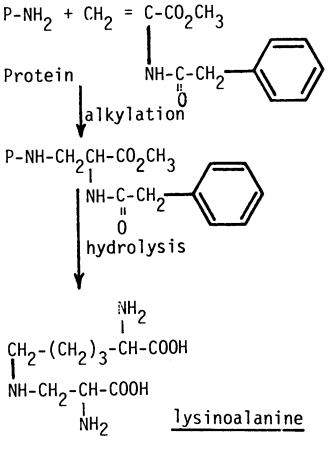


Fig. 2. Formation of a lysinoalanine residue from a protein amino group and N-phenylacetamidodehydroalanine methyl ester.

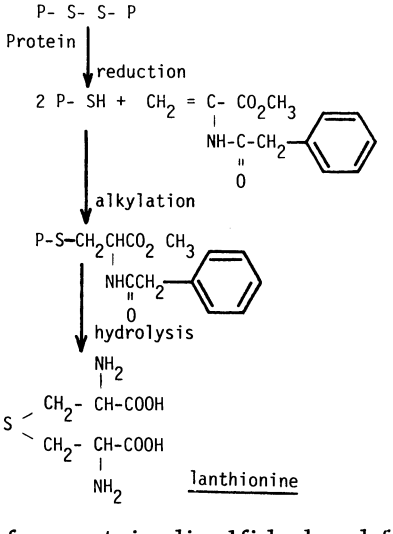


Fig. 3. Reduction of a protein disulfide bond followed by alkylation to form two lanthionine residues.

various protein functional groups. In previous papers we described factors that influence reaction rates of SH groups in amino acids, peptides, and related model compounds with dehydroalanine and dehydroalanine methyl ester (Snow et al., 1975 a, b). Here, we describe reactions of dehydroalanine derivatives with selected native and reduced proteins and related polyamino acids.

REACTION OF PROTEINS WITH DEHYDROALANINE EXPERIMENTAL

Synthesis of Reagents

Acetamidoacrylic acid methyl ester (N-acetyl dehydroalanine methyl ester. Acetamidoacrylic acid (Aldrich) (10.0 g, 80 mM) was suspended in 200 ml ether to which was added an ether solution of diazomethane with vigorous stirring at 0°C until the yellow of diazomethane did not disappear immediately. The ether solution was then filtered and evaporated to dryness on a rotary evaporator at 30°C. The residue was recrystallized twice from a small amount of ether. The crystalline product melted at 46.5-48.5°. Infrared and nuclear magnetic resonance (NMR) spectra were consistent with the assigned structure. NMR shows that excess diazomethane converts the ester to 3-acetamidopyrazoline-3-carboxylic acid methyl ester. This compound, which presumably arises from addition of diazomethane to the double bond, was not studied further.

Phenylacetamidoacrylic acid. This compound was synthesized from phenylacetamide (K&K) according to the procedure of Kildisheva et al. (1955). M.p. 178-180°C.

Phenylacetamidoacrylic acid methyl ester. The ester was prepared from phenylacetamidoacrylic acid and diazomethane. The recrystallized sample (from petroleum ether) melted at 59-61°.

Anal. calcd. for $C_{12}H_{13}NO_3$ (219.24): C, 65.74; H, 5.98; N, 6.39
Found: C, 65.90; H, 5.91; N, 6.38

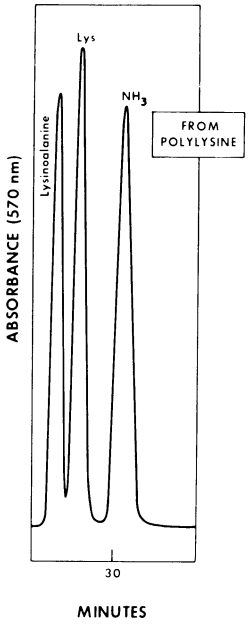
Amino Acid Analysis

Ten mg samples of protein were hydrolyzed in 15 ml 6 N HCl (constant boiling) in sealed, evacuated flasks at 110° for 24 hr. The hydrolyzates were analyzed with a Phoenix Amino Acid Analyzer.

Lysinoalanine derived from polylysine was eluted on the short basic column, LAL derived from proteins on the long (physiological) basic column, and lanthionine isomers (Friedman and Noma, 1975) were resolved on the physiological column for acidics and neutrals. The following ion-exchange conditions were used:

	Short Hydrolyzates		Long Physiological	
Resin Type	Basics	Acidics & Neutrals	Basics	Acidics & Neutrals
	Durrum Resin DC-2	Beckman Custom Research Spherical Resin Type PA-35	Durrum Resin	Durrum Resin
Column dimensions	0.9 x 13 cm	0.9 x 69 cm	0.9 x 23 cm	0.9 x 69 cm
Column height	6.0 cm	50.0 cm	18.0 cm	58.0 cm
Flow rates: buffer ninhydrin	70 ml/hr 35 ml/hr	70 ml/hr 35 ml/hr	70 ml/hr 35 ml/hr	70 ml/hr 35 ml/hr
Temperature	52.5°C	52.5°C	52.5°-62.5°C	31.4°-62.5°C
Buffer pH	5.35	3.50-4.40	4.00-5.00	3.28-4.40
Na^+ conc.	0.35	0.20	0.43-0.38	0.165-0.27
Analysis time	70 min	125 min	180 min	210 min

Elution positions of lysinoalanine and lanthionine are illustrated in Figures 4 and 5.



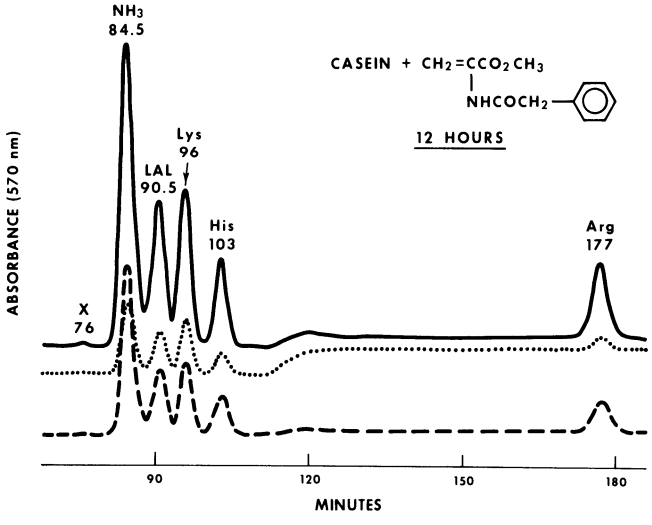


Fig. 4. Elution position of lysinoalanine.

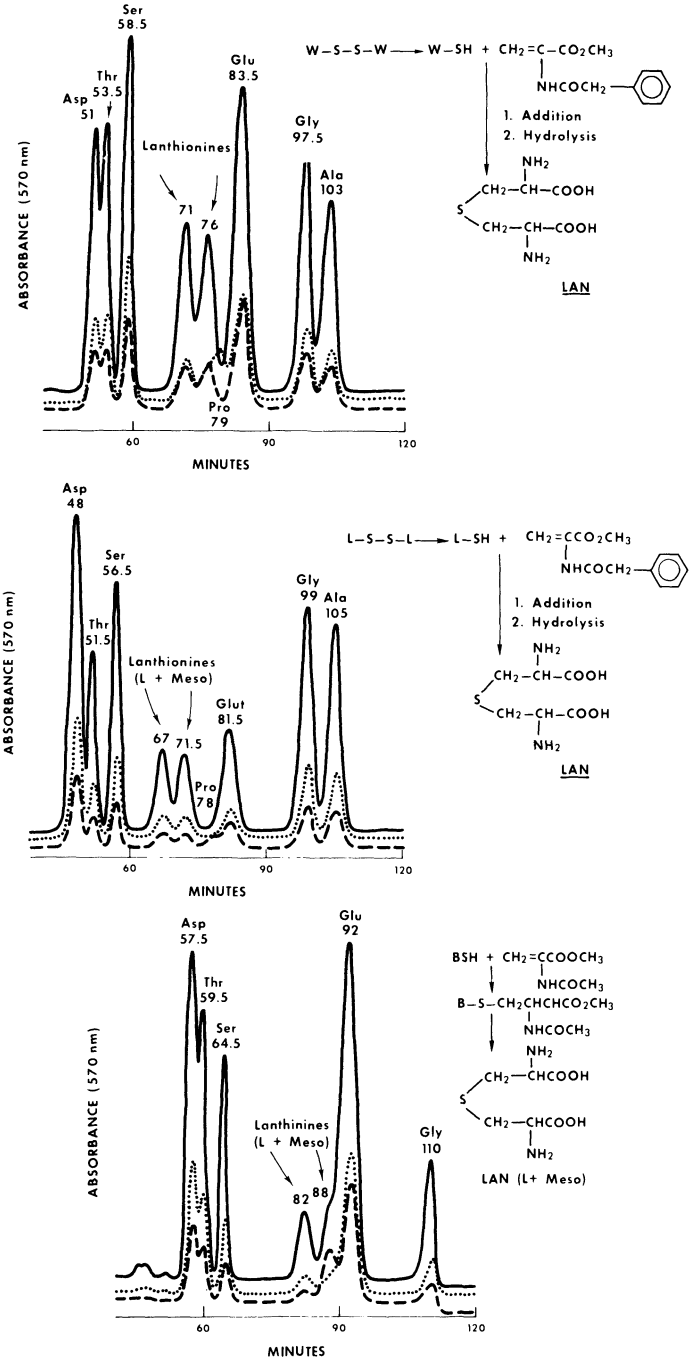


Fig. 5. Ion exchange chromatography of reduced wool (WSH), lysozyme (LSH), and bovine serum albumin (BSH) alkylated with dehydroalanine derivatives.

Chemical Modification Experiments

α-N-acetyl-L-lysine. Dehydroalanine methyl ester (11.6 mg) and α-N-acetyl-L-lysine (Sigma) (13.2 mg) were dissolved in 10 ml 0.01 M sodium borate solution (pH 9.1) a nitrogen atmosphere of and allowed to react for 24 hr. Amino acid analysis revealed the presence of two peaks in about equal amounts. These had the elution positions of lysine and lysinoalanine.

Polylysine. Polylysine (Sigma) (198 mg) was treated with phenylacetamidoacrylic acid methyl ester (562 mg) in a mixture of 25 ml pH 10 (0.01 M) borate buffer and 5 ml DMSO at room temperature for 5 hr under a nitrogen atmosphere. The reaction mixture was dialyzed against water for two days and then lyophilized. Amino acid analysis of a 7.5 mg sample revealed the presence of lysinoalanine and lysine in about equal amounts.

Proteins (Native). Proteins (lysozyme, ribonuclease, casein) (10 mg) were treated with 10 mg of N-acetyl-dehydroalanine methyl ester in 20 ml 0.01 M pH 10 sodium borate containing 25% DMSO (v/v) under N_2 at 38° for 24 hr. The samples were dialyzed, freeze-dried, and submitted for amino acid analysis.

A similar study was conducted out in which acetyl dehydroalanine methyl ester was replaced by N-phenylacetamidodehydroalanine methyl ester.

Rate studies with casein were carried out as follows: Casein (0.504 g) and N-phenylacetamidodehydroalanine methyl ester were dissolved in a mixture of 250 ml 0.01 M sodium borate and 250 ml DMSO at room temperature under an atmosphere of nitrogen. Aliquots (10 ml) were withdrawn from the reaction mixture, dialyzed against 0.01 M acetic acid to quench the reaction, lyophilized, hydrolyzed, and submitted for amino acid analyses.

Immersion of the dialysis bag containing the protein solution into a 20-liter water tank dilutes the diffusible contents of the bag several-hundred-fold and lowers its pH. The net effect is to decrease the rate of the reaction by several orders of magnitude since the reaction of an amino group with the double bond of dehydroalanine would be expected to be directly proportional to the pH of the reaction medium and to follow second order kinetics (Friedman and Wall, 1966; Snow et al., 1975).

Proteins (Reduced). Tributylphosphine (Aldrich) was redistilled at reduced pressure. About 400 mg of protein was dissolved or suspended in a mixture of 100 ml n-propanol and 100 ml 0.1 M Tris pH 7.6 buffer (Friedman and Noma, 1970). To the protein solution was added 2 ml tri-n-butylphosphine and 400 mg N-phenylacetamidodehydroalanine methyl ester. Aliquots (25 ml) were withdrawn from the reaction mixture at various

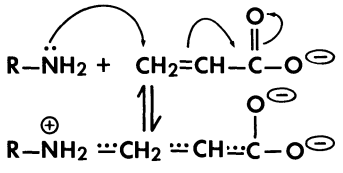
time periods, dialyzed against distilled water, lyophilized, and subjected to amino acid analysis.

RESULTS AND DISCUSSION

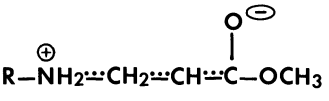
Reactivities of dehydroalanines. Initial studies carried out with N-acetyldehydroalanine (N-acetamidoacrylic acid) showed that the amino groups of proteins reacted only to a neglegible extent with the double bond of this compound to form lysinoalanine side chains. This result is not surprising inasmuch as earlier studies (Friedman and Wall, 1966) showed that the related acrylic acid also reacts slowly with amino groups in amino acids, in marked contrast to the observed facile addition of such amino groups to the double bond of methyl acrylate. (This reaction is the basis for a proposed method for estimating available lysine in proteins (Finley and Friedman, 1971)).

Reactivity of acrylic acid (as well as the corresponding N-acetamido derivative) is probably so slow because the transition state is destabilized by charge repulsion as shown in Figure 6. No such charge repulsions are possible with the methyl esters. For this reason, most of our studies utilize N-acetyldehydroalanine methyl ester and the related N-phenylacetamidodehydroalanine methyl ester (Table 1).

Reactions with protein amino groups. After treatment of casein, bovine serum albumin (BSA), lysozyme, wool, and polylysine with acetamido- and phenylacetamideo acrylic acid methyl esters at pH 9-10, dialysis, hydrolysis, and amino acid analysis disclosed transformations of various amounts of lysine to lysinoalanine residues (Table 2). Rate studies revealed that



CHARGE REPULSION DURING FORMATION OF TRANSITION STATE WITH ACRYLIC ACID



NO CHARGE REPULSION WITH METHYL ACRYLATE

Fig. 6. Transition states for nucleophilic addition of an amino group to acrylic acid and acrylic acid methyl ester.

Table 1

Lysinoalanine Formation with Proteins

Reagent	Proteins		
	Lysozyme	Ribonuclease	Casein
Acetyldehydroalanine methyl ester (A)	0.471	1.896	0.193
Phenylacetamidodehydroalanine methyl ester (B)	0.867	3.790	0.523
Ratio (B)/(A)	1.841	2.000	2.710

[a]Numbers are molar ratios of LAL to leucine. Proteins were treated for 24 hr as described in the experimental section.

Table 2

Half-Cystine, Lanthionine, Lysine, and Lysinoalanine (LAL) Contents of Reduced, Alkylated Proteins

Protein[a]	1/2 Cys	Lan (L+meso)	Lys	LAL
BSA	0.53	0.00	0.91	0.00
BSA-X[b]	0.00	0.93	0.91	0.14
Lysozyme	0.87	0.00	0.71	0.00
Lysozyme-X[b]	0.00	1.86	0.59	0.51
Wool	1.40	0.00	0.36	0.00
Wool-X[b]	0.07	2.51	0.40	0.25

[a]All number are molar ratios to leucine.

[b]Disulfide bonds were reduced to SH groups with tributylphosphine and the SH groups alkylated for 4 hr, except for wool which was treated for 24 hr, with N-phenylacetamidodehydroalanine methyl ester to form lanthionine derivatives.

complete transformation is not possible with only a single addition of reagent under these conditions (Table 3). Since the reaction is second-order, a higher concentration of dehydroalanine should effect a more complete conversion of lysine to lysinoalanine side chains. Complete reaction would be desirable since it could provide a convenient way to estimate available and unavailable lysine in a single determination, as by the ethyl vinyl sulfone procedure (Friedman and Finley, 1975a, b). Incomplete reaction is accounted for by partial hydrolysis of the esters to their less reactive acrylic acid derivatives. This hydrolysis can be minimized by carrying out the alkylations in aqueous-DMSO or aqueous-ethanol solutions (Snow et al., 1975a).

Table 3

Basic Amino Acid Content (μM/g) of Alkylated Casein[a]

Amino acid	Reaction time (hr) 0	1	2	4	12	24
LAL	0.0	142	160	153	220	242
Lys	505	326	311	288	286	277
His	166	139	140	145	156	159
NH_3	1026	1101	1124	1005	1016	1049
Arg	190	151	159	156	178	139

[a] Casein was treated with N-phenylacetamidodehydroalanine methyl ester for various time periods as described in the Experimental Section.

Reactions with protein SH groups. Studies of the reactivities of protein SH groups generated by reducing disulfide bonds with tributylphosphine (Friedman and Noma, 1970) revealed that N-phenylacetamidodehydroalanine methyl ester completely alkylated the protein SH groups at pH 7.6 in aqueous n-propanol buffer, as shown by nearly quantitative recovery of lanthionine (Table 2). This approach can be exploited to develop a procedure to estimate (cystine + cysteine) content of proteins. However, caution must be exercised because many processed food proteins could contain a considerable amount of lanthionine (Cuq et al., 1974).

CONCLUDING REMARKS

The results of this study demonstrate that lysinoalanine and lanthionine side chains can be introduced into proteins under conditions that avoid strong alkaline treatment. These results imply that dehydroalanine is an intermediate in forming lanthionine and lysinoalanine crosslinks under the influence of alkali. They also suggest that such modified proteins are not only appropriate for studying the nutritional safety of crosslinked amino acid residues but also have desirable functional properties.

ACKNOWLEDGMENT

We thank Amy T. Noma for the amino acid analyses.

REFERENCES

Cuq, J. L., Provansal, M., Chortier, L., Besancon, P., and Cheftel, C. (1974). Effects of oxidative and alkaline treatments of food proteins on the nutritional availability of methionine and lysine, Proc. IVth Int. Congr. Fd. Sci. and Technol., Madrid, Spain.

Finley, J. W. and Friedman, M. (1973). Chemical methods for available lysine. Cereal Chem., 50, 101-105.

Finley, J. W. and Friedman, M. (1976). Products derived from the alkali treatment of proteins. 172nd American Chemical Society Meeting, Abstracts, p. AGFD 46.

Friedman, M. and Finley, J. W. (1975a). Reactions of proteins with ethyl vinyl sulfone. Int. J. Peptide and Protein Res., 7, 481-486.

Friedman, M. and Finley, J. W. (1975b). Vinyl compounds as reagents for available lysine and proteins. In "Protein Nutritional Quality of Foods and Feeds," M. Friedman (Editor), Dekker, New York, Part 1, pp. 503-520.

Friedman, M. and Noma, A. T. (1975). Methods and problems in chromatographic analysis of sulfur amino acids. In "Protein Nutritional Quality of Foods and Feeds," M. Friedman (Editor), Dekker, New York, Part 1, pp. 521-548.

Friedman, M. and Wall, J. S. (1966). Additive linear free energy relationships in reaction kinetics of amino groups with α,β-unsaturated compounds. J. Org. Chem., 31, 2888-2894.

Friedman, M. and Noma, A. T. (1970). Cystine content of wool. Text. Res. J., 40, 1073-1078.

Kildisheva, O. V., Rasteikene, L. P. and Knunyants, I. L. (1955). Reactions of mercapto amino acids. Communication 4. N-Acyl-2,3-dihaloalanines. Bull. Acad. Sci. USSR, Div. Chem. Sci., 231-239.

Snow, J. T., Finley, J. W. and Friedman, M. (1975a). A kinetic study of the hydrolysis of N-acetyldehydroalanine and N-acetyldehydroalanine methyl ester. Int. J. Peptide and Protein Res., 7, 461-466.

Snow, J. T., Finley, J. W. and Friedman, M. (1975b). Relative reactivities of sulfhydryl groups with N-acetyldehydroalanine and N-acetyldehydroalanine methyl ester. Int. J. Peptide and Protein Res., 8, 57-64.

16

NUTRITIONAL SIGNIFICANCE OF CROSS-LINK FORMATION DURING FOOD PROCESSING

Richard F. Hurrell and Kenneth J. Carpenter

Department of Applied Biology, University of Cambridge, Cambridge CB2 3DX

ABSTRACT

When proteins are severely heated, in the presence or absence of sugars, the fall in nutritional value appears to be largely, although not completely, explained by reduced protein digestibility. All amino acids are considerably reduced in availability; which is in contrast to early Maillard damage which occurs under mild conditions of heating or storage in the presence of reducing sugars and affects mainly lysine. It seems that cross-linkages are formed in the severely heated protein or protein-sugar mixes and that these reduce the rate of protein digestion.

When freeze-dried chicken muscle (15% H_2O content) was severely heated its protein showed a great fall in digestibility but its value for supporting growth in the rat had fallen even more. Aspartyl-lysine and glutamyl-lysine cross-linkages, which had been formed during heating, appeared to be as digestible as the total N component and it seems that once they are released from the protein chain they can be absorbed and utilized.

INTRODUCTION

Most of the protein we eat has been heat-treated in some way before consumption; so too have the protein concentrates, such as fish meals or soyabean meals, which are used to supplement livestock rations. In general, cooking increases digestibility, however in some instances excessive heat can reduce the nutritive value of food proteins. The nutritive value of a protein depends on the physiological availability of its constituent amino acids. Any

reactions occurring during heat treatment, which lead to an impaired digestion, absorption or utilization of any amino acid which is likely to be limiting in the diet, could therefore reduce the nutritive value of the protein. In practical diets, the amino acids most likely to be limiting are the sulphur amino acids (methionine and cystine), lysine and tryptophan. The foodstuff manufacturer therefore needs to know what reactions can take place during heat processing and storage and what their nutritional consequences are.

MAILLARD REACTIONS

Heat damage to food proteins may involve reactions between the protein and other food ingredients or reactions between the amino acid side chains of the protein itself. It has long been known that even under mild conditions of heating, and during prolonged storage, Maillard reactions can occur between ε-amino groups of the protein and sugar aldehyde groups (Figure 1). After mild heating, lysine is the only amino acid significantly reduced in availability and there is only a small reduction in N digestibility (Henry, Kon Lea and White, 1948; Henry and Kon, 1950). Such damage has been shown to occur during production and storage of milk powders where lysine reacts with the reducing sugar lactose. The sugar link with lysine units is thought to be mainly in the deoxyketosyl form (Erbersdobler, 1976) which is unavailable to the rat as a source of lysine (Finot, 1973). It seems that, due to hindered trypsin action, only 10% of the lysine units substituted at the ε-amino group with fructose are released from the protein chain (Erbersdobler, 1976). Once released fructosyl-lysine can be absorbed by passive diffusion (Erbersdobler, Husstedt, Alfke, Brandt and Chelius, 1974). It is not utilized and appears mainly in the urine. The undigested

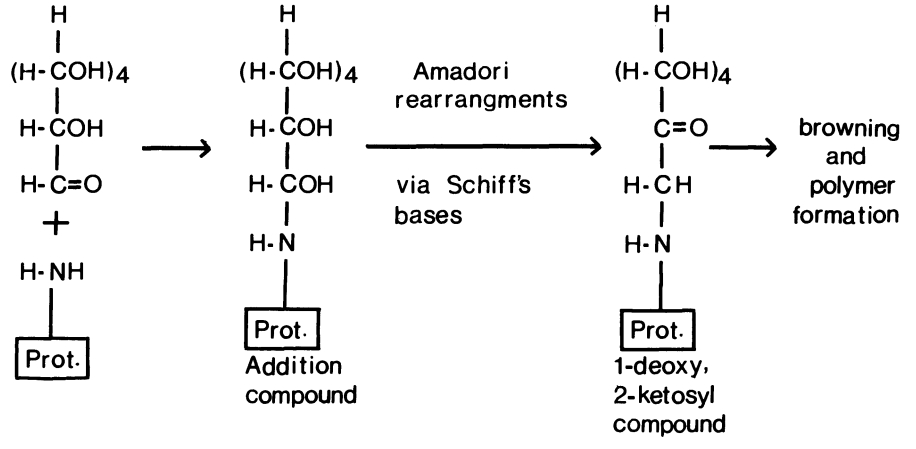


Figure 1. Simplified scheme of the Maillard reactions.

peptide-bound moiety and those free units not absorbed from the intestine can be degraded by micro-organisms in the hind gut (Erbersdobler, Gunsser and Weber, 1970).

With increasing severity of heat treatment, damage can occur in the presence or absence of carbohydrates. In their presence, Maillard reactions go past the deoxyketosyl stage, going on to form brown pigments or melanoidins. This affects not only the availability of lysine but the availability of all amino acids and the digestibility of the protein as a whole. Advanced Maillard reactions can destroy large proportions of lysine and arginine and to a lesser extent, tryptophan, cystine and histidine (i.e. they are not recovered on hydrolysis of the protein) (Evans and Butts, 1949; Miller, Hartley and Thomas, 1965). It can also reduce the enzymic release of these and of all other amino acids after enzymic hydrolysis in vivo or in vitro. Figure 2 (from Erbersdobler, 1976) shows the recovery of amino acids from in vitro enzymic hydrolysates of casein-glucose-water mixtures dried at different temperatures. The mixture containing 10% glucose dried overnight at 90° showed serious damage to lysine only, whereas the mixture containing 20% glucose dried at 105° showed a much more general damage.

It has been suggested that profuse enzyme-resistant cross-linkages are formed in the protein via advanced Maillard products

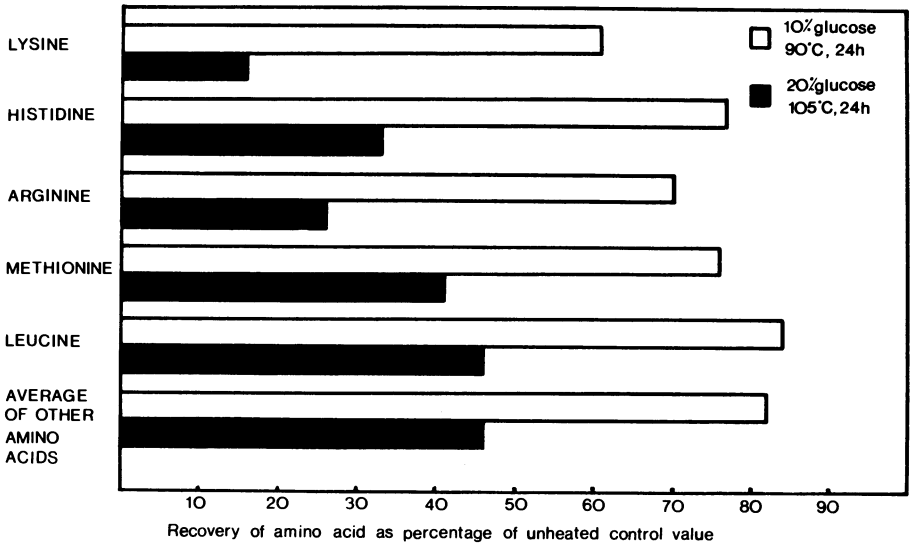


Figure 2. Recovery of amino acids from in vitro enzymic hydrolysates of casein-glucose mixtures dried at different temperatures (Erbersdobler, 1976).

TABLE 1

Protein quality values on freeze-dried cod muscle heated with 10% glucose at 14% moisture for 27h at 85°. (Miller, Carpenter & Milner, 1965)

	A unheated	B heated with glucose	B/A
RAT			
N digestibility (%)	96	41	0.43
Net protein utilization (%)	91	18	0.20
CHICK			
Available lysine (g/16gN)	11.2	0.2	0.02
Available methionine (g/16gN)	3.6	0.4	0.11

and that these reduce the rate of protein digestion (Valle-Riestra and Barnes, 1970). However the reduction in digestibility does not always explain the decreased nutritive value of the protein. Table 1, from Miller, Carpenter and Milner (1965) shows some protein quality values for freeze-dried cod muscle heated with 10% glucose. After heating for 27h at 85° the N digestibility fell to 43% of its original value but the NPU and the availabilities of lysine and methionine to the chick fell much more. There was no destruction of methionine and the observed 35% destruction of lysine still does not completely explain its low availability. It is possible that amino acids were absorbed from the gut in non-metabolizable forms and excreted in the urine. The formation of toxic substances may also contribute to the reduced nutritive value of materials containing products of the Maillard reaction (Adrian and Frangne, 1973). Advanced Maillard damage could occur during the production of soya-bean meals and groundnut meals.

PROTEIN-PROTEIN REACTIONS

In the absence of reducing sugars or other carbonyl compounds, protein heat damage as measured by loss of ε-amino lysine and loss of nutritional value, occurs at a much slower rate (Carpenter, 1973). However, after really severe or prolonged heating conditions both reactive lysine and nutritional value can be considerably reduced. Lysine is not destroyed as in Maillard damage and is almost completely recovered on acid hydrolysis (Hurrell and Carpenter, 1974), as are all the other amino acids except cystine. The main reason for a reduced nutritive value in such materials appears to be a reduced digestibility.

It has been suggested that heat causes the formation of new enzyme resistant cross-linkages within the protein molecules so reducing its digestibility and the biological availability of its constituent amino acids (Mauron, 1972; Ford, 1973). There are

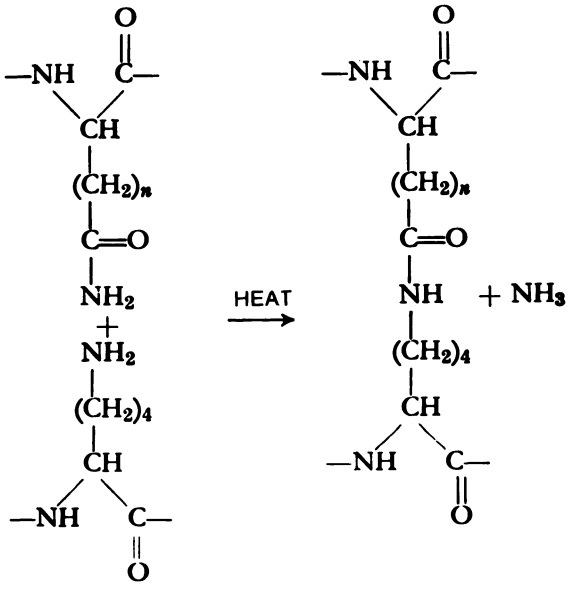


Figure 3. Formation of isopeptide bond by reaction of the ε-amino group of lysine with the amide group of asparagine (n = 1) or of glutamine (n = 2).

many possible cross-linking reactions between the different amino acid side chains and one of the most likely seems to be the formation of new isopeptide bonds by reaction of the ε-amino group of lysine with either the carboxyl groups of aspartic or glutamic acids (Mecham and Olcott, 1947) or more likely with the amide groups of glutamine and asparagine (Figure 3) (Bjarnason and Carpenter, 1970). This would explain the observed fall of FDNB-reactive lysine which occurs in severely heated proteins. The term isopeptide is used to differentiate these bonds from the normal peptide link. Other cross-linkages may result from the degradation of cystine. Heat can cause fission of the disulphide bond, yielding dehydroalanine which may condense with cystine to form lanthionine, or with the ε-amino group of lysine to form lysinoalanine (Horn, Jones and Ringel, 1941; Bohak, 1964; Ziegler, 1964).

In collaboration with Professor Asquith's group in Belfast, we have studied the nutritional changes in heated proteins in which cross-linkages had been formed (Hurrell, Carpenter, Sinclair, Otterburn and Asquith, 1976). Solvent-extracted chicken muscle containing 15% moisture was heated for different times at 121° so as to cause a large decrease in its reactive lysine content as measured by fluorodinitrobenzene (FDNB)(Table 2). Total lysine

TABLE 2

Levels of lysine, lysine isopeptides and lanthionine

(mg/g crude protein) recovered from heated proteins.

	Chicken muscle			Bovine Plasma Albumin		
	hrs at 121°			hrs at 121°		
	0	8	27	0	8	27
Total lysine	96	88	89	124	115	114
Bound lysine	3	13	26	2	19	39
FDNB-lysine	89	73	61	122	96	75
Aspartyl-lysine+	0	2.0	4.5	0	0	0
Glutamyl-lysine+	0	2.0	4.6	0	0	0
Lanthionine	0	0	0	0	13.2	14.2

+ calculated as lysine equivalents

recovered after simple acid hydrolysis was not greatly reduced, even after 27h heating, indicating that in the absence of carbohydrates, lysine is not destroyed by heating. Bound lysine was determined as that lysine which was unreactive to FDNB but was released by acid-hydrolysis of the FDNB-treated material (Roach, Sanderson and Williams, 1967). It was found to increase with heat treatment. Aspartyl-lysine and glutamyl-lysine cross-linkages were not detected in enzymic digests of the unheated chicken muscle but they were isolated from enzymic digests of the heated material in similar quantities which increased with duration of heat treatment. Lanthionine was not formed. With bovine plasma albumin (BPA) heated under the same conditions the opposite occurred (Table 2). Rather surprisingly no isopeptides were detected even though the reactive lysine had fallen considerably with a corresponding increase in bound lysine. Lanthionine cross linkages had been formed but lysinoalanine was not detected in either material, nor would it be expected to be formed when heating at neutral or acid pH (Bjarnason and Carpenter, 1970).

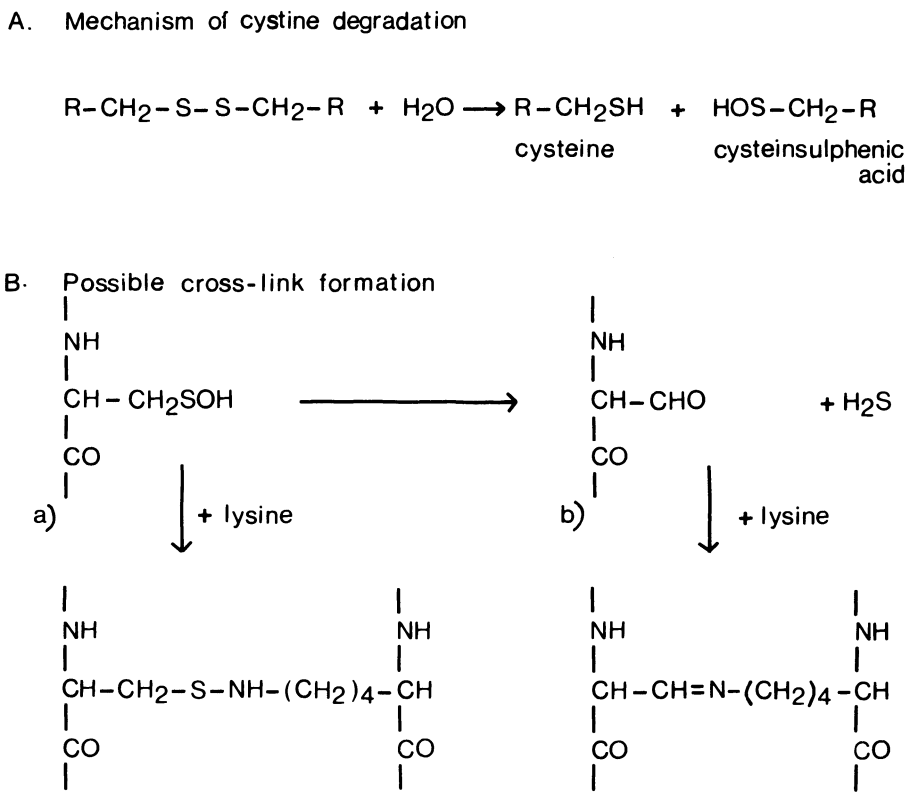


Figure 4. Possible cross-linkages via lysine and cystine degradation products.

In the heated chicken muscle, the recovered isopeptides accounted for only 30-50% of those lysine units made inaccessible to FDNB. It is possible that more of the 'bound' lysine was present in the isopeptide form than was released by in vitro digestion as the digestion procedure was found to be incomplete. Lysine however may have taken part in other reactions that block its ε-amino group. This would explain the loss of ε-amino groups from heated BPA. No lysinoalnine was detected, but there are other possible cross-linkages involving lysine and cystine degradation products (Figure 4) and BPA has a high cystine content. Philips (1936) proposed a cross-linkage via lysine and the aldehyde formed via the degradation of cysteinsulphenic acid whereas Speakman (1933) suggested a sulphenamide cross-linkage by direct reaction of lysine with cysteinsulphenic acid. It is possible also that the peptide chain could be ruptured at dehydroalanine to form an amide group and a pyruvic acid derivative (Figure 5) (Bjarnason and Carpenter, 1970). The amide group can then react with lysine with the liberation of ammonia. Both H_2S and NH_3 have been detected in heated BPA (Bjarnason and Carpenter, 1970).

Digestion of heated proteins. The chicken muscle heated for 8h and 27h at 121° was fed to rats and ileal and faecal digestibilities

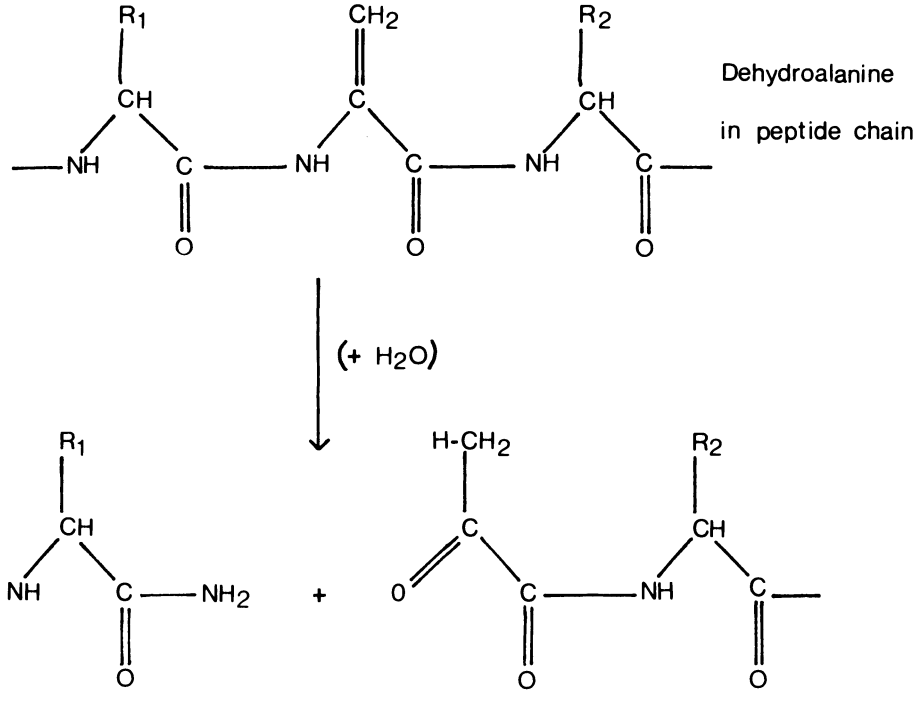


Figure 5. Hydrolysis of peptide chain at dehydroalanine.

TABLE 3

Digestibility and NPR values for unheated and heated chicken muscle fed to rats (Hurrell et al, 1976)

	A unheated	B heated 27h at 121°	B/A
Ileal digestibility (%)			
Nitrogen	88	57	0.65
Aspartyl-lysine	-	83	
Glutamyl-lysine	-	76	
Faecal digestibility (%)			
Nitrogen	98	80	
Lysine	99	82	
Aspartyl-lysine	-	93	
Glutamyl-lysine	-	97	
Net Protein Ratio	5.5	1.9	0.35

were studied. Ileal values were obtained by analysing the undigested material from the lower end of the small intestine thus avoiding bacterial fermentation. It had been expected that the isopeptide linkages that had been formed during heating would be less well digested than the rest of the protein molecule and that they would accumulate in the ileal and faecal contents of rats given the heated material. Surprisingly, the results (Table 3) seem to indicate that the isopeptides are slightly more efficiently digested by the rat than is the rest of the protein molecule. The ileal nitrogen digestibility fell from 88% in the unheated material to 57% after heating for 27h at 121°, however the ileal digestibilities

of aspartyl-lysine and of glutamyl-lysine were 83% and 76% respectively. Faecal digestibility values followed a similar pattern. Nitrogen and lysine digestibilities had been reduced from almost 100% to about 80% after heating but the isopeptide digestibilities had fallen to a lesser extent. It could be that some of the isopeptides were destroyed by bacteria or additional enzymes present in the digesta, although attempts were made to correct for this by using the recovery of free glutamyl-lysine added at the start of the in vitro digestion procedure (66%) as a correction factor.

We cannot, however, put too much emphasis on the isopeptide digestibility values since we know the in vitro enzymic digestion procedure for their detection was incomplete. However it appeared to be just as incomplete for the faeces and ileal contents as for the original heated chicken muscle. Also the proportion of lysine in the faecal protein was not higher than that present in the food protein, so that the faecal digestibility of lysine was not reduced to a greater extent than that of other amino acids. With other severely heated proteins, the ileal digestibility of lysine has also been shown to fall by a similar extent to that of nitrogen and of the other amino acids (Varnish and Carpenter, 1975) and this would be inconsistent with the isopeptide-linked lysine in proteins being indigestible. Unlike fructosyl-lysine, (the Maillard compound), free glutamyl-lysine has been shown to be completely available to the rat as a source of lysine (Mauron, 1972; Waibel and Carpenter 1972). It seems that those amino acids involved in cross-link formation are neither digested nor absorbed at a slower rate.

The hypothesis is, therefore, that cross-link formation reduces the rate of protein digestion, possibly due to preventing enzyme penetration or by blocking the sites of enzyme attack. It should be remembered that the food protein only has a limited time in the digestive tract and it seems that if more time was available, as in some in vitro digestion studies, more protein would be digested. Even if amino acids are released from the protein in the intestine their absorption may be hindered by undigested peptides which seem to block the sites of amino acid absorption (Buraczewski, Buraczewska and Ford, 1967).

But (again referring to Table 3) it also appears that the large decrease in nutritional value as measured by NPR was only partly accounted for by the reduced ileal digestibility of the protein. Similar findings have been reported in heated pork muscle (Donoso, Miller, Lewis and Payne, 1962) and heated fish meals (Carpenter, 1973). The reasons for the reduced utilization of protein are unclear. There is virtually no destruction of amino acids except cystine and all amino acids and N appear to undergo similar falls in ileal digestibility. With severely heated blood meal we have

TABLE 4

Availability of amino acids in chicken muscle heated 116° for 27h.

(Varnish & Carpenter, 1975)

(Each value as % of unheated control value)

Method	Lysine	Methionine	Tryptophan
Ileal digestibility	52	62	48
Chick growth assay	56	66	44

found that faecal digestibility of lysine was 50% and that chick availability (by growth assay) was zero (Waibel, Cuperlovic, Hurrell and Carpenter, 1976). Ileal digestibilities are normally only about 10% lower than faecal digestibilities. However, in another study with chicken muscle heated for 27h at 116° without prior solvent extraction we found that decreased ileal digestibility values completely explained the falls in biological availability of lysine, methionine and tryptophan (Table 4) (Varnish and Carpenter 1975). However, this does appear to be the 'odd man out' and has yet to be confirmed.

To explain reduced protein utilization, it has been suggested that amino acids may be absorbed from the gut in non-metabolizable forms and excreted in the urine (Ford and Shorrock, 1971). Racemization is a possible mechanism whereby an amino acid may be chemically modified and rendered biologically unavailable (Bjarnason and Carpenter, 1970; Hayase, Kato and Fujimaki, 1973). The racemized amino acid may be absorbed but broken down in the body along with the excess of other amino acids, the carbon skeleton being used as an energy source and the N excreted. However it is still unclear which amino acid or amino acids, if any, are responsible for this reduced N utilization and it may be that the lowered nutritive value is explained by the formation of toxic compounds, such as have been identified after Maillard damage or simply by the 'physiological stress' to the rat at receiving such indigestible material (Tanaka, Amaya, Lee and Chichester, 1974).

To summarise, it would appear that isopeptides, and possibly also the other cross-linkages formed during excessive heating of

protein in the presence or absence of carbohydrates, reduce the rate of protein digestion possibly due to a steric hindrance or by blocking the sites of enzyme attack. Once the lysine-isopeptides are released from the protein however they can be absorbed and utilized. Although reduced protein digestibility appears to explain the major part of the falls in nutritive value and in amino acid availability that occur in heated proteins, it does not usually explain it completely.

REFERENCES

Adrian, J. and Frangne,R. (1973). Maillard reaction. 8. Effect of pre-melanoidins on digestibility of nitrogen in vivo and on proteolysis in vitro. Annls Nutr. Aliment., 27, 111.

Bjarnason, J. and Carpenter, K. J. (1970). Mechanisms of heat damage in proteins. 2. Chemical changes in pure proteins. Br. J. Nutr., 24, 313.

Bohak, Z. (1964). N^{ε}-(DL-2-amino-2-carboxyethyl)-L-lysine, a new amino acid formed on alkali treatment of proteins. J. Biol. Chem., 239, 2878.

Buraczewski, S., Buraczewska, L. and Ford, J. E. (1967). The influence of heating of fish proteins on the course of their digestion. Acta biochim. pol., 14, 121.

Carpenter, K. J. (1973). Damage to lysine in food processing: its measurement and its significance. Nutr. Abstr. Rev., 43, 424.

Donoso, G., Lewis, O. A. M., Miller, D. S. and Payne, P. R. (1962). Effect of heat treatment on the nutritive value of proteins: chemical and balance studies. J. Sci. Fd Agric., 13, 192.

Erbersdobler, H. (1976). Amino acid availability. In, 'Protein Metabolism and Nutrition', D. J. A. Cole, K. N. Boorman, P. J. Buttery, D. Lewis, R. J. Neale and H. Swan (Editors). Butterworths, London.

Erbersdobler, H., Gunsser, I. and Weber, G. (1970). Degradation of fructoselysine by the intestinal flora. Zentrl. Vet. Med., 17, 573.

Erbersdobler, H., Husstedt, W., Alfke, F., Brandt, A. and Chelius, H. H. (1974). The absorption and distribution of fructose-lysine. Z. Tierphysiol. Tierernährung. Futtermittelk., 33, 202.

Evans, R. J. and Butts, H. A. (1949). Inactivation of amino acids by autoclaving. Science, 109, 569.

Finot, P. A. (1973). Non-enzymic browning. In, 'Proteins in Human Nutrition', J. W. G. Porter and B. A. Rolls (Editors). Academic Press, London.

Ford, J. E. (1973). Some effects of processing on nutritive value. In, 'Proteins in Human Nutrition', J. W. G. Porter and B. A. Rolls (Editors). Academic Press, London.

Ford, J. E. and Shorrock, C. (1971). Metabolism of heat-damaged protein in the rat. Influence of heat damage on the excretion

of amino acids and peptides in the urine. Br. J. Nutr., 26, 311.

Hayase, F., Kato, H. and Fujimaki, M. (1973). Racemization of amino acid residues in proteins during roasting. Agric. Biol. Chem., 37, 191.

Henry, K. M. and Kon, S. K. (1950). Effect of reaction with glucose on the nutritive value of casein. Biochim. biophys. Acta, 5, 455.

Henry, K. M., Kon, S. K., Lea, C. H. and White, J. D. C. (1948). Detrioration on storage of dried skim milk. J. Dairy Res., 15, 292.

Horn, M. J., Jones, D. B. and Ringel, S. J. (1941). Isolation of a new sulphur-containing amino acid (lanthionine) from sodium carbonate-treated wool. J. Biol. Chem., 138, 141.

Hurrell, R. F. and Carpenter, K. J. (1974). Mechanisms of heat damage in proteins. 4. The reactive lysine content of heat-damaged material as measured in different ways. Br. J. Nutr., 32, 589.

Hurrell, R. F., Carpenter, K. J., Sinclair, W. J., Otterburn, M. S. and Asquith, R. S. (1976). Mechanisms of heat damage in proteins. 7. The significance of lysine-containing isopeptides and of lanthionine in heated proteins. Br. J. Nutr., 35, 383.

Mauron, J. (1972). Influence of industrial and household handling on food protein quality. In,'International Encyclopedia of Food and Nutrition, Vol. 2., Protein and Amino Acid Functions, E. J. Bigwood (Editor). Pergamon Press, Oxford.

Mecham, D. K. and Olcott, H. S. (1947). Effect of dry heat on proteins. Ind. Engng Chem. ind. (int.) Edn, 39, 1023.

Miller, E. L., Carpenter, K. J. and Milner, C. K. (1965). Availability of sulphur amino acids in protein foods. 3. Chemical and nutritional changes in heated cod muscle. Br. J. Nutr., 19, 547.

Miller, E. L., Hartley, A. W. and Thomas, D. C. (1965). Availability of sulphur amino acids in protein foods. 4. Effect of heat treatment upon total amino acid content of cod muscle. Br. J. Nutr., 19, 565.

Philips, H. (1936). Cross-linkage formation in keratins. Nature, Lond., 138, 121.

Roach, A. G., Sanderson, P. and Williams, D. R. (1967). Comparison of methods for the determination of available lysine value in animal and vegetable protein sources. J. Sci. Fd Agric., 18, 274.

Speakman, J. B. (1933). Reactivity of the sulphur linkage in wool. Nature, Lond., 132, 930.

Tanaka, M., Amaya, J., Lee, T. C. and Chichester, C. O. (1974). Effects of the browning reaction on the quality of the protein. Proceedings of the 4th International Congress of Food Science and Technology, Madrid, Spain.

Valle-Riestra, J. and Barnes, R. H. (1970). Digestion of heat-damaged egg albumin by the rat. J. Nutr., 100, 873.

Varnish, S. A. and Carpenter, K. J. (1975). Mechanisms of heat damage in proteins. 6. The digestibility of individual amino acids in heated and propionylated proteins. Br. J. Nutr., 34, 339.

Waibel, P. E. and Carpenter, K. J. (1972). Mechanisms of heat damage in proteins. 3. Studies with ε-(γ-L-glutamyl)-L-lysine. Br. J. Nutr., 27, 509.

Waibel, P. E., Cuperlovic, M., Hurrell, R. F. and Carpenter, K. J. (1976). Processing damage to lysine and other amino acids in the manufacture of blood meal. J. Agric. Fd Chem., accepted for publication.

Ziegler, K. (1964). New cross-links in alkali-treated wool. J. Biol. Chem., 239, 2713.

17

THE FORMATION, ISOLATION AND IMPORTANCE OF ISOPEPTIDES IN HEATED PROTEINS

Michael Otterburn, Michael Healy and William Sinclair

The Queen's University of Belfast

21 Chlorine Gardens, Belfast BT9 5DL, N. Ireland

ABSTRACT

The separation and resolution of the isopeptides N^{ε}(γ-L-glutamyl)-L-lysine and N^{ε}(β-aspartyl)-L-lysine, formed in heated proteins, has been successfully achieved. The method demands a well characterised ion-exchange column and the use of pH 3.40 lithium citrate buffer (0.2N Li^{+}). Due to variations in particle size and percentage crosslinkages in the ion-exchange resin a computer assisted buffer gradient system has been developed. This system affects resolution of both isopeptides in 7h. The use of leucyl-glycine as an internal standard facilitates quantitative estimation of the isopeptides.

This separative method has been used to analyse a series of heated protein samples and to estimate the quantities of isopeptides formed. The ability of a protein to form isopeptide links is discussed as well as the implication of such links on the reactivity and digestibility of proteins.

INTRODUCTION

It is now generally accepted that the effect of heat on proteins, in the absence of carbohydrates and fats, can impare their nutritional value. Heat-induced chemical changes and modifications to proteins have been studied, the most significant effect of which seems to be the formation of chemically and enzyme resistant cross-links in the protein matrix (Asquith and Otterburn, 1969, 1971). The presence of such crosslinks within the proteins' structure tends

to reduce the digestibility of the protein and renders some constituent amino acids biologically unavailable (Mauron, 1972; Ford, 1973).

The formation of ω-ε bonds (isopeptides) between lysyl and glutamyl or aspartyl residues (or their amides) was first postulated for wool keratin (Astbury and Woods, 1934). It was not, however, until the work of Mecham and Olcott (1947) that any experimental evidence was offered for the formation of such moieties in heated proteins.

Through the work of Lorand, Downey, Gotoh, Jacobsen and Tokura (1968), Pisano, Finlayson and Peyton (1968) and Matačić and Loewy (1968), on the fibrinogen → fibrin transformation, the existence of N^{ε}(γ-L-glutamyl)-L-lysine (G-L) in polymerized fibrin was established. This moiety along with N^{ε}(β-L-aspartyl)-L-lysine (A-L) was isolated from keratin, thus fulfilling Astbury's early postulate (Asquith, Otterburn, Buchanan, Cole, Fletcher and Gardner, 1970). Later work established for the first time that both these crosslinks were formed during the heating of proteins (Asquith and Otterburn, 1971; Asquith, Otterburn and Gardner, 1971).

The purpose of this investigation is to describe the analytical techniques involved in monitoring A-L and G-L in proteins, and to discuss the formation of these moieties in various protein systems. The significance and importance of such crosslinks will also be examined.

EXPERIMENTAL

Materials and Reagents

Proteins. Lactalbumin, casein, zein, lysozyme and egg albumen were obtained from British Drug Houses Ltd. Bovine plasma albumin (Cohn Fraction V) was supplied by Koch-Light Ltd.

Bovine haemoglobin and plasma were provided by Regal Foods Ltd., Craigavon (N.I.).

Chicken muscle X 902E and X 903 and all other protein samples were provided by Dr. K.J. Carpenter of Cambridge University.

Enzymes. Pepsin 3X crystallized was obtained from B.D.H. Ltd., aminopeptidase M from Hexoran Ltd. and pronase from Calbiochem. Inc. Prolidase was prepared from swine kidneys by the method of Davis and Smith (1957).

Chemicals. N^{ε}(γ-L-glutamyl)-L-lysine (G-L) was purchased from Cyclo Chemicals Inc.

N^{ε}(β-L-aspartyl)-L-lysine (A-L) was synthesized by the method of Zahn and Pätzold (1963).

Leucyl-glycine was purchased from Nutritional Biochemical Corporation Inc. All other chemicals used were B.D.H. Analar grade materials.

Buffers. Buffers were prepared according to the basic recipies in the Technicon Auto Analyser handbook (1973).

Lithium buffers were prepared from lithium hydroxide monohydrate, and citric acid monohydrate.

Pentachlorophenol and n-octanoic acid were added to all buffers to inhibit microbial growth.

Methods

Amino Acid Analysis. All analyses were performed on a Technicon Auto Analyser, employing a 6 x 1300 mm column packed with 'Chroma-Beads' Type A resin. The column was maintained at 60° throughout the analyses.

Tungstate Density Measurements. The extent of crosslinkages in ion-exchange resins was determined by the sodium tungstate density method of Hamilton (1963).

Buffer Gradient Calculations. Using a teletype input to an ICL 1907 digital computer, buffer gradients were calculated.

Enzymic Digestion of Proteins. The protein samples were reduced with tributyl phosphine or dithiothreitol and the formed thiol groups blocked with acrylonitrile or iodoacetic acid. The resulting samples were enzymically digested using pepsin, pronase, amino peptidase M and prolidase as described by Cole, Fletcher, Gardner and Corfield (1971).

Heat Treatments of Proteins. All proteins were equilibrated to 15% moisture content and heated in sealed ampoules under nitrogen at 85°, 115° or 121° (Carpenter, Morgan, Lea and Parr, 1962).

X 902E, X 903 chicken muscle, X 949 and X 953 bloodmeals, lactalbumin and casein samples were provided by Dr. K.J. Carpenter from earlier work (Bjarnason and Carpenter, 1970).

Animal Experiments. Feeding trials were carried out on X 902E chicken muscle protein using CFY strain 21-d-old female rats (Anglia Laboratory Animals, Huntingdon, Cambs.). The animal experiments were carried out by Dr. R.F. Hurrell as reported in Hurrell, Carpenter, Sinclair, Otterburn and Asquith (1976).

RESULTS AND DISCUSSION

Since the original isolation of G-L and A-L from a heat treated protein-keratin (Asquith and Otterburn, 1971) recent work has shown the presence of the isopeptides in a variety of other heated proteins (Hurrell, Carpenter, Sinclair, Otterburn and Asquith, 1976). Other workers have also shown the presence of G-L in certain heated milk proteins (Schmitz, Zahn, Klostermeyer, Rabbel and Watanabe, 1976).

The major difficulties in the identification and isolation of the isopeptides has always been twofold, (i) to break down the protein without damaging the crosslinks and (ii) to separate in a satisfactory manner the isopeptides from the other compounds which elute in the neutral region from an ion-exchange resin. As far as the first point is concerned, due to the acid labile character of G-L and A-L the degradation of the protein must be carried out enzymically or microbiologically. At the present time there are various suitable enzyme systems available (Cole, Fletcher, Gardner and Corfield, 1971; Milligan, Holt and Caldwell, 1971; and Schmitz, 1975); all tend to employ three or four proteases and peptidases in sequence, all use a reduction and blocking sequence if the protein is very high in cystine. Only the work of Schmitz (1975) uses a pre-digestion with pepsin or pronase prior to any reduction and blocking. In the present work the method of Cole, Fletcher, Gardner and Corfield (1971) has been used, although corroborative use has been made of the method of Schmitz.

As far as the resolution of the isopeptides from an ion-exchange resin is concerned, in the original work of Asquith and Otterburn (1971) total resolution of both moieties was achieved using sodium citrate buffers on a normal Technicon 21h buffer system. The isopeptides eluted in the neutral region between leucine and tyrosine. However, it was found after a considerable period of time that resolution of G-L and A-L was being affected by increasing peak widths and excessively high column pressures. This loss in resolution was traced to physical damage of the resin. Replacement of the resin (Chromabeads A) gave improved peak shape and resolution for the analyses of protein acid and enzymic digests. However, there had been a drastic change in the elution characteristics of the isopeptides; thus A-L eluted simultaneously with isoleucine whereas G-L was fully resolved between isoleucine and leucine. The changed elution patterns was attributed to two factors; the differences in

particle size of the old and new resins and secondly the degree of crosslinking in the resin. This latter parameter was measured by Hamilton's Tungstate method (1963) and gave values of 6.0% and 8.2% crosslinking for old and new resins respectively.

It became apparent from these observations that modification of the buffer eluant system would be necessary in order to compensate for the varying characteristics of different batches of resin. Thus in order to obtain a more flexible elution system which could be applied to other resins two approaches were adopted for attaining resolution of the isopeptides. These were: (a) modification of the existing buffer gradients and (b) changing the salt component of the buffer.

(a) Buffer Gradient Modification

Variation of the buffer gradient seemed the most feasible approach for attaining complete resolution of both G-L and A-L. In the Technicon amino acid analyser the buffer gradient is produced by an Autograd consisting of nine interconnected chambers arranged such that liquid flows in only one direction, passing from chamber 9 through 8 to 7 ... to 1 and thence to the column. The shape of the gradient may be altered by varying the initial pH of the buffers in the individual chambers. One is presented with the problem of selecting initial pH values so that the required gradient is achieved. Peterson and Sober (1959) developed the theory and practice of multi-chambered Autograds but their calculation for buffer gradients was very laborious and impracticable. Burns, Curtis and Kacser (1965) modified the theory and presented a set of equations which were suitable for the calculation of buffer gradients by means of a digital computer:

$$\frac{dV}{dt} = -\frac{1}{9} \cdot F \qquad (1)$$

$$\frac{d\,C_9}{dt} = 0 \qquad (2)$$

$$\frac{d\,C_8}{dt} = \frac{1}{9} \cdot \frac{F}{V} \cdot (C_9 - C_8) \qquad (3)$$

$$\frac{d\,C_7}{dt} = \frac{2}{9} \cdot \frac{F}{V} \cdot (C_8 - C_7) \qquad (4)$$

$$\vdots \qquad \vdots$$

$$\frac{d\,C_1}{dt} = \frac{8}{9} \cdot \frac{F}{V} \cdot (C_2 - C_1) \qquad (10)$$

where V = volume in any chamber at time "t" (ml)
t = time from start of run (min)
F = flow rate out of chamber 1 (ml min^{-1})
C_1, C_2 ..... C_9 = pH in chamber 1, 2 ..... 9 at time "t".

An ICL 1907 digital computer was available for application of the technique. ICL subroutine F4RUNK (1968) was employed to solve the differential equations using the fourth-order Runge-Kutta process (Froberg, 1965). The equations were integrated over the time interval "T" required to empty the chambers. An integration step length $\delta t = \frac{T}{100}$ proved satisfactory and, starting with initial volume V and initial pH values C_1, C_2 ..... C_9, solutions were found at times δt, 2 δt .....100 δt.

Basically the Runge-Kutta method is as follows:- consider the single equation

$$\frac{dC}{dt} = f\ (t, C) \tag{11}$$

At any time t_o let C have the value C_o, i.e.

$$C_{(t_o)} = C_o \tag{12}$$

At time $(t_o + \delta t)$ the value of C is:-

$$C_{(t_o + \delta t)} = C_o + \frac{1}{6}\ (k_1 + 2k_2 + 2k_3 + k_4) \tag{13}$$

where $k_1 = \delta t\ f\ (t_o, C_o)$ (14)

$k_2 = \delta t\ f\ (t_o + \frac{1}{2}\ \delta t, C_o + \frac{1}{2}\ k_1)$ (15)

$k_3 = \delta t\ f\ (t_o + \frac{1}{2}\ \delta t, C_o + \frac{1}{2}\ k_2)$ (16)

$k_4 = \delta t\ f\ (t_o + \delta t, C_o + k_3)$ (17)

Thus, given V and C_9 initially the above equation can be determined at time δt for equations (1) and (2). Equations (3) to (10) then give C_8, C_7 ..... C_1 at time δt. This procedure was repeated to calculate C_1 at times 2 δt, 3 δt ..... 100 δt. The buffer gradient produced by the initial pH values was represented by a plot of C_1 versus δt.

Calculation of the ionic equilibria produced by mixing buffer solutions of a tribasic acid are extremely complex. For the case in hand, where the total concentration of citric acid was constant, the

pH obtained by mixing several buffers was calculated as the weighted average of the individual solutions.

In practice, the computer was supplied with the following information. (a) The initial volume in each chamber; (b) flow rate; and (c) initial pH values of individual chambers. The pH of the buffer leaving chamber 1 at 0%, 2% ..... 98% of the total elution time was calculated and printed out in a matter of seconds. Thus a rapid means of evaluating the gradients produced by any given set of initial pH values was available. By trial and error it was possible to substitute values until the desired modification to a gradient was achieved. In general, a selected alteration to an existing gradient could be obtained after 10 to 12 variations of initial pH values.

The objective was to increase the elution volume between isoleucine, leucine and tyrosine, thereby facilitating complete resolution of both isopeptides. For this purpose it was necessary to alter the Technicon 21h buffer gradient (Figure 1) employed by Asquith and Otterburn (1971). N.B. The salt gradient was maintained constant during alterations to pH gradients. The original 21h pH gradient was maintained until just prior to the elution pH of isoleucine and a plateau was introduced between pH 4.1 and pH 4.4 corresponding to the elution pH of isoleucine and tyrosine respectively. The original shape of the gradient was then resumed (Figure 2). Table 1 lists the composition of the Autograd for a 21h pH gradient in the pH region 4.1 - 4.4.

This gradient had no influence upon the elution of isoleucine through to tyrosine but was found to have greatly increased the separation between histidine and arginine. In fact it was necessary to continue the gradient to pH 6.00 (instead of pH 5.00) in order to elute arginine.

It was apparent that the critical pH region for elution of isoleucine and tyrosine occurred much earlier than their elution pH values. Burns, Curtis and Kacser (1965) obtained improved resolution between phenylalanine and ammonia by introducing a plateau between the respective elution pH values. However, they also found it necessary to radically increase the rate of slope of the early portion of the gradient and applied similar variations to the salt gradient.

It was decided that the critical pH region for resolution of isopeptides could best be found by introducing a plateau at earlier and earlier stages in the gradient while at the same time maintaining the original slope up to, and after, the plateau. Changes in the elution pattern would be due solely to the influence of the plateau and would not be complicated by other variations in

TABLE 1

Composition of Autograd for Standard and Modified 21h pH Buffer Gradients

Chamber	Normal Technicon	Modified in Region pH 4.1 to 4.4	Modified in Region pH 3.3 to 3.5
1	2.875	2.875	2.85
2	2.875	2.875	3.30
3	2.875	2.875	1.20
4	2.875	1.80	5.90
5	3.89	6.00	2.20
6	4.70	4.00	3.00
7	5.00	4.00	4.00
8	5.00	4.00	5.00
9	5.00	6.00	5.00

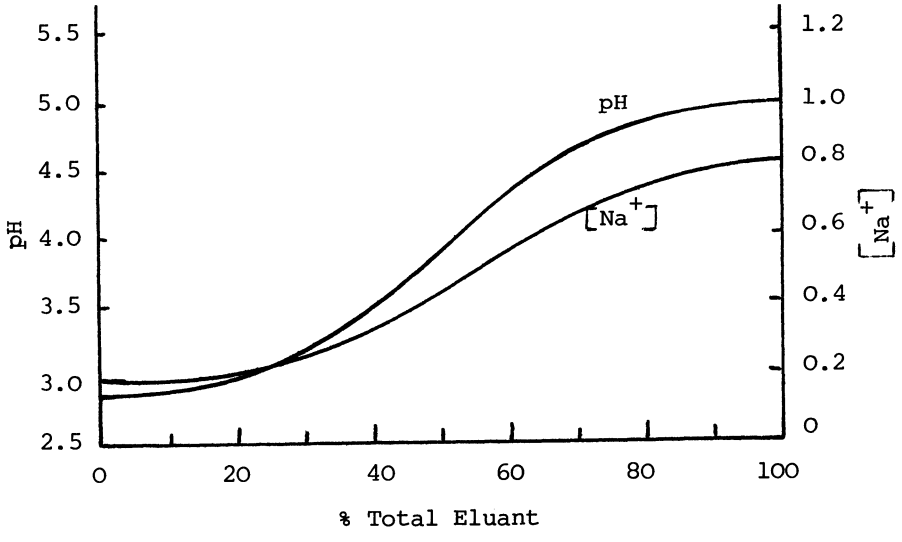


Fig. 1. Standard 21h Buffer Gradients for Amino Acid Analysis.

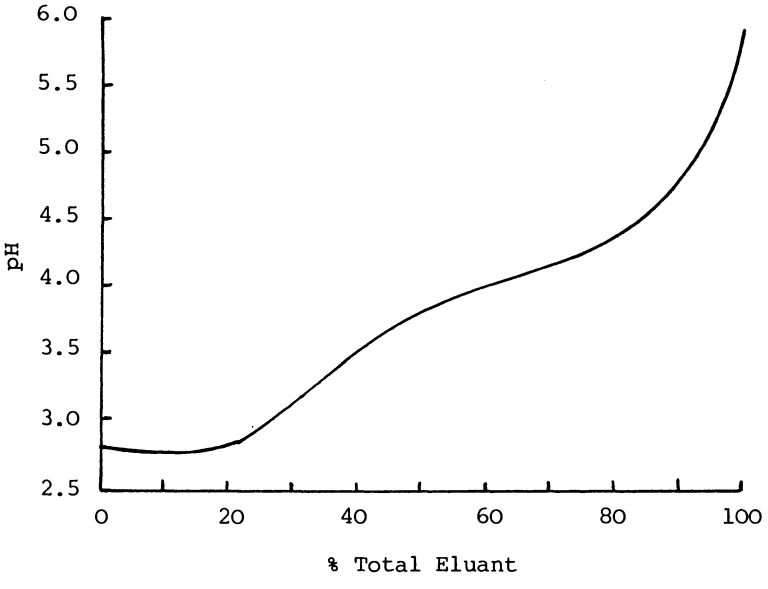


Fig. 2. Standard Buffer Gradient modified in the Region pH 4.1 - 4.4.

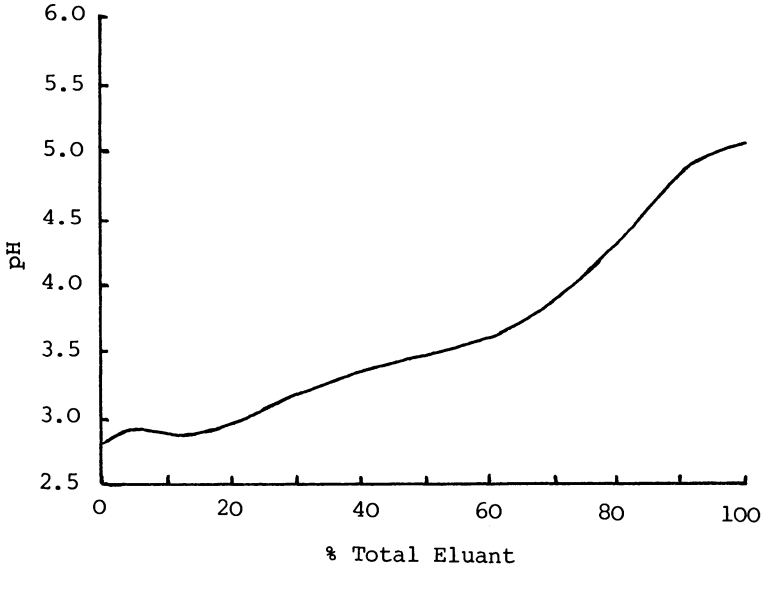


Fig. 3. Standard Buffer Gradient modified in the Region pH 3.3 - 3.5.

the gradient. Application of this procedure demonstrated that a plateau between pH 3.3 and pH 3.5 (Figure 3) yielded the desired increase in elution volume between isoleucine and tyrosine. Table 1 also lists the composition of the Autograd required for a 21h pH gradient modified in the region pH 3.3 to 3.5. The latter gradient resolved A-L, between isoleucine and leucine but G-L eluted simultaneously with leucine. Examination of very similar gradients also gave significant increase in elution volume between isoleucine and tyrosine but none were successful in resolving both isopeptides.

The logical extension to this approach was the investigation of constant pH buffers having neither a pH nor salt gradient but chosen with pH values near the critical region (salt concentration was maintained at 0.2 N Na^+). The procedure was unsuccessful and adequate resolution of both A-L and G-L was not obtained.

The preceding work depended upon alteration of partition coefficients of individual amino acids by variation of the mobile phase. Greatest differences occurred in the critical pH region which had been readily identified by systematic examination of specially constructed pH buffer gradients. Inability to resolve both isopeptides indicated that further changes in partition coefficients would best be achieved by altering the salt component of the buffer system.

(b) The Use of Lithium Buffers

It seemed unlikely that variation of the sodium ion concentration would yield sufficiently large changes in partition coefficients. However, sulphonic acid ion-exchange resins exhibit differing affinities for alkali metal ions. The most notable of these is the difference in selectivity coefficients of lithium and sodium i.e. 0.79 and 1.56 respectively for 8.0% crosslinked resin (Dean, 1969). The values refer to equivalents of ion adsorbed from 1 ml of solution per 1 g of dry resin in the H form. It is believed that hydration of lithium ions accounts for this difference (Dean, 1969; Cotton and Wilkinson, 1972).

Various authors have utilised lithium buffers to achieve resolution of asparagine and glutamine (Benson, Gordon and Patterson, 1967; Peters, Berridge, Cummings and Lin, 1968; Perry, Stedman and Hansen, 1968; Mondino, 1969). Others have developed high speed analyses in which resolution was maintained by employing lithium buffers (Atkin and Ferdinand, 1970).

Accordingly, lithium was substituted for sodium in the standard Technicon 21h gradient and it was apparent that major changes in

partition coefficients had occurred in the isopeptide region. It had previously been established using sodium citrate buffers that the region pH 3.3 - 3.5 was critical. This region also proved to be the critical pH region for lithium buffers. Investigation of constant pH buffers (0.2 N Li^+) proved successful and complete resolution of both isopeptides was achieved with pH 3.40 lithium citrate buffer. A-L was fully resolved between leucine and tyrosine and G-L eluted between tyrosine and phenylalanine (Figure 4).

Although the change from sodium to lithium was the major factor in achieving resolution with the new resin, the influence of pH cannot be overlooked. Minor variations of pH resulted in loss of baseline separation and at pH 3.38 overlapping of isopeptides occurred (Figure 5). The need for accurate control of pH highlighted a difficulty encountered with the electrode system normally employed for adjusting the pH of sodium buffers. The combined glass and reference electrode gave erroneous pH values in lithium solutions. When reimmersed in calibration buffer, the time taken for the electrode to regain its accuracy and speed of response was dependent on the length of time of immersion in the lithium solution. The problem was associated with the porous-frit liquid junction of the reference electrode. No difficulties were experienced with reference electrodes constructed with ground-glass-sleeve liquid junctions. A calomel electrode of this type was used regularly for ten months before replacement was necessary.

The method employed by Asquith, Otterburn, Buchanan, Cole, Gardner and Fletcher (1970) did not include an internal standard. In their procedure, as in the current technique with lithium buffers, norleucine eluted simultaneously with A-L. Other authors (Harding and Rogers, 1971) utilised α-amino-β-guanidinopropionic acid which eluted just before arginine. This compound was not eluted from the column with pH 3.40 lithium buffer and leucyl-glycine was adopted as an internal standard. The dipeptide was fully resolved from all other components and eluted close to the isopeptides (Figures 4 and 5). Relative response factors were 6.10 and 5.96 for A-L and G-L respectively.

Thus complete resolution of A-L and G-L was achieved. Quantitative analyses were carried out in 7h using pH 3.40 lithium citrate buffer (0.2 N Li^+) and employing leucyl-glycine as an internal standard.

Isopeptide Analyses of Proteins

Once a reproducible method of separation and analysis of G-L and A-L had been established, the method was used to investigate

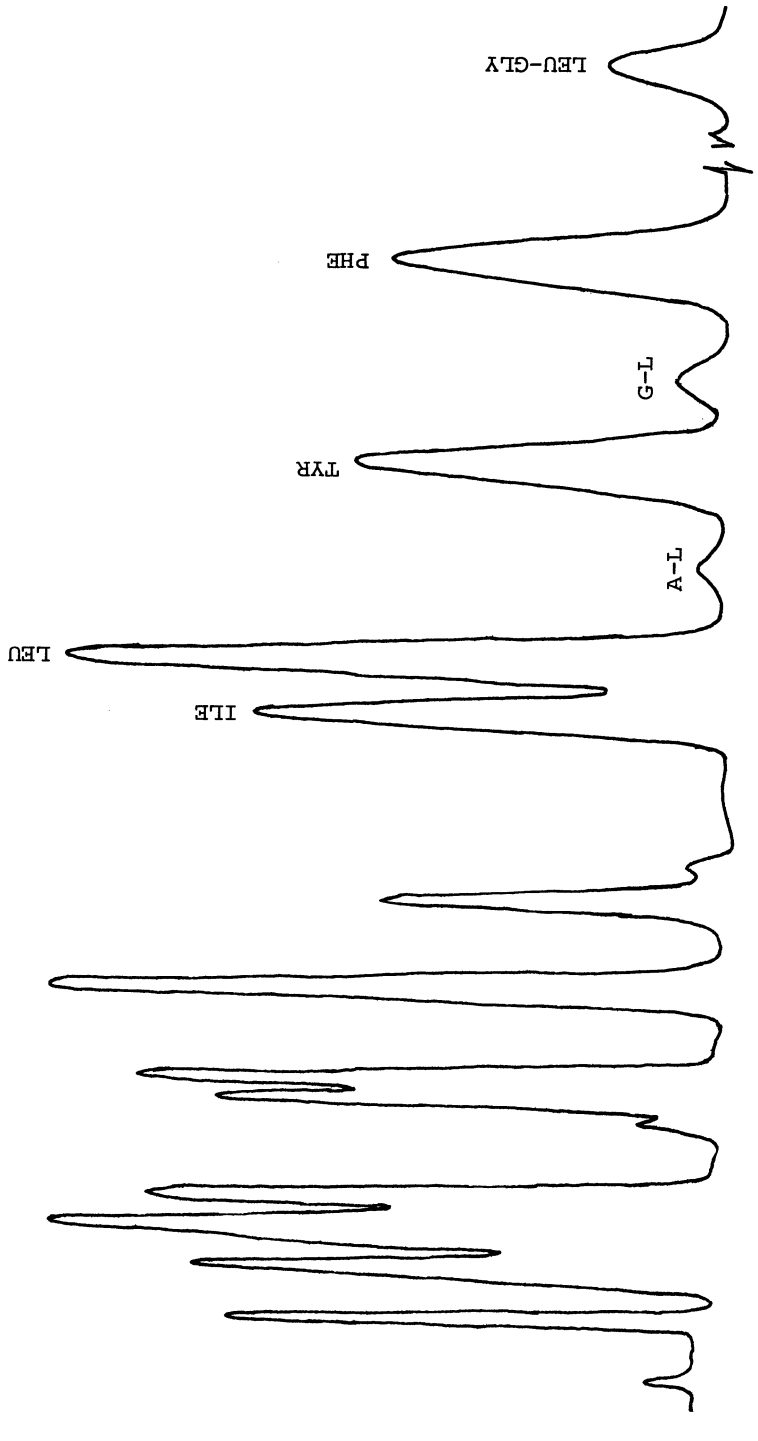


Fig. 4. Chromatogram of Isopeptide Analysis.

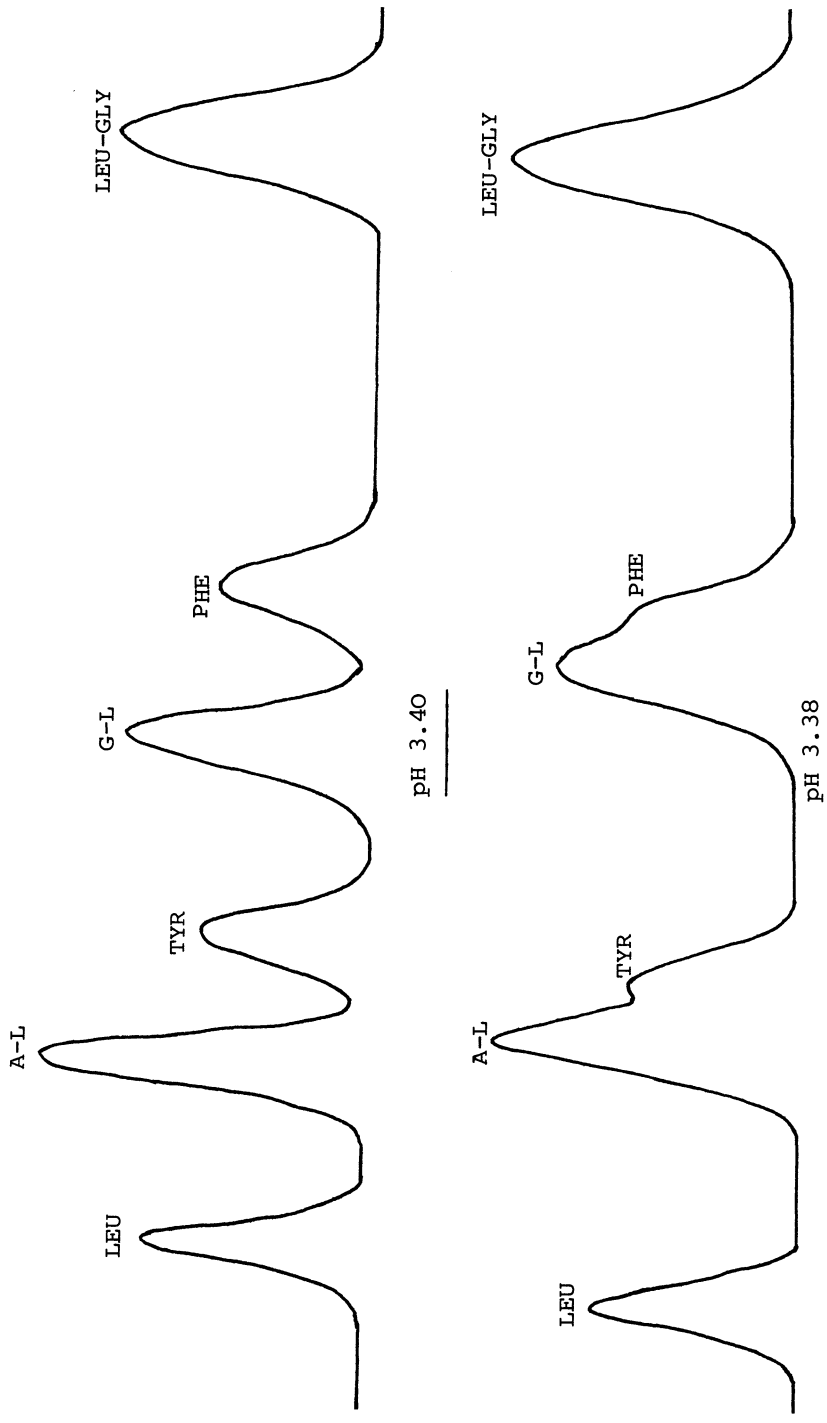


Fig. 5. Dependence of Separation of Isopeptides upon pH

the isopeptide content of a series of proteins (heat damaged and native).

The ease of formation of isopeptide crosslinks in any specific protein should be dependent on two factors. Firstly, the content of aspartic and glutamic acids (or their amides) in relation to the proportion of lysine present and secondly, the proximity of carboxyl (or amide) and amino side groups. Differing quantities of aspartyl or glutamyl residues should define the tendency of the protein to form A-L and G-L respectively. However, the proximity of reactive side chains will be determined by the stereochemistry of the particular protein molecule. To date relatively few secondary and tertiary structures have been determined. Another difficulty as far as the spatial arrangement of the amino acid residues is that, when proteins are subjected to heat, it is very probable that large conformational changes occur, thus disturbing the original shape of the molecule.

The proportion of the aspartyl, glutamyl and lysyl residues in the proteins used are shown in Table 2. In the unheated proteins no isopeptides were located. This was not surprising as far as the highly purified proteins were concerned, but the presence of some G-L was expected in the industrial blood samples. All the samples were subjected to heat treatments and then enzymically hydrolysed by the method of Cole, Fletcher, Gardner and Corfield (1971). Each digest was then separated on an Autoanalyser using lithium citrate buffers (pH 3.40) as described earlier in this paper. The results of the isopeptide analysis are shown in Table 2. It is apparent from the results that in the case of chicken muscle X 902E, where specific protein had been heated for different times, more prolonged heating results in an increase in the amounts of isopeptide links formed, as was previously observed with keratin (Asquith and Otterburn, 1971).

From the quantities of isopeptides formed during the heat treatments it would appear that there is no obvious correlation between the production of G-L and A-L and the proportions of the reactive amino acid residues present in the proteins. Thus in bovine plasma albumin, whilst there is a high proportion of the necessary residues, no isopeptides were detected. Gelatin has a relatively high glutamyl content and again no isopeptides were located. The industrial sample of bovine haemoglobin yielded significant quantities of both isopeptides after heating at 121° for 27h. Similarly, samples of whole blood (bovine) damaged by industrial drying processes (blood meal X 949 and X 953) also contained both G-L and A-L. The relative quantities of the isopeptides in these samples did not seem to follow a consistent pattern.

TABLE 2

Isopeptides in Heated Proteins (g/16 gN)

	Protein	Time of heating (h)	Temp. °C	Aspartic	Glutamic	Lysine	A-L	G-L
	BPA Cohn fraction V	8	121	9.99	13.21	12.21	0	0
	BPA Cohn fraction V	27	121	9.99	13.21	12.21	0	0
*	Bovine plasma	27	121	9.49	12.76	12.11	0	0
*	Bovine haemoglobin	27	121	9.73	7.14	9.85	0.75	0.19
+	Blood meal X 949	Low temperature		9.20	8.92	9.50	0.33	0.32
+	Blood meal X 953	High temperature		9.62	9.16	7.56	0.25	0.44
	Egg albumen	57	115	9.24	13.05	6.82	0.27	0.12
	Zein	57	115	5.06	22.16	0.05	0.11	0
	Lactalbumin	27	85	10.07	15.46	10.37	0.04	0.06
	Lactalbumin	57	115	10.07	15.46	10.37	1.04	0.74
	Lysozyme	27	121	16.80	4.92	5.75	0.43	0
	Casein	27	115	6.80	19.99	7.88	0.14	0.48
	Chicken muscle X 902E	4	121	10.5	14.7	9.7	0.3	0.2
	" " "	8	121	10.5	14.7	9.7	0.4	0.4
	" " "	27	121	10.5	14.7	9.7	0.8	0.9
	Gelatin	27	121	6.42	11.79	4.47	0	0

* Ex-Regal Foods Ltd., Craigavon (N.I.)

\+ Industrial samples of heat damaged blood meal

In the case of zein a low isopeptide content, even on prolonged heating, should be expected in view of the low original lysine content. However, it was surprising that the isopeptide which was formed was entirely A-L, especially in view of the high proportion of glutamic acid in the native protein. Indeed, most of the proteins examined seemed preferentially to form A-L on heating. Only casein, with a high glutamic acid content, and chicken muscle protein were exceptions to this finding. This was particularly surprising as in the in vivo formation of isopeptide links, G-L is the moiety usually observed (Lorand, Downey, Gotoh, Jacobsen and Tokura, 1968; Pisano, Finlayson and Peyton, 1968; Matačič and Loewy, 1968).

Thus, spatial relationships of the residues to one another in the structure of the protein is apparently the controlling factor in permitting isopeptide formation when the protein is heated. Of particular interest with regard to this aspect is the preferential formation of A-L as a result of heating egg white lysozyme. The complete amino acid sequence of this protein is known together with its secondary and tertiary structures (Phillips, 1967). Examination of these properties shows that, due to folding of the protein chain, lysine residues at the 33, 96, 97 and 116 positions are located almost directly opposite asparagine or aspartic acid residues. Such an arrangement obviously enhances the formation of A-L. Only two glutamic acid residues occur in the molecule and their positioning is less likely to result in isopeptide formation.

The fact that isopeptide links are formed in egg albumen is of particular interest in so far as this material is commonly used as foodstuff, often after heating at temperatures in excess of 121°. The formation of isopeptide links may have some bearing on the denaturation changes observed under these conditions.

The presence of extraneous non-protein material can have a significant effect on the formation of isopeptides. Chicken muscle protein (X 903) heated in the presence of natural fats showed a reduction in the quantities of isopeptide crosslinks formed. Further, it was observed that the presence of the lipid material hindered complete enzymic digestion of the sample to such an extent that free isopeptides could not be detected in the partial hydrolysates so obtained. Presumably this is a physical effect in which the presence of lipids prevents penetration into the protein by aqueous solutions of proteases and peptidases. Extraction of such lipid material from the heated samples prior to enzymic digestion resulted in clear hydrolysates in which the free isopeptides were detected and quantitatively estimated. It can be seen from Table 3 that the quantities of isopeptides formed in the X 903 protein were very low and correspond to approximately 4h heating of the purified X 902E muscle. (cf. Table 2).

TABLE 3

Influence of Natural Fats and Methyl Stearate on Isopeptide Formation g/16g N

SAMPLE	A-L	G-L
X 902E unheated (fat removed)	0.00	0.00
X 902E 27h @ 121° (fat removed)	0.80	0.87
X 903 unheated (fat present)	0.00	0.00
X 903 27h @ 116° (fat present)	0.27	0.15
X 902E + 5% methyl stearate unheated	0.00	0.00
X 902E + 5% methyl stearate 27h @ 121°	0.26	0.21
X 902E + 15% methyl stearate unheated	0.00	0.00
X 902E + 15% methyl stearate 27h @ 121°	0.34	0.27

Natural fats may have hindered isopeptide formation by engaging in a competitive reaction with the ε-amino groups of lysine or by acting as a hydrophobic barrier between the polar amino and carboxyl groups. To clarify this point unheated X 902E (i.e., fat extracted) chicken muscle was slurried with chloroform solutions of methyl stearate. After the solvent had been removed the samples of muscle protein containing 5% and 15% methyl stearate were equilibrated to 14% moisture and heated in sealed tubes for 27h at 121°. The fatty acid ester was solvent extracted from the samples prior to isopeptide analysis. The results are shown in Table 3; these indicate that methyl stearate had a similar effect to the natural fats and hindered isopeptide formation.

The preceding work shows that isopeptide bonds can be formed in pure proteins by the action of heat. The significance of their formation has been summarised in the context of protein chemistry by Asquith, Otterburn and Sinclair (1972). However, as far as nutrition is concerned their importance could lie in two areas: (i) the 'binding' of lysyl residues, and (ii) the formation of crosslinks which may inhibit or reduce the rate of *in vivo*

digestion of the heat modified protein. In a recent paper, the influence of heat induced crosslinks upon the nutritional properties of chicken muscle protein was examined (Hurrell, Carpenter, Sinclair, Otterburn and Asquith, 1976). In this work chicken muscle protein X 902E and X 903 were used in rat feeding trials in order to determine the ileal and faecal digestibilities of the protein. Table 4 shows the analyses of the various lysine fractions in the dietary protein and the ileal and faecal samples.

It was anticipated from this work that the inaccessible lysine (i.e., lysine which is unavailable for reaction with F.D.N.B.) and the lysine contained within G-L and A-L would have significantly lower digestibility values than the overall protein. Thus, these high lysine fractions within the heated protein should accumulate in the ileal and faecal contents. The results of the feeding trials are shown in Table 5. These results indicate that the isopeptides are as digestible if not more so than the rest of the protein. An explanation of these results probably lies in the stability of the isopeptides to enzymic and bacterial attack during digestion.

Studies were carried out to examine the % recovery of synthetic G-L to each stage of the in vivo digestion procedure. The results are shown in Table 6. These results show that 75.9% recovery of the G-L can be achieved. If, however, a bactericide is used, such as thymol, almost 96% recovery is found. Although it is accepted that in vivo digestion and in vitro hydrolysis are not identical Table 6 does show that the G-L is relatively stable to the proteases and peptidases used. This work seems to confirm the findings of Waibel and Carpenter (1972), who found that G-L itself was totally available as a source of lysine and that hydrolysis probably occurred within the intestinal wall.

The recovery studies on the isopeptide was then extended to include incubating G-L with extracts from various sections of intestines from rats. This was carried out in order to determine whether residual bacteria, microflora etc. could hydrolyse the isopeptide link. The incubation results are shown in Table 7. From the results it appears that G-L itself is susceptible to attack by intestinal bacteria etc. Because of these findings it would be necessary to correct each sample for isopeptide loss. It was not, however, considered prudent to apply a general correction factor of 66% to all ileal and faecal samples. The studies on the stability of the isopeptides indicated that the results obtained for G-L and A-L in the ileal and faecal samples were low and their digestibilities correspondingly high. It is most unlikely that G-L and A-L were more digestible than the intact protein or its associated lysine fractions. Thus it can be concluded from the nutritional work that the formation of G-L and A-L isopeptide links

TABLE 4

Analysis of Samples from Rat Feeding Trials with X 902E and X 903 Chicken Muscle (w/w %)

	Sample	Total Lys. %	Inacces. Lys. %	Acces. Lys. %	A-L %	G-L %	Protein %	Chromic Oxide %
*Dietary Protein	Protein-free	0	0	0	0	0	0	0.3
	Unheated	9.7	0.6	9.1	0	0	100	0
	8h @ 121°C	9.0	1.5	7.5	0.35	0.37	100	0
	27h @ 121°C	8.9	2.8	6.1	0.80	0.87	100	0
*Ileal Sample	Protein-free	nd	nd	nd	nd	nd	11.1	3.35
	Unheated	nd	nd	nd	nd	nd	21.1	2.92
	8h @ 121°C	nd	nd	nd	nd	nd	28.5	2.16
	27h @ 121°C	nd	nd	nd	0.09	0.13	37.6	2.10
*Faeces Sample	Protein-free	0.30	0.04	0.26	0	0	6.9	4.21
	Unheated	0.56	0.08	0.48	0	0	10.9	4.80
	8h @ 121°C	0.86	0.16	0.70	0.013	0.005	16.1	4.56
	27h @ 121°C	2.20	0.65	1.55	0.047	0.022	31.1	3.69
+Dietary Protein	Protein-free	0	0	0	0	0	0	0.3
	Unheated	9.6	0.7	8.9	0	0	100	0
	27h @ 116°C	9.0	1.8	7.2	0.27	0.15	100	0
+Ileal Sample	Protein-free	0.55	0.03	0.52	0	0	14.4	3.62
	27h @ 116°C	2.47	0.38	2.09	0	0	33.0	2.73
+Faeces Sample	Protein-free	0.56	0.04	0.52	0	0	14.7	3.66
	27h @ 116°C	1.41	0.24	1.17	0	0	26.9	3.53

nd = Not Determined

* = X 902E

+ = X 903

TABLE 5

% Digestibility *Values from Rat Feeding Trials with X 902E and X 903 Chicken Muscle

Sample		Total Lys.	Inacces. Lys.	Acces. Lys.	A-L	G-L	Protein
Ileal X 902	Unheated	nd	nd	nd	-	-	88
	8h @ 121°C	nd	nd	nd	nd	nd	70 (80)
	27h @ 121°C	nd	nd	nd	84	79	56 (64)
Faecal X 902	Unheated	99	96	99	-	-	98
	8h @ 121°C	96 (97)	95 (99)	96 (97)	98	99	94 (96)
	27h @ 121°C	82 (83)	83 (86)	82 (83)	95	98	80 (82)
Ileal X 903	27h @ 116°C	75 (85)	78 (88)	75 (85)	100	100	76 (85)
Faecal X 903	27h @ 116°C	91 (93)	91 (97)	92 (93)	100	100	89 (91)

* Figures in brackets represent each value expressed as a % of the corresponding value for the unheated control.

TABLE 6

% Recovery of ε-N-(γ-Glutamyl)-Lysine from the Enzymatic Hydrolysis Procedure

Hydrolysis stage (without thymol)	% Recovery
Tributyl phosphine	91.5
Acrylonitrile	87.9
Buffer	93.5
Pepsin	93.6
Pronase	74.7
Aminopeptidase M	93.8
Prolidase	93.6
Complete digestion	*75.9
Complete digestion in the presence of thymol	95.8

* This value decreased to 11.0% on storage for 50 days at +4°C.

nd = Not Determined

TABLE 7

% Recovery of ε-N-(γ-Glutamyl)-Lysine after Incubation with Rat Intestinal Bacteria

Intestinal Region	Culture Medium	% Recovery without a Bactericide	% Recovery with Thymol	% Recovery with Mercuric Chloride
Duodenum	Nutrient broth	75	86	96
Jejunum	Nutrient broth	95	94	95
Ileum	Nutrient broth	62	59	90
Whole intestines	Minimal	0	nd	93
Whole intestines	Minimal + 0.04% glucose	0	nd	91
Whole intestines	Nutrient broth	0	nd	95

during heat treatments to proteins probably reduce the rate of digestion of the protein, either by insolubilization, preventing enzyme penetration, or by the masking of site of enzyme attack. However, G-L and A-L themselves appear to be as digestible as the protein as a whole.

ACKNOWLEDGEMENTS

We would like to express our thanks to the following people: Professor R. S. Asquith for his valuable and continued support and advice; and Dr. K. J. Carpenter and Dr. R. F. Hurrell for carrying out all feeding trials mentioned in this work. We would also like to thank the A.R.C. (G.B.) for a grant to W.J.S.

REFERENCES

Asquith, R. S. and Otterburn, M. S. (1969). Basic amino acids in heated keratin. J. Textile Inst., 60, 208.

Asquith, R. S., Otterburn, M. S., Buchanan, J. H., Cole, M., Fletcher, J. C. and Gardner, K. L. (1970). The identification of N^{ϵ}-(γ-L-Glutamyl)-L-lysine cross-links in native wool keratins. Biochim. Biophys. Acta., 221, 342.

Asquith, R. S. and Otterburn, M. S. (1971). Self-crosslinking in keratin under the influence of dry heat. Applied Polymer Symposium, 18, 277.

Asquith, R. S., Otterburn, M. S. and Gardner, K. L. (1971). The identification of N^{ϵ}(β-L-Asparty1)-L-lysine in native and heated keratin. Experientia, 27, 1388.

Asquith, R. S., Otterburn, M. S. and Sinclair, W. J. (1974). Isopeptide crosslinks - their occurrence and importance in protein structure. Angew. Chem. Int. Edit., 13, 514.

Astbury, W. T. and Woods, H. J. (1934). X-ray studies of hair, wool and related fibres. Phil. Trans. Roy. Soc. (London), A 232, 333.

Atkin, G. E. and Ferdinand, W. (1970). Accelerated amino acid analysis: studies on the use of lithium citrate buffers and the effect of n-propanol, in the analysis of physiological fluids and protein hydrolyzates. Anal. Biochem., 38, 313.

Auto-analyser handbook (1973). Technicon Instrument Co. Ltd., Basingstoke, England.

Benson, J. V., Gordon, M. J. and Patterson, J. A. (1967). Accelerated chromatographic analysis of amino acids in physiological fluids containing glutamine and asparagine. Anal. Biochem., 18, 228.

Bjarnason, J. and Carpenter, K. J. (1970). Mechanisms of heat damage in proteins. Part 2: Chemical changes in pure proteins. Brit. J. Nutr., 24, 313.

Burns, J. A., Curtis, C. F. and Kacser, H. (1965). A method of the production of a desired buffer gradient and its use for the chromatographic separation of argino-succinate. J. Chromatog., 20, 310.

Carpenter, K. J., Morgan, C. B., Lea, C. H. and Parr, L. J. (1962). Chemical and nutritional changes in stored herring meal. Part 3: Effects of heating at controlled moisture contents on the binding of amino acids in freeze-dried herring press cake and in related model systems. Brit. J. Nutr., 16, 451.

Cole, M., Fletcher, J. C., Gardner, K. L. and Corfield, M. C. (1971). A study of enzymatic hydrolysis applicable to the examination of processed wools. Applied Polymer Symposium, 18, 147.

Cotton, F. A. and Wilkinson, G. (1972). "Advanced Inorganic Chemistry". 3rd Edition, Interscience, New York and London.

Davis, N. C. and Smith, E. L. (1957). Purification and some properties of prolidase of swine kidney. J. Biol. Chem., 224, 261.

Dean, J. A. (1969). "Chemical Separation Methods." Van Nostrand, London and New York.

Ford, J. E. (1973). "Proteins in Human Nutrition," p.515; Porter, J. W. G. and Rolls, B. A., Editors, Academic Press, London.

Froberg, C. E. (1965). "Introduction to Numerical Analysis." Addison and Wesley, London and New York.

Hamilton, P. B. (1963). Ion exchange chromatography of amino acids. A single column, high resolving, fully analytical procedure. Anal. Chem., 35, 2055.

Harding, H. W. J. and Rogers, G. E. (1971). ε(γ-glutamyl)-lysine crosslinkage in citrulline-containing protein fractions from hair. Biochemistry, 10, 624.

Hurrell, R. F., Carpenter, K. J., Sinclair, W. J., Otterburn, M. S. and Asquith, R. S. (1976). Mechanisms of heat damage in proteins. Part 7: The significance of lysine-containing isopeptides and of lanthionine in heated proteins. Brit. J. Nutr., 35, 383.

International Computers Ltd. (I.C.L.). "Scientific Sub-Routines," F4RUNK, (1968).

Lorand, L., Downey, J., Gotoh, T., Jacobsen, A. and Tokura, S. (1968). The transpeptidase system which crosslinks fibrin by γ-glutamyl ε lysine bonds. Biochem. Biophys. Res. Commun., 31, 222.

Matačić, S. and Loewy, A. G. (1968). The identification of isopeptide crosslinks in insoluble fibrin. Biochem. Biophys. Res. Commun., 30, 356.

Mauron, J. (1972). "Encyclopaedia of Food and Nutrition, Vol. 2; Protein and Amino Acid Functions, p. 417"; Bigwood, E. J., Editor. Pergamon Press, Oxford.

Mecham, D. K. and Olcott, H. S. (1947). The effect of dry heat on proteins. Ind. Eng. Chem., 39, 1023.

Milligan, B., Holt, L. A. and Caldwell, J. B. (1971). The enzymic hydrolysis of wool for amino acid analysis. Applied Polymer Symposium, 18, 113.

Mondino, A. (1969). A new system of automatic amino acid analysis. Part II. J. Chromatog., 39, 262.

Perry, T. L., Stedman, D. and Hansen, S. (1968). A versatile lithium buffer elution system for single column automatic amino acid chromatography. J. Chromatog., 38, 460.

Peters, J. H., Berridge, B. J., Cummings, J. G. and Lin, S. C. (1968). Column chromatographic analysis of neutral and acidic amino acids using lithium buffers. Anal. Biochem., 23, 459.

Peterson, E. A. and Sober, H. A. (1959). Variable gradient device for chromatography. Anal. Chem., 31, 857.

Phillips, D. C. (1967). Lysozyme and the development of protein crystal chemistry. Seventh International Congress of Biochemistry, Tokyo. p.63.

Pisano, J., Finlayson, J. S. and Peyton, M. P. (1968). Crosslink in fibrin polymerized by factor XIII. Science, 160, 892.

Schmitz, I. (1975). Dissertation, T. H. Aachen (W. Germany).

Schmitz, I., Zahn, H., Klostermeyer, H., Rabbel, K. and Watanabe, K. (1976). Zum vorkommen von isopeptidbindungen in erhitztem milcheiweiss. Z. Lebensm. Unters.-Forsch., 160, 377.

Waibel, P. E. and Carpenter, K. J. (1972). Mechanisms of heat damage in proteins. Part 3: Studies with ε-(γ-L-glutamyl)-L-lysine. Brit. J. Nutr., 27, 509.

Zahn, H. and Pätzold, W. (1963). Synthese von oligamiden aus L-glutaminsäure und L-lysin. Chem. Ber., 96, 2566.

HEAT INDUCED CROSSLINKS IN MILK PROTEINS AND CONSEQUENCES FOR THE MILK SYSTEM

Henning Klostermeyer and Ernst H. Reimerdes

Institut für Chemie
Bundesanstalt für Milchforschung
D 2300 Kiel

ABSTRACT

The protein system of milk is rather unusual, there are nearly no interchain crosslinks found. Even intrachain crosslinks, especially disulfide bridges, are present only in about every fourth protein molecule. Heating causes dramatic changes in the structure of milk proteins, resulting in the formation of polymeric networks. The contribution of individual milk proteins, namely the ß-lactoglobulins, α-lactalbumin and $\varkappa$-casein, to the formation of crosslinks is studied with respect to heating temperature and time, pH and atmosphere. Measured are changes in molecular weights and in the SH/SS-levels as well as the formation of dehydroalanine, lysinoalanine, lanthionine and isopeptide bonds. Some practical aspects of crosslinking in milk proteins are discussed.

THE MILK SYSTEM

Bovine milk is a very complex biological system containing water (about 87%), lactose (about 4.9%), proteins (about 3.5 %) and minor components (about 0.7%) as well as protein coated lipid droplets (about 3.6%). Normally each single protein is found in a number of genetic variants. About 80% of the milk proteins belong to the casein complex,

consisting of α_{s1}-, ß- and $\varkappa$-casein (ratio in the range of 4:3:1), the proteolytic degradation products of ß-casein (γ-, T-, RS-caseins) and the so-called minor caseins as there are α_{s3}-, α_{s4}-, α_{s5}-caseins. The caseins form large aggregates, the micelles, including calcium ions and low molecular weight anions. Some special chemical features have to be mentioned in order to understand the behaviour and the reactivity of the caseins. The caseins are extremely hydrophobic proteins and therefore the micelles dissociate at low temperatures according to the entropy effect. Beside this they are phosphoproteins (therefore calcium ion binding) and the proline content is relatively high while that of cysteine and cystine is very low; this means, these proteins have very low amount of tertiary structures and there is no denaturation in the general way.

On the other hand the so-called whey proteins are highly structured proteins, partially they are blood serum components, partially milk own proteins. The major components are the ß-lactoglobulins and α-lactalbumin with about 10% and 4% of the milk proteins respectively. ß-Lactoglobulin contains two disulfide bridges and one thiol group per molecule and normally it is associated as a dimer. α-Lactalbumin is stabilized by four disulfide bridges and is a glycoprotein with varying amounts of carbohydrates.

A useful entry to the literature on milk and milk proteins are the key references Webb, Johnson and Alford (1974), McKenzie (1970/71), and Mulder and Walstra (1974).

TYPICAL HEAT INDUCED CHANGES IN THE MILK PROTEIN SYSTEM

Definitely the heat treatment of milk is a serious problem during cheese manufacturing especially with renneting of the milk. During renneting the enzyme chymosin splits one special peptide bond in $\varkappa$-casein, resulting in para-$\varkappa$-casein and a glycopeptide fragment the so-called glycomacropeptide. Para-$\varkappa$-casein causes coagulation together with the residual micellar proteins. In heat treated milk this proteolytic step is much slower according to the complex formation between whey proteins, especially ß-lactoglobulin, and $\varkappa$-casein (Wilson and Wheelock, 1972; Wheelock

and Kirk, 1974, Wiechen and Knoop, 1974). It sounds reasonable to assume direct crosslinking of $\varkappa$-casein and ß-lactoglobulin with disulfide bridges created by thiol/disulfide exchange, which means that the rennin-sensitive Phe-Met-bond is no longer available for the enzyme. This mechanism has not been established till now (McKenzie, 1970/71). This should be possible by isolation and characterization of definite $\varkappa$-casein/ß-lactoglobulin reaction products. Another explanation for the hindrance of $\varkappa$-casein proteolysis might be coating of the casein micelles by polymerized denatured whey proteins, without involving $\varkappa$-casein in complex formation by covalent bonds.

The complex formation of ß-lactoglobulin and $\varkappa$-casein during heat treatment of milk is of technical use in the manufacture of so-called coprecipitates of caseins and whey-proteins (Southward and Goldman, 1975). During the single processes for the preparation of coprecipitates the thiol/disulfide exchange reactions are increased on purpose by preliminary heating skim milk at alkaline pH (up to pH 8.5) with temperatures above the denaturation temperature of whey proteins. During this treatment only SH- and SS-containing peptides will be crosslinked. Therefore the total protein precipitation is performed either at the isoionic point of the caseins or by addition of calcium ions; in the latter case this result in a protein complex, which contains the whey proteins, $\varkappa$-casein and the α_s-minor caseins crosslinked by covalent disulfide bridges while the caseins are complexed by ionic calciumphosphate bridges according to the amount of serine phosphates in these molecules.

Furthermore this insolubility and heterogeneity of coprecipitates made it impossible to look for their composition; one of the best ways to get a solution of these complex molecules as single proteins is achieved by oxidation with performic acid (Klostermeyer and Offt, 1976). This is in agreement with a high contribution of disulfide bridges during crosslinking.

If there is extensive heat treatment other crosslinking reactions involving lactose (Maillard reaction) are complicating the system and analysis. This reaction is wellknown, e.g. from browning of condensed milk and it might be one of the

reasons why milk powder, especially roller dried powder is no longer used in cheese making. But according to their limited swelling properties high heat milk powders are of excellent usefulness, as the coprecipitates, for the preparation of some baker's ware. These insoluble proteins show almost no reactivity with the thiol and disulfide bridges of the grain proteins which are important during the baking process. Soluble proteins show reactivity in this direction which will cause damage of the texture of the baked products.

Another indication for secondary reactions during heat treatment is the cooking flavour found in heated milk and milk protein products, which indicates the degradation of the S-containing amino acids. As a reaction product of this degradation lysinoalanine has be found, but the heating conditions were much stronger than during processing in practice (Freimuth et al., 1974).

POTENTIAL SOURCES FOR CROSSLINKING REACTIONS

According to our present knowledge there are no intermolecular crosslinkings of the proteins in native milk. Recently there were found small amounts of isopeptides in bovine colostrum (Klostermeyer, Rabbel, and Reimerdes, 1976). The most important sources for temperature induced crosslinking reactions should be found in the cysteine/cystine content of the proteins. However, in general milk processing is performed at more or less acidic pH-ranges, this means there will take place probably only very limited disulfide exchange reactions or ß-eliminations. Beside this we have to recognize that the caseins, the major milk proteins average to 0.09% sulfur in form of cysteine or cystine, which is very low.

Definitely α_{s1}-casein and ß-casein and their fragments do not contain cysteine/cystine residues, while $\varkappa$-casein has two half-cystines, which are not very reactive (Beeby, 1974) and a carbohydrate moiety, while the so-called minor caseins contain one or two disulfide bridges (Hoagland, Thompson and Kalan, 1971). The amount of these minor caseins equals about the concentration of $\varkappa$-casein in milk (Nijhuis, Klostermeyer and Reimerdes, 1976). This means on a molecular base that only any eight to tenth casein molecule is able

to form crosslinkings according to disulfide bridges. Calculated on the base of the whole milk protein system any third or fourth molecule might contribute to polymerization in this way.

Considering the ß-elimination reactions during heat treatment, however, resulting in dehydroalanine, all casein components are potential partners for intermolecular crosslinking reactions. This will not only be due to the formation of dehydroalanine from sulfur containing proteins - in this case most of the caseins will be passive reacting partners during the dehydroalanine-protein reaction - but also to eliminations at the phosphorylated serine residues, which means any casein is able to form active dehydroalanine groups and therefore active crosslinking abilities (Manson and Carolan, 1972).

Furthermore all milk proteins are able to take part in isopeptide formation and last not least all of them are able to perform crosslinking reactions according to the Maillard reaction (degradation products of sugars as crosslinking reagents). The formation of salt bridges by the phosphate groups of the caseins and calcium-ions is not summarized under so-called crosslinkings, even if this association or formation of aggregates is extremely important for milk processing.

Till today there are no systematic evaluations of the contribution of the several sources to the crosslinking reactions in milk and milk products during the different ways of processing.

THE REACTIVITY OF INDIVIDUAL MILK PROTEINS DURING HEAT TREATMENT

Our present work is concerned with the behaviour of the SH- and SS-groups of the individual milk proteins. We are studying the changes in SH- and SS-content with single highly purified milk proteins during heat treatment dependent on temperature, pH, time and atmosphere. In connection with these reactions we follow the formation of dehydroalanine, lysinoalanine and lanthionine as well as the formation of isopeptides and the molecular weights of the resulting polymers. SH- and SS-groups are determined polarographically

(Mrowetz and Klostermeyer, 1972), dehydroalanine, lysinoalanine and lanthionine are determined after acid hydrolysis according to Watanabe and Klostermeyer (1976b). The procedure for the isopeptide determination (Schmitz et al., 1976) was also developed in our laboratory. Molecular weigth determinations were performed by column chromatography with molecular sieves.

The first studies were performed with pure ß-lactoglobulin A, with this genetic variant there are found much less results in literature than with the more temperature, sensitive ß-lactoglobulin B. For two reasons ß-lactoglobulins are very interesting for these studies, namely they are the largest amount of sulfur containing protein components of milk and they contain thiol as well as disulfide groups. The following studies were performed with pure α-lactalbumin because there were no data available according to its behaviour during heat treatment. Further studies were performed with the sulfur containing caseins, especially $\varkappa$-casein. These caseins are highly unstructured proteins, according to the low content of tertiary structure they do not show normal denaturation behaviour and we expect different reactivities during heat treatment. Especially with the minor caseins there were large problems with the purification, during this research we developed special techniques (Nijhuis and Klostermeyer, 1975; Nijhuis, Klostermeyer and Reimerdes, 1976).

ß-Lactoglobulin

Heat induced changes in milk are significantly correlated to the reactions of the ß-lactoglobulins. It is well known that milk containing only the genetic variant ß-lactoglobulin A is more heat stable than ß-lactoglobulin B containing milk (Gough and Jenness, 1962). The thermal stability of milk is comparable to the different heat stability of the genetic ß-lactoglobulin variants, which might be due to their different association behaviour (Zimmerman, Barlow and Klotz, 1970). The SH-groups of ß-lactoglobulins are normally burried in the dimer complex and therefore of low reactivity. Heat treatment of ß-lactoglobulin results in an increase of dimer dissociation; this reaction, which follows several steps, results in the denaturation of the ß-lactoglobulin monomer and in polymer formation (see e.g.

Sawyer, 1968; Roels, Préaux and Lontie, 1973). On the other hand blocking the SH-groups in ß-lactoglobulin by SH-reagents did not cause a stabilization of the monomer but results in a total suppression of the polymer formation (Roels, Préaux and Lontie, 1973; Watanabe and Klostermeyer, 1976a).

Thus during milk processing blocking of the SH-groups would be very important and one of the possibilities to achieve this might be the oxidation of the functional SH-groups. During our heating experiments with ß-lactoglobulin A in the presence and absence of oxygen in the system we got indeed very different results. Heating the protein under nitrogen (75°C, pH 6.9, 40 min.) resulted in only small amounts of residual dimers and high-polymer protein material. There were no polymer intermediates found. Heating in the presence of oxygen caused polymers of lower molecular weight too, the composition is not known (Watanabe and Klostermeyer, 1976a).

During the same experiments the losses of sulfur from cysteine and cystine residues were quite different. In the presence of oxygen 0.58 mol/ß-lactoglobulin dimer was eliminated which amounts to 5.8% of the protein sulfur while under nitrogen only 0.2% of the protein sulfur was eliminated. Changing the heating temperature to 95°C causes sulfur elimination of 12.2% and 0.9% respectively.

At the same time in the oxygen containing system there were found o.13 moles dehydroalanine, 0.1 mol lysinoalanine and no lanthionine. The results change dramatically if the pH is slightly increased from pH = 6.9 to pH = 7.3, 13.1% of the amino acid sulfur is eliminated after 20 min. In the case of pH = 9.5 the effect is even more significant, 38.7% of the sulfur is lost.

The change in sulfur content naturally influences the amount of the other components caused by heat treatment, resulting in about 1 mol dehydroalanine, 3,5 moles lysinoalanine and 0.04 moles lanthionine/dimer in the pH 9.5 experiment. In the case of lanthionine 50% were meso-lanthionine and 50% were found in the L-form. Checking the amount of lysine after the heating procedure only 23 moles/dimer are still available instead of 30 moles/dimer in native ß-lactoglobulin. Since ß-lactoglobulin does not contain any carbohydrates, at the

moment there is no explanation for this heating effect.

The temperature dependency of these reactions is very significant. There are two critical temperature ranges, one around 70°C at which ß-lactoglobulin denatures and a second one at about 120°C at which the cystine degradation increases very much, while the change in SH content is relatively small. Therefore dehydroalanine mainly derives from cystine not from cysteine.

Isopeptides were not found during normal heating procedures, but during dry heating of ß-lactoglobulin we were able to show formation of isopeptides. The amount of these crosslinkings is too small in order to have any effect on food processing. Surprisingly there were found only Glu Lys-isopeptides while Asp Lys was not formed under the above conditions (Schmitz et al., 1976).

We have also done a direct comparison of the heat induced degradation between the ß-lactoglobulins A and B. As expected, the effects were somewhat higher with the B variant, in average within the range of 5 to 10%. Actually the differences were much higher in short time heating experiments but rather small after long time heating (Watanabe and Klostermeyer, 1976c).

α-Lactalbumin

There are almost no results on the effect of heat treatment on pure α-lactalbumin. One of the reasons is the problem to get larger amounts of homogeneous pure material for these studies. The first results of the direct comparison of α-lactalbumin with ß-lactoglobulin (contrarily to the ß-lactoglobulins α-lactalbumin is free of thiol groups!) looks very promising.

α-Lactalbumin is more heat sensitive than the ß-lactoglobulins. The observed effects are much stronger under oxygen free conditions than in an open air system. The relative differences in the results from the two heating conditions are smaller than with ß-lactoglobulin. The losses of sulfur are definitely higher and in correlation the amount of dehydroalanine formed is two to three times higher than with ß-lactoglobulin. According to these results and effects the

amount of secondary reactions is much higher, this means the crosslinking is very large and the product shows enterely different technological properties in relation to heat treated ß-lactoglobulin. With increasing temperatures the thiol content of ß-lactoglobulin is decreasing while with α-lactalbumin in the range of 70°C to 80°C the thiol formation starts from zero content and only above 120°C the formation increases dramatically. This at the same time is the temperature were the disulfide degradation increases significantly. There is further research in progress concerning the influence of the carbohydrate moiety during heat treatment, - α-lactalbumin is a glycoprotein - and the molecular weight and polymer distribution, caused by heat. In comparison we studied the heat treatment of lysozyme solutions, a protein which is structurally related to α-lactalbumin; the results are very similar to those gotten with the milk protein (Schnack, Reimerdes and Klostermeyer, 1976).

TABLE 1

Effect of N-Ethylmaleinimide (NEMI, 20 mg/100 ml Milk) on the Rennet Coagulation of Untreated and Highly Heated (10 Min., 80°C) Milk

Material	Clotting Time min	Curd Quality[a]	SH-Groups mg/100 ml
Native Milk			
Unheated	8	44	0.53
Unheated + NEMI	8	43	0.15
Heated	17	6	0.44
Heated + NEMI	10	26	0.07
Pasteurized Milk			
Original	15	24	0.42
Original + NEMI	16	23	0.10
Heated	21	8	0.38
Heated + NEMI	15	21	0.09
Heated + $CaCl_2$	14	14	-
Heated + $CaCl_2$ + NEMI	9	30	-

a Relative values, measured with a Hellige-Thrombelastograph (Freiburg, W.Germany)

SOME PRACTICAL CONCLUSIONS

According to these results there are heat induced changes of economical and practical importance during milk processing. Furthermore, these changes depend significantly on the disulfide content of the milk protein system. This means also that it might be possible to avoid negative changes and properties e.g. by thiol blocking agents. This is in agreement with the results of Wilson, Wheelock and Kirk (1974) who were able to show that the addition of such reagents before heating kept the original renneting properties, this even during UHT-conditions (140°C, Thomasow and Klostermeyer, 1976). This will be of great practical interest, because during UHT-treatment spores of Clostridium will be killed; this means it will be possible to avoid nitrate addition during cheese making, if by the addition of thiol blocking agents it is possible to keep the original renneting properties of milk during UHT-sterilization. Unfortunately at the moment there is no SH-blocking reagent available, which might be added to food products. On the other hand in the literature there were several proposals to add such compounds to milk before heat treatment in order to avoid cooking flavour. The potential applications of such compounds show much more possibilities. Tab. 1 demonstrates the influence of the thiol blocking reagent N-ethylmaleinimide on the clotting time of heat treated and raw milk.

Another point of practical importance is the influence of crosslinking on the proteolytic digestion of the milk proteins, especially if during heat treatment amino acids as lanthionine and lysinoalanine are formed and involved in crosslinking. During our research (Klostermeyer, Rabbel and Reimerdes, 1975) we found a dramatic change of the proteolytic digestibility in vitro after heat treatment in the temperature range of 70°C to 80°C. This effect is used in order to show the heat treatment of an unknown milk product especially of the pasteurizing temperature.

Partially crosslinking effects show negative properties during food processing. On the other hand crosslinking reactions are of great advantage in food texture studies. In this case the low sulfur content of 0.9% cysteine/cystine sulfur is a great disadvantage of the milk proteins. Therefore

milk proteins in the moment do not seem suitable for the preparation of textured proteins on the base of disulfide crosslinkings. In spite of a series of patents and the fairly cheap availability of milk proteins, there is no product comparable to the textured foods from plant proteins. At the moment we are studying the influence of additional SH-groups, introduced syntetically to caseins, on the crosslinking and texturing properties of these proteins (Klostermeyer and Reimerdes, 1976). Crosslinkings on the bases of lysinoalanine, lanthionine or the Maillard reaction cause products which show decreased biological availibility and therefore a lower biological value (Hurrell et al., 1976; Anonymus, 1976). Therefore futural attempts should depend on the disulfide bridging properties.

ACKNOWLEDGEMENT

H.K. thanks the Deutsche Forschungsgemeinschaft for supporting research on heated food proteins and for a travelling grant.

REFERENCES

Anonymus (1976). Processed protein foods and lysinoalanine. Nutrition Reviews, 34, 120-122.

Beeby, R. (1974). The half-cystines of bovine α-casein. Biochim. Biophys. Acta, 359, 346-350.

Fox, P.F. and Hoynes, M.C.T. (1974). Heat stability of milk: influence of colloidal calcium phosphate and ß-lactoglobulin. J. Dairy Res., 42, 427-435.

Freimuth, U., Schlegel, B., Gahner, E. and Nötzold, H.(1974). Die Bildung von Lysinoalanin in alkalibehandelten Milchproteinen. Nahrung, 18, K5 - K8.

Gough, P. and Jenness, R. (1962). Heat denaturation of ß-lactoglobulin A and B. J. Dairy Sci. 45, 1033-1039.

Hoagland, P.D., Thompson, M.P. and Kalan, E.B. (1971). Amino Acid Composition of α_{s3}-, α_{s4}- and α_{s5}-Casein. J. Dairy Sci., 54, 1103-1110.

Hurrell, R.F., Carpenter, K.J., Sinclair, W.J., Otterburn, M.S. and Asquith, R.S. (1976). The significance of lysine-containing isopeptides and of lanthionine in heated proteins. Br. J. Nutr., 35, 383-395.

Klostermeyer, H. and Offt, S. (1976). Unpublished.

Klostermeyer, H. and Reimerdes, E.H. (1976). In preparation.

Klostermeyer, H., Rabbel, K. and Reimerdes, E.H. (1975). Hitzeinduzierte Veränderungen der proteolytischen Verdaubarkeit von Milcheiweiß. Milchwissenschaft, 30, 194-199.

Klostermeyer, H., Rabbel, K. and Reimerdes, E.H. (1976). Vorkommen der Isopeptidbindungen N^{ε}-(ß-Aspartyl-)lysin und N^{ε}-(γ-Glutamyl-)lysin in Rindercolostrum. Hoppe-Seyler's Z. Physiol. Chem., in press.

Manson, W. and Carolan, T. (1972). The alkali-induced elimination of phosphate from ß-casein. J. Dairy Res., 39, 189-194.

McKenzie, H.A.; Editor (1970/1971). "The Milk Proteins - Chemistry and Molecular Biology". 2 Vol., Academic Press, New York and London.

Mrowetz, G. and Klostermeyer, H. (1972). Polarographische Bestimmung der Disulfidgruppen in Milch. Polarographische Bestimmung der Thiolgruppen in Milch. Z. Lebensmittel-unters. Forsch., 149, 74-78, 133-139.

Mulder, H. and Walstra, P. (1974). "The milk fat globule". CAB/Pudoc, Wageningen.

Nijhuis, H., Klostermeyer, H. and Reimerdes, E.H. (1976). Separation of α_{s2}-casein and the α_s-minor caseins in form of their S-sulfonates. Milchwissenschaft, to be published.

Ralston, G.B. (1972). The decrease in stability of ß-lactoglobulin on blocking the sulphydryl group. Compt. Rend. Lab. Carlsberg, 38, 499-512.

Roels, H., Préaux, G. and Lontie, R. (1973). Polarimetric and chromatographic investigation of the anaerobic alkaline denaturation of bovine ß-lactoglobulin A and B. Evidence for thiol-disulfide exchange. Biochemie, 55, 421-430.

Sawyer, W.H. (1968). Heat Denaturation of Bovine ß-Lactoglobulins and Relevance of Disulfide Aggregation. J. Dairy Sci., 51, 323-329.

Schmitz, I., Zahn, H., Klostermeyer, H., Rabbel, K. and Watanabe, K. (1976). Zum Vorkommen von Isopeptidbindungen in erhitztem Milcheiweiß. Z. Lebensmittelunters. Forsch., 160, 377-381.

Schnack, U., Reimerdes, E.H. and Klostermeyer, H. (1976). In preparation.

Southward, C.R. and Goldman, A. (1975). Co-precipitates - a review. N.Z. J. Dairy Technol., 10, 101-112.

Thomasow, J. and Klostermeyer, H. (1976). Beeinflussung der Labgerinnung hocherhitzter Milch durch N-Äthylmaleinimid. Milchwissenschaft, in press.

Watanabe, K. and Klostermeyer, H. (1976 a). Heat induced changes in sulfhydryl and disulfide levels of ß-lactoglobulin A and the formation of polymers. J. Dairy Res., in press.

Watanabe, K. and Klostermeyer, H. (1976 b). Bildung von Dehydroalanin, Lysinoalanin und Lanthionin durch hitzeinduzierten Abbau von Cystein- und Cystinresten in ß-Lactoglobulin A. Z. Lebensmittelunters.Forsch., in preparation.

Watanabe, K. and Klostermeyer, H. (1976 c). Heat induced degradations of ß-lactoglobulin A and B. Milchwissenschaft, in press.

Webb, B.H., Johnson, A.H. and Alford, J.A.; Editors (1974). "Fundamentals of Dairy Chemistry". Second Edition. The Avi Publ. Comp., Inc., Westport.

Wheelock, J.V. and Kirk, A. (1974). The role of ß-lactoglobulin in the primary phase of rennin action on heated casein micells and heated milk. J. Dairy Res., 41, 367-372.

Wiechen, A. and Knoop, A.-M. (1974). Untersuchungen über das Verhalten von ß-Lactoglobulin und α-Lactalbumin beim Erhitzen der Milch mit Hilfe der ^{131}J-markierten Milchproteine. Milchwissenschaft, 29, 65-70.

Wilson, G.A. and Wheelock, J.V. (1972). Factors affecting the action of rennin in heated milk. J. Dairy Res., 39, 413-419.

Wilson, G.A., Wheelock, J.V. and Kirk, A. (1974). The effect of reduction and alkylation on the primary phase of rennin action on unheated and heated milk. J.Dairy Res.,41,37-44.

Zimmerman, J.K., Barlow, G.H. and Klotz, I.M. (1970). Dossication of ß-lactoglobulin near neutral pH. Arch. Biochem. Biophys., 138, 101-109.

19

THE COMPLETE ENZYMIC HYDROLYSIS OF CROSSLINKED PROTEINS

Brian Milligan and Leo A. Holt

Division of Protein Chemistry, C.S.I.R.O.,

Parkville, Vic. 3052, Australia

ABSTRACT

Procedures used for the complete enzymic hydrolysis of proteins are reviewed. The successful application of complete enzymic hydrolysis in the detection of naturally occurring isopeptide crosslinks and various other types of chemically introduced crosslinks is described. The method may fail if the level of crosslinking is too high, or if crosslinking is accompanied by racemization. Although it is usual to cleave disulphide crosslinks prior to enzymic digestion, the necessity for this in all cases is questioned.

INTRODUCTION

Although acid hydrolysis is the most useful method for quantitatively converting a protein into its constituent amino acids for determination of the amino acid composition, it has some disadvantages. One of these, the decomposition of tryptophan, has recently been overcome by replacing the traditionally used 6 M hydrochloric acid with 3 M *p*-toluenesulphonic acid (or methanesulphonic acid) containing tryptamine.[1] Another disadvantage, the conversion of asparagine and glutamine to aspartic acid and glutamic acid, will always prevent the determination of the individual contents of these four amino acids by analysis of acid hydrolysates. Any other acid-labile groups present, *e.g.* isopeptide crosslinks, will also be destroyed, and thus escape detection.

Acid hydrolysis is also unsuitable for preparing some chemically-modified proteins for analysis due to reversion of the modified amino acid residues during hydrolysis.

Some of the above problems can be overcome by using enzymic hydrolysis, although this technique is by no means without disadvantages of its own. Early attempts to hydrolyse proteins completely with enzymes were thwarted by the failure of the available enzymes to cleave peptide bonds adjacent to proline residues, and it was only after prolidase had been isolated and characterized[2] that a general method for the complete enzymic hydrolysis of proteins was devised.[3] Variants of this method were subsequently developed for hydrolysing wool,[4-7] collagen,[8,9] and other proteins.[5]

The original enzymic hydrolysis method of Hill and Schmidt[3] entails incubation of the protein with papain, and then with a mixture of prolidase and leucine aminopeptidase. Pronase[4,5,7] and pepsin[6] have been used instead of papain, and aminopeptidase-M[6,7] instead of leucine aminopeptidase, but prolidase is essential if good yields of all of the amino acids present are to be obtained. Collagen, because of its unique structure, is more difficult to hydrolyse than other proteins but over 90% of the peptide bonds can be hydrolysed by successive incubation with collagenase, papain and a mixture of peptidases (including prolidase) isolated from hog-kidney microsomes.[8,9]

The main advantage of enzymic hydrolysis is that it may release certain amino acid derivatives from proteins intact, whereas these derivatives would be destroyed under the conditions of acid hydrolysis. For example, the isolation of ε-(γ-glutamyl)-lysine from insoluble fibrin,[10-12] wool[4,6,13] and the medulla of mammalian hair[14,15] can only be achieved with the aid of enzymic hydrolysis. The isopeptide bond linking the ε-amino group of lysine to the γ-carboxyl group of glutamic acid is resistant to proteolytic enzymes[16] but not to acid hydrolysis.

A further advantage of enzymic hydrolysis over acid hydrolysis is that, in principle, it permits determination of the complete amino acid composition, including aspartic acid, asparagine, glutamic acid, glutamine and tryptophan. In practice, not all amino acids are released quantitatively. For example, digestion of proteins with Pronase, prolidase and leucine aminopeptidase consistently gives lower yields of glycine, proline, aspartic acid and asparagine than of the hydrophobic amino acids, valine, leucine, isoleucine, phenylalanine and tryptophan, which are released in near-quantitative yield.[5] Glutamine is probably released in good yield, but partly cyclizes to pyrrolid-2-one 5-carboxylic acid under the conditions of digestion.

Enzymic hydrolysis is much more complicated and time-consuming than acid hydrolysis and it is never likely to be used routinely for the amino acid analysis of proteins. Some of the enzymes used, especially prolidase, are expensive and not particularly stable. Also, the analysis of enzymic hydrolysates is more complex than that of acid hydrolysates due to the presence of asparagine and glutamine in the former. A modified buffer system (using lithium instead of sodium salts) is required to resolve these amino acids from serine and threonine during ion-exchange chromatography.[5,17]

HYDROLYSIS OF PROTEINS CONTAINING DISULPHIDE CROSSLINKS

It is generally believed that the disulphide bonds in cystine-containing proteins must be cleaved before enzymic hydrolysis if satisfactory yields of amino acids are to be obtained. Hill and Schmidt[3] obtained poor yields of cystine and the adjacent amino acids from insulin and ribonuclease A after digestion with papain, leucine aminopeptidase and prolidase. They overcame the problem by converting the cystine residues to cysteic acid residues by oxidation with performic acid. Later workers found that wool could be hydrolysed enzymically to its constituent amino acids in reasonable yield only if the disulphide crosslinks were cleaved before[4-6] or during[7] digestion.

We have found that simple cystine peptides, such as cystinylbisglycine and bisglycylcystine are hydrolysed by Pronase, and therefore it seems that the difficulties encountered in the hydrolysis of cystine-containing proteins are steric in nature. Although oxidation or reduction/alkylation of cystine residues removes the steric influence of the disulphide crosslinks, the new amino acid residues so introduced may be more difficult to release enzymically than the other "natural" residues present.

The most suitable method of disulphide cleavage probably depends upon the particular enzymes used. For example, Hill and Schmidt[3] oxidized proteins with performic acid prior to digestion with papain, prolidase and leucine aminopeptidase and obtained good yields of most amino acids. In contrast, Milligan *et al.*[4] found that the yields of amino acids released from oxidized wool were much lower than those from reduced and carboxymethylated wool when Pronase, prolidase and leucine aminopeptidase were used. Reduction/cyanoethylation has also been used to prepare wool proteins for enzymic hydrolysis.[4,6] The yields of amino acids were not quite as good as from reduced and carboxymethylated wool (using Pronase, prolidase and leucine aminopeptidase),[4] and the procedure suffers from the disadvantage that some lysine residues may be cyanoethylated during the treatment.[6]

We have recently observed that the yields of amino acids released by enzymic hydrolysis of lysozyme with Pronase, leucine aminopeptidase and prolidase were no worse than those released from S-carboxymethyl and S-sulpho lysozyme. This result suggests that it may not always be necessary to cleave disulphide crosslinks before carrying out enzymic hydrolysis. However, some amino acids were released in only 60-70% yield (from all three proteins), and the experiment needs to be repeated using more active enzyme preparations to confirm the above result.

HYDROLYSIS OF PROTEINS CONTAINING ISOPEPTIDE CROSSLINKS

The occurrence of isopeptide crosslinks in proteins has been demonstrated by the isolation of ε-(γ-glutamyl)lysine from enzymic hydrolysates of insoluble fibrin,[10-12] the medulla from various hairs and quills[14,15] and reduced/alkylated wool.[4,6,13,18] It has been claimed that ε-(β-asparty1)lysine residues are also present in native wool[18] but this crosslink has not been detected in other proteins. Milligan *et al.*,[4] and Schmitz *et al.*[7] found ε-(β-asparty1)lysine in enzyme digests of heated wool, but not in native wool. The occurrence and nutritional significance of isopeptide crosslinks in proteins has recently been reviewed by Asquith *et al.*[19]

Medullary proteins are characterized by a very high ε-(γ-glutamyl)lysine content, as high as 25-30 residues/1000 residues in medulla of rat, rabbit and guinea-pig hair.[14] Harding and Rogers[15] have proposed that these crosslinks are responsible for the extreme insolubility of medullary proteins in the usual protein solvents. Surprisingly, medullary proteins dissolve very rapidly on incubation with proteases, even with such highly specific ones as trypsin,[15] whereas a fibrous protein, *e.g.* wool, containing a similar number of disulphide crosslinks would probably be unaffected. Possibly the longer isopeptide crosslink may allow for greater chain movement than can occur with the shorter disulphide crosslink, so permitting greater access to the enzymes. The degree of crystallinity of the protein no doubt also influences the ease of digestion.

Quite large numbers of isopeptide crosslinks are introduced into wool on prolonged heating at 140°C.[20] The amounts formed at temperatures above 140°C cannot be determined by analysis of enzyme digests, due to the complete resistance of the wool (even after disulphide cleavage) to proteolysis. This resistance to hydrolysis has been attributed to the presence of isopeptide crosslinks, but in view of the behaviour of medullary proteins, it may be due to the introduction of lanthionine and lysinoalanine residues and the racemization which occurs concomitantly (see later).

HYDROLYSIS OF PROTEINS CONTAINING CHEMICALLY INTRODUCED CROSSLINKS

A wide variety of bifunctional reagents has been used to introduce crosslinks into proteins.[21,22] When alkylating agents are used, the crosslinks are usually stable to acid, and thus the sites and extent of crosslinking can be determined by isolating crosslinked pairs of amino acids from an acid hydrolysate.[23] However, if acylating agents are used the crosslinks do not survive acid hydrolysis, and amino acid analysis reveals no differences between the treated and original protein. In certain cases, *e.g.* when formaldehyde is used as the crosslinking agent, some types of crosslink may be broken during hydrolysis, and others formed.

An indirect method, in which the modified protein is treated with 1-fluoro-2,4-dinitrobenzene (FDNB) prior to hydrolysis and amino acid analysis, is sometimes used to determine the sites and extent of reaction of proteins with reagents that produce acid-labile protein derivatives.[24-27] Unfortunately this method has several disadvantages, expecially when crosslinking agents are used, because the introduction of crosslinks may affect the accessibility of functional groups in the protein to FDNB. Moreover, this method cannot distinguish between crosslinking and monofunctional reaction of the reagent with the protein.

Enzymic hydrolysis, when possible, followed by the isolation and identification of crosslinked pairs of amino acids, provides much more conclusive evidence for the location and extent of crosslinking. This method has recently been applied to wool that has been crosslinked by treatment with formaldehyde,[6,28] and with the bifunctional acylating agent, di-(*p*-nitrophenyl)sebacate.[4] It was shown that both reagents introduced crosslinks between lysine residues.[4,28]

A major problem in these studies was the difficulty in achieving complete hydrolysis, or even complete dissolution of the wool in some cases. For example, formaldehyde-treated wool could be dissolved completely only by oxidizing with performic acid and then digesting with enzymes.[28] Only part of the wool dissolved if reduction/carboxymethylation[28] or reduction/cyanoethylation[6] were used. Thus one cannot attribute the low yield of an amino acid in the hydrolysate to reaction of its side chain with the crosslinking agent. Wool that had been crosslinked by treatment with di-(*p*-nitrophenyl)sebacate dissolved readily on enzymic hydrolysis if first reduced and carboxymethylated.[4] The greater ease with which enzymes hydrolyse this treated wool may be due to the fact that the crosslinks are much longer than in formaldehyde-treated wool. This would permit much greater chain flexibility and thus facilitate enzyme/substrate complex formation.

Wool that has been treated with sodium carbonate[6] cannot be hydrolysed completely with enzymes. This effect is undoubtedly due in part to crosslinking by the lysinoalanine[29] and lanthionine[30] residues introduced by alkaline treatments. In addition to the steric restrictions imposed by crosslinking, the racemization which occurs concomitantly[31-34] no doubt also inhibits proteolysis. Some of the enzymes present in Pronase hydrolyse peptides containing hydrophobic D-amino acid residues but the rate of hydrolysis is much less than that of the corresponding L-enantiomers.[35-36] Enzymic hydrolysis of cyanide-treated wool (after reduction/carboxymethylation) also gives poor yields of most amino acids. As with alkali-treated wool, both racemization and the introduction of lanthionine crosslinks[37] are probably responsible.

Racemization and steric hindrance due to crosslinking are not the only factors that inhibit the enzymic hydrolysis of chemically-modified proteins. Proteolytic enzymes are designed by nature to hydrolyse proteins containing quite a small range of "natural" amino acid residues. It is therefore to be expected that the effectiveness of these enzymes might be impaired when they are confronted by "un-natural" amino acid residues. This may be the reason why converting the tryptophan residues in apomyoglobin to 2-(*o*-nitrophenylsulphenyl)tryptophan or kynurenine residues drastically reduces the ease with which peptide bonds involving these residues are cleaved.[38]

Despite the above limitations, enzymic hydrolysis is a very useful tool, in that it may provide the only direct evidence possible regarding the occurrence and location of crosslinks in proteins.

ACKNOWLEDGMENT

We thank Dr. C.M. Roxburgh for the amino acid analyses.

REFERENCES

1 Liu, T.S., and Chang, Y.H. (1971). *J. Biol. Chem.*, 246, 2842.
2 Davis, N.C., and Smith, E.L. (1957). *J. Biol. Chem.*, 224, 261.
3 Hill, R.L., and Schmidt, W.R. (1962). *J. Biol. Chem.*, 237, 389.
4 Milligan, B., Holt, L.A., and Caldwell, J.B. (1971). *Appl. Polym. Symp.*, No.18, 113.
5 Holt, L.A., Milligan, B., and Roxburgh, C.M. (1971). *Aust. J. Biol. Sci.*, 24, 509.

6 Cole, M., Fletcher, J.C., Gardner, K.L., and Corfield, M.C. (1971). *Appl. Polym. Symp.*, No. 18, 147.

7 Schmitz, I., Baumann, H., and Zahn, H. (1976). 'Proc. Int. Wool Text. Res. Conf., 5th, Aachen, 1975' in "Schriftenr. Dtsch. Wollforsch. Inst. Tech. Hochschule Aachen", in press.

8 Bensusan, H.B., Dixit, S.N., and McKnight, S.D. (1971). *Biochim. Biophys. Acta*, 251, 100.

9 Bensusan, H.B. (1972). *Biochim. Biophys. Acta*, 285, 447.

10 Pisano, J.J., Finlayson, J.S., and Peyton, M.P. (1968). *Science*, 160, 892.

11 Matacić, S. and Loewy, A.G. (1968). *Biochem. Biophys. Res. Commun.*, 30, 356.

12 Lorand, L., Downey, J., Gotoh, T., Jacobsen, A., and Tokura, S. (1968). *Biochem. Biophys. Res. Commun.*, 31, 222.

13 Asquith, R.S., Otterburn, M.S., Buchanan, J.H., Cole, M., Fletcher, J.C., and Gardner, K.L. (1970). *Biochim. Biophys. Acta*, 207, 342.

14 Harding, H.W.H. and Rogers, G.E. (1972). *Biochim. Biophys. Acta*, 257, 37.

15 Harding, H.W.J. and Rogers, G.E. (1971). *Biochemistry*, 10, 624.

16 Kornguth, M.L., Neidle, A., and Waelsch, H. (1963). *Biochemistry*, 2, 740.

17 Benson, J.V. Jr., Gordon, M.J., and Patterson, J.A. (1967). *Analyt. Biochem.*, 18, 228.

18 Asquith, R.S., Otterburn, M.S., and Gardner, K.L. (1971). *Experientia*, 27, 1388.

19 Asquith, R.S., Otterburn, M.S., and Sinclair, W.J. (1974). *Angew. Chem. Int. Ed. Engl.*, 13, 514.

20 Asquith, R.S. and Otterburn, M.S. (1971). *Appl. Polym. Symp.*, No. 18, 277.

21 Fasold, H., Klappenberger, J., Meyer, C., and Remold, H. (1971). *Angew. Chem. Int. Ed. Engl.*, 10, 795.

22 Wold, F. (1967). *Methods Enzymol.*, 11, 617.

23 Zahn, H. and Meienhofer, J. (1956). *Melliand Textilber.*, 37, 432.

24 Zahn, H., Rouette, H.K., and Schade, F. (1965). "Int. Wool Text. Res. Conf, 3rd, Paris, 1965", Sect. II, p. 495, Institut Textile de France, Paris.

25 Leach, S.J., Milligan, B., Inglis, A.S., Frazer, L.A., and Holt, L.A. (1968). "Symposium on Fibrous Proteins Australia 1967", W.G. Crewther, ed., Butterworths (Australia), Sydney, p. 373.

26 Hille, E. (1962). *Text.-Prax.*, 17, 171.

27 Asquith, R.S. and Chan, D.K. (1971). *J. Soc. Dyers Colour.*, 87, 181.

28 Caldwell, J.B. and Milligan, B. (1972). *Text. Res. J.*, 42, 122.

29 Bohak, Z. (1964). *J. Biol. Chem.*, 239, 2878.
30 Horn, M.J., Jones, D.B., and Ringel, S.J. (1941). *J. Biol. Chem.*, 138, 141.
31 Neuberger, A. (1948). *Adv. Protein Chem.*, 4, 297.
32 Nyilasi, J. (1957). *Acta Chim. Acad. Sci. Hung.*, 10, 353.
33 Hill, J. and Leach, S.J. (1964). *Biochemistry*, 3, 1814.
34 Provansal, M.M.P., Cuq, J.A., and Cheftel, J. (1975). *J. Agric. Food Chem.*, 23, 938.
35 Nomoto, M., Narahashi, Y., and Murakami, M. (1960). *Biochem. J. (Japan)*, 48, 906.
36 Dutta, A.S. and Giles, M.B. (1976). *J. Chem. Soc. Perkin Trans. 1*, 244.
37 Zahn, H., Kunitz, F.-W., and Hildebrand, D. (1960). *J. Text. Inst.*, 51, T740.
38 Holt, L.A. and Milligan, B. (1973). *Aust. J. Biol. Sci.*, 26, 871.

20

CROSS-LINKING OF PROTEIN BY PEROXIDASE

Mark A. Stahmann

Department of Biochemistry
University of Wisconsin-Madison
Madison, Wisconsin 53706

ABSTRACT

The reaction products of peroxidase, a hydrogen donor and hydrogen peroxide decreased the amount of lysine recovered from proteins after acid hydrolysis. Oxidation of peroxidase treated proteins with performic acid prior to hydrolysis formed α-amino adipic acid indicating that the peroxidase or the quinones formed by peroxidase had oxidatively deaminated some lysyl residues of the protein to form lysyl aldehyde. Gel filtration and polyacrylamide gel electrophoresis revealed dimers, trimers and higher protein polymers that were not detected when peroxidase was omitted. Since some of the protein polymers were not dissociated by gel electrophoresis in the presence of dodecyl sulfate, urea and mercaptoethanol, it suggests that the free radicals or quinones formed by peroxidase had interacted with or cross-linked protein molecules by the formation of covalent bonds. Oxidative enzymes like peroxidase and polyphenol oxidase may lower the nutritive value of proteins by the oxidative deamination of lysine, reaction with cysteine and methionine and by cross-linking protein molecules to reduce their susceptibility to enzymatic hydrolysis.

INTRODUCTION

Cross links between polypeptide chains of elastin, or fibrin are thought to be formed by specific enzymes such as lysyl oxidase (Siegel, Sheldon, and Martin, 1973) or the cross-linking enyzme

of fibrin (Matacic and Lowry, 1968). Free radical peroxidation from a lipid oxidizing system caused polymerization of proteins (Roubal and Tappel, 1966). The reaction of o-quinones formed by o-diphenol oxidase with proteins was studied by Pierpoint (1969) who showed that it involved thiol and ε-amino groups but protein polymers were not seen. This paper is a summary of work now in press which shows that horseradish peroxidase, hydrogen peroxide and various hydrogen donors act upon proteins to deaminate some ε-amino groups of lysyl residues of proteins (Stahmann and Spencer, 1976) and to form cross-linked protein polymers (Stahmann, Spencer and Honold, 1976).

OXIDATIVE DEAMINATION OF THE ε-GROUP OF LYSYL RESIDUES OF PROTEINS BY PEROXIDASE

Loss of Lysine by Peroxidase. Peroxidase, hydrogen peroxide and catechol formed products which reacted with casein and β-lactoglobulin to cause a substantial reduction in the lysine that could be recovered after acid hydrolysis. Fifty-two percent of the lysine was lost from a reaction mixture containing 0.03 M catechol, 0.01 M hydrogen peroxide, 0.002 mg/ml peroxidase and 5 mg/ml casein in 0.01 M phosphate buffer, pH 7.5 within 48 hr. When peroxidase was omitted the loss was only 4%. Peroxidase caused a 60% loss of lysine from β-lactoglobulin under similar conditions. Inclusion of benzene sulfinic acid, a quinone trapping agent, prevented loss of lysine.

Formation of α-Amino Adipic Acid. When an acid hydrolysate of the above reaction mixtures containing peroxidase was chromatographed on an amino acid analyzer, a small new peak was detected. When the reaction mixture was oxidized with performic acid before hydrolysis, three to five times more of the new ninhydrin positive compound was observed. This new compound was shown to be identical with authentic α-amino adipic acid by cochromatography and mass spectroscopy. Its formation is shown in Figure 1.

In a similar way, peroxidase formed α-amino adipic acid from polylysine; the amino adipic acid formed accounted to from 20% to 40% of the lysine lost. Alpha-amino adipic acid was recovered from peroxidase treated casein, cytochrome C, histone, high lysine histone and polylysine. The amount of amino acipic acid produced varied with the concentration of peroxidase, substrates and time of reaction; the difference was much less than would be expected from classical kinetics. The amount of α-amino adipic acid recovered was small; in the case of histone it was less than 5% of the lysine in the histone.

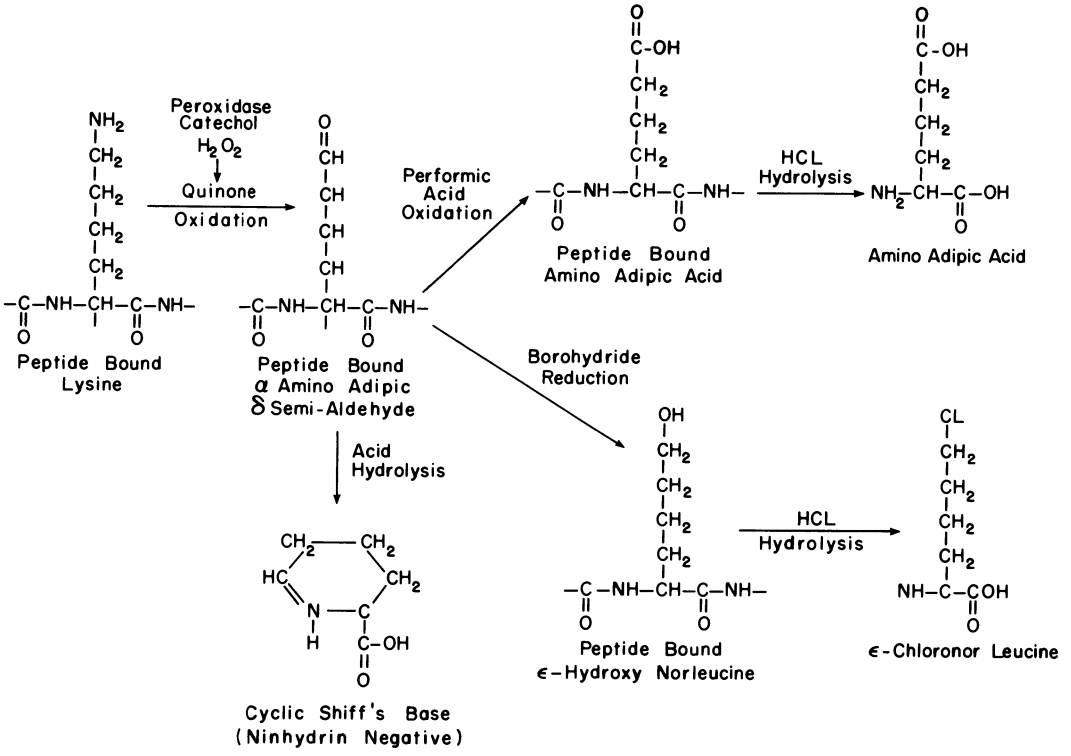


Figure 1. Reactions of peroxidase and peptide bound lysine. α-Aminoadipic acid was isolated from hydrolysates of several proteins which had been reacted with peroxidase and then oxidized with performate. Characterization was by cochromatography and mass spectroscopy. A peak that eluted in the position reported for ε-chloronorleucine was observed after borohydride reduction and hydrolysis with hydrochloric acid.

Evidence for the intermediate formation of lysyl aldehyde was obtained by reduction with sodium borohydride of peroxidase treated β-lactoglobulin followed by acid hydrolysis and ion-exchange chromatography. A new ninhydrin positive peak was seen in the position reported for ε-chloro-norleucine which is formed from ε-hydroxy-norleucine during hydrolysis with hydrochloric acid. A positive test for aliphatic aldehyde was obtained from peroxidase treated histone with N-methyl benzothiazolone. We conclude that the ε-amino group of some of the lysyl residues were oxidatively deaminated to an aldehyde by the peroxidase system to form lysyl aldehyde which could be further oxidized to α-aminoadipic acid or reduced to ε-hydroxy-norleucine.

In one experiment, α-aminoadipic acid was formed from polylysine when high molecular weight polylysine was separated from the peroxidase by a dialysis membrane. The peroxidase thus apparently oxidized catechol to o-quinone which diffused through the membrane and then oxidatively deaminated some ε-amino groups of the polylysine to an aldehyde. Such an oxidation of primary amines by 3,5-tert-butyl o-quinone has been reported (Corey and Achiwa, 1969). The fact that benzenesulfinic acid protected the lysine by trapping o-quinone is consistent with this mechanism.

Only one enzyme, lysyl oxidase, has been reported to deaminate the lysine side chains of proteins. We have shown that a peroxidase enzyme system will also oxidatively deaminate lysyl residues of proteins. The resulting aldehyde group on the surface of the protein could then react with another protein molecule to form dimmers, trimers and higher protein polymers.

CROSS-LINKING OF PROTEINS BY PEROXIDASE

Conventional Acrylamide Gel Electrophoresis. The deamination studies were done in an attempt to understand how soluble proteins could be cross-linked in vitro by peroxidase. In our earlier cross-linking experiments, the model system contained commercial peroxidase (Worthington RZ 1 or Sigma RZ 3), hydrogen peroxide, a hydrogen donor and a purified soluble protein. The hydrogen donors included benzidine, p-anisidine, o-phenylene diamine, guaiacol, catechol or pyrogallol. The proteins studied were commercial, crystalline cytochrome C, bovine serum albumin, catalase, ovalbumin, β-lactoglobulin, and pepsin. Peroxidase one other protein, one hydrogen donor and hydrogen peroxide were contained in a given reaction mixture in 0.01 M tris (hydroxymethyl) amino methane buffer pH 7.5. The changes observed in the band patterns after acrylamide gel electrophoresis is illustrated for ovalbumin in Figure 2 and summarized for five proteins in Table 1.

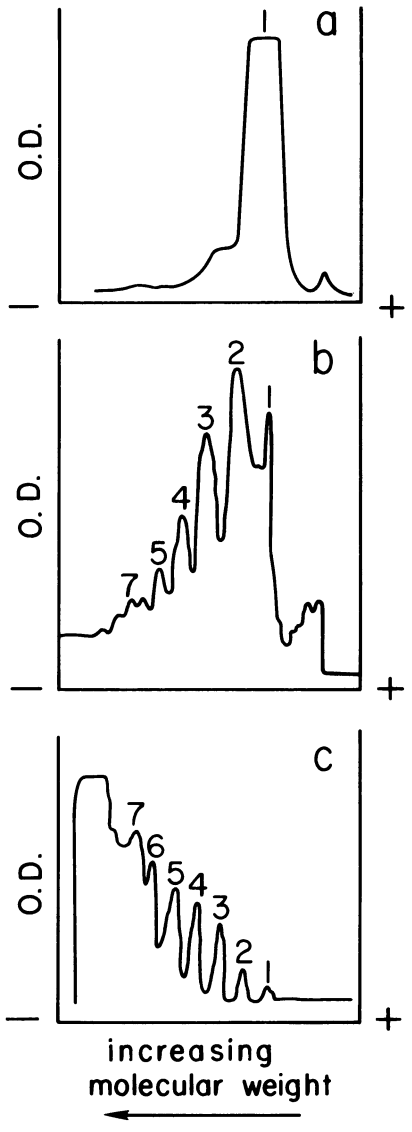


Fig. 2. Electrophoresis of polymerized ovalbumin. Optical density scans were made of gels from: (a) native ovalbumin; ovalbumin (5 mg/ml) treated with peroxidase 90.1 mg/ml) and either (b) 0.03 M p-anisidine and 0.01 M hydrogen peroxide or (c) 0.05 M p-anisidine and 0.017 M hydrogen peroxide. Sample (b) was predominantly dimer, trimer, and remaining monomer. Sample (c) was mostly very high molecular weight polymers.

TABLE 1

Changes in Gel Electrophoresis Patterns of Soluble Proteins Caused by Peroxidase and Various Hydrogen Donors[a]

Hydrogen donor[b]	Cytochrome C	BSA	Catalase	Ovalbumin	β-Lactoglobulin
Benzidine	xx	xxx	xxx	xx	xxx
p-Anisidine	xxx	xxx	0	xxx	xxx
o-Phenylenediamine	xxx	xxx	xx	xxx	xxx
Guaiacol	x	x[c]	x	x[c]	xx[c]
Catechol	xxx	xx	xx	xx	xx
Pyrogallol	xx	x	xx	x	x

[a]Changes observed in acrylamide gel electrophoresis patterns of reaction mixtures as compared with the native protein pattern; 0 = no change; x = some new material migrating more slowly than native protein; xx = much material migrating slower than native protein band, appreciably less material in native protein band; xxx = most material at tops of large pore and/or small pore gels, and complete loss of native protein band.

[b]The reaction mixtures contained hydrogen donor, hydrogen peroxide (0.01M), soluble protein (1 mg/ml), and peroxidase (0.25 mg/ml).

[c]New, discrete bands observed migrating more slowly than native protein band(s).

In many cases some new bands that migrated more slowly than the native protein (0) were seen and are indicated in Table 1 by x or xx. When most of the protein remained at the top of the gel, it is indicated as xxx. Since much of the peroxidase treated proteins moved through the gel more slowly than the native protein or remained at the top of the gel, it suggested that the proteins were cross-linked into higher aggregates or protein polymers that were larger than the native proteins. However, this experiment did not rule out the possibility that changes in the net charge might be the cause for altered mobility so additional tests were made to prove that the molecular weight had increased.

Molecular Weight Determination of Polymers of Ovalbumin Using Gels of Varying Acrylamide Concentrations. A reaction mixture containing ovalbumin, peroxidase, p-anisidine and hydrogen peroxidase was incubated at pH 7.5 for several hours to form a deep red opaque solution. Aliquots were studied by gel electrophoresis using varying concentrations of acrylamide (Hedrick and Smith, 1968). Many protein bands showed a lower mobility (R_f) relative to the dye in gels of higher acrylamide concentration due to increased frictional sieving. When the logarithms of the R_f's of the various bands were plotted against the acrylamide concentrations, a family of straight lines were obtained (Figure 3). The molecular weight of the species comprising each band is a linear function of the slope of this line (Hendrick and Smith, 1968).

Table 2 lists the observed molecular weights of polymers of ovalbumin produced by reaction of native ovalbumin with peroxidase The observed molecular weight of the first band (monomer) from the treated ovalbumin was about 10% higher than that of the native ovalbumin. This may indicate some binding of the oxidation products of anisidine to the protein. We conclude that the bands from the reaction mixture are monomer, dimer, trimer, tetramer, pentamer, hexamer and heptamer of ovalbumin. The red color of the bands suggests that the increase in molecular weight of the monomer from the reaction mixture (55,000) compared to the native protein (50,000) may be due to oxidation products binding to the ovalbumin.

Detection of Cross-Linking by Gel Filtration Chromatography. Native cytochrome C was eluted from a column of Sepharose G-100 after about three void volumes. However, after the cytochrome was treated with peroxidase, catechol and hydrogen peroxide, most of the cytochrome C was then eluted from the same column with only one void volume. Since only very large molecules (M.W. over 100,000) will be totally excluded from Sephadex G-100 and will then elute with only one void volume, it indicated that the new peak seen after cytochrome C was reacted with peroxidase contained very high molecular weight aggregates of cross-linked cytochrome.

Ovalbumin, serum albumin and β-lactoglobulin was studied by thin layer chromatography using Sephadex G-200 before and after each protein was reacted with peroxidase, p-anisidine and hydrogen peroxidase. In all cases, the peroxidase treated proteins migrated much further on the Sephadex G-200 thin layer plates than the same unreacted native protein. Since only very high molecular weight molecules (M.W. over 800,000) are totally excluded from Sephadex G-200, the material that moved farther than the unreacted native protein was highly cross-linked or aggregated protein.

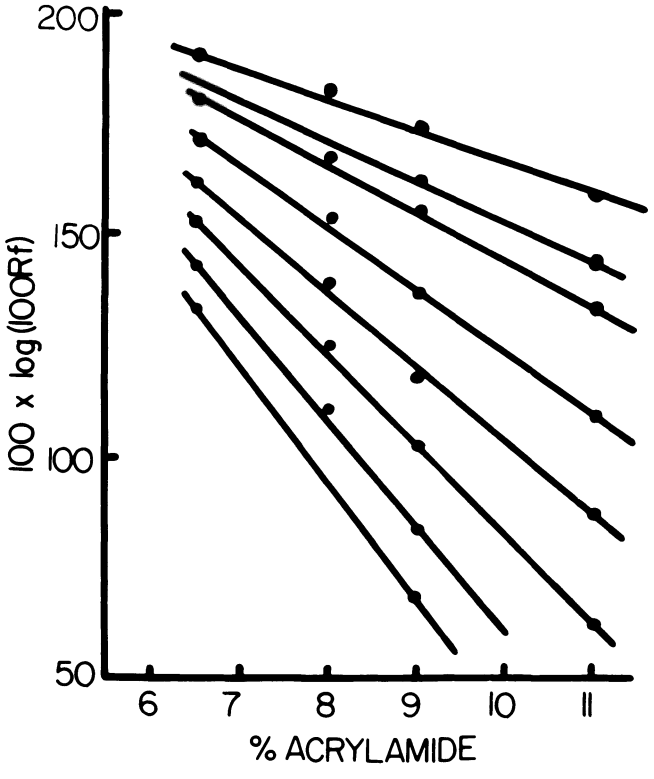


Fig. 3. Electrophoretic migration of polymers of ovalbumin produced by peroxidase in gels of different acrylamide concentrations. The protein bands exhibited a lower mobility (Rf) relative to bromphenol blue when electrophoresed in gels of higher acrylamide concentration, due to the increased frictional sieving experienced by the migrating species. When the logarithms of the Rf's of a band in gels of different acrylamide concentrations were plotted against the percent acrylamide a straight line was obtained; the molecular weight of the species comprising the band is a linear function of the slope of this line.

Demonstration of Cross-Linking by Dodecylsulfate Polyacrylamide Gel Electrophoresis. Several reaction mixtures of a soluble protein, hydrogen donor, hydrogen peroxide and peroxidase were studied by acrylamide gel electrophoresis in the presence of dodecylsulfate,urea and mercaptoethanol (Dunker and Rueckert, 1969). Dodecyl sulfate denatures, dissociates, and binds to the individual polypeptide chains of proteins so that their migration through the gel is a function of only the molecular weights of their individual polypeptide chains. Using proteins of well established molecular weights, a standard curve relating log of molecular weight and

TABLE 2

Observed Molecular Weights of Polymers of Ovalbumin Produced by Reaction of Ovalbumin with Peroxidase[a]

Species	Slope[b]	Calculated molecular weight[c]	Molecular weight/assumed polymer number[d]
Native ovalbumin[e]	6.4	50,000	50,000
Peroxidase treated ovalbumin, band 1	6.7	55,000	55,000
band 2	9.0	92,000	46,000
band 3	10.6	118,000	39,500
band 4	16.2	209,000	52,000
band 5	20.2	273,000	55,000
band 6	23.5	326,000	54,000
band 7	25.6	361,000	52,000

[a]Five mg/ml ovalbumin was treated with 0.3 mg/ml horseradish peroxidase, 0.04 M p-anisidine, and 0.04 M hydrogen peroxide in aqueous buffer, pH 7.5.

[b]Calculated from Fig. 3.

[c]Calculated from the relationship: Molecular Weight = 16,170 x slope - 53,400

[d]Assuming band 1 is monomer, band 2 is dimer, band 3 is trimer, etc.

[e]A small amount of high molecular weight material was observed in native obalbumin gels, probably corresponding to naturally occurring dimer (slope = 10.0) and tetramer (slope = 15.6). No trimer or polymers of molecular weight greater than 210,000 daltons were observed in native protein gels.

mobility relative to bromophenol blue was prepared. With this curve it was possible to estimate the molecular weights of the polypeptide chain in the bands that were seen in the gels before and after the protein was reacted with peroxidase. Treatment of the soluble proteins with peroxidase caused formation of many new bands (Table 3).

The estimated molecular weights of the monomer of β-lactoglobulin and cytochrome C from the reaction mixtures were higher than that of the native protein; these differences may be due to binding of oxidation products. The additional bands from the reaction mixture of cytochrome C and β-lactoglobulin represent molecular weights appropriate for successive additions of approximately 11,000 and 18,000 daltons, respectively. This is what we would expect if each successive band represents a polymer to which one additional protein molecule had been cross-linked.

That some covalent cross-linking was produced by peroxidase was demonstrated by the protein bands appropriate for the di, tri, and higher polymers after the proteins in reaction mixtures were denatured with dodecyl sulfate and subjected to gel electrophoresis in the presence of dodecyl sulfate, urea and mercaptoethanol. However, non-covalent cross-links such as hydrogen or hydrophobic bonds must also be involved and could account for the much higher molecular weights observed in the absence of dodecyl sulfate (Table 2) and for the total exclusion of some peroxidase treated proteins from the gels.

DISCUSSION

REDUCTION IN NUTRITIVE VALUE OF PROTEINS BY OXIDASES

The oxidative deamination of lysyl residues in proteins by peroxidase will reduce the lysine content of proteins and hence lower their nutritive value. The reactive quinones formed by peroxidase or polyphenol oxidase may also react with the sulfhydryl groups of cysteine and the thioether of methionine as well as with the ε-amino groups of lysine in proteins. These quinones are strong oxidizing agents; they may oxidize some of the methionine residues to the sulfoxide or react with the thioether to form a sulfonium compound (Vithayathil and Murthy, 1972). Since the sulfur containing amino acids and lysine are often the most limiting amino acids in plant proteins, a destruction of only a small part of these essential amino acids by the oxidase enzymes may markedly lower the nutritive value of protein in plant foods or feeds. Inasmuch as many plant tissues contain both peroxidase and polyphenol oxidase as well as phenolic compounds that may form quinones, care should be taken to reduce oxidase action in the processing of plant foods and feeds.

TABLE 3

Observed Molecular Weights of Polymers of Soluble Proteins Produced by Reaction of Native Protein with Peroxidase[a]

Band Number	Cytochrome C (catechol[b])	β-Lactoglobulin (catechol[b])	B.S.A. (p-anisidine[b])
1	20,000	21,000	66,000
2	31,000	35,400	145,000
3	42,000	54,000	210,000
4	54,000	74,000	265,000
5	60,000	91,000	300,000
6		112,000	

[a]Known molecular weight of the monomer of these well characterized native proteins are cytochrome - 12,500, β-Lactoglobin - 17,500, bovine serum albumin (B.S.A.) - 66,000. All other molecular weights were calculated from the mobilities of bands on SDS acrylamide gels.

[b]Hydrogen donor used with peroxidase and hydrogen peroxide to cause polymerization of native proteins.

Cross-linking of proteins will also reduce their susceptibility to enzymatic hydrolysis. The tanning process that converts hide tissue to leather by the formation of cross-links makes the proteins in the leather much less susceptible to enzymatic hydrolysis. We are now investigating the effect of a peroxidase system (peroxidase, hydrogen peroxide and a phenol) upon the hydrolysis of proteins by the digestive enzymes. In preliminary experiments we measured the release of free amino acids by the digestive enzymes, pepsin and pancreatin (Akeson and Stahmann, 1964; Stahmann and Woldegiorgis, 1975) from lysozyme before and after reaction with peroxidase. chlorogenic acid and hydrogen peroxide. The release of most amino acids was not reduced by treatment with peroxidase; release of lysine, tryptophan and histidine was significantly reduced. Methionine release showed the greatest reduction. Only about one fifth as much methionine was released when lysozyme was treated with hydrogen peroxide and peroxidase; addition of chlorogenic acid reduced the enzymatic release of methionine by 90%. No free cystine was released by the digestive enzymes. Horigome and Kandatsu (1968) found that caseins allowed to react with caffeic acid, isochlorogenic acid and phenolic compounds of red clover leaves in the presence of o-diphenol oxidase were inferior to control casein in biological value, digestibility and available lysine.

Although the exact mechanism by which the peroxidase system forms cross-links between protein molecules and reduces their nutritive value is not known, we believe that the oxidative deamination of lysyl residues at the surface of the protein and a binding of or reaction with the oxidation products of the hydrogen donor to the protein are both involved. The resulting aldehydes formed from lysine residues may react with a lysine residue of a second protein molecule to form a dimer. The two protein molecules would be held together by the Shiff's base that is formed. The reduction product of this Shiff's base would form N-(5-amino-5-carboxypententanyl)-lysine (lysinonorleucine) which has been reported in elastin (Franzblau, Sinex and Faris, 1965). Two lysyl aldehyde residues also could condense by aldol condensation.

It is known that the enzymatic action of peroxidase involves the intermediate formation of free radicals; such free radicals also may be involved in the polymerization of proteins by the peroxidase. We have preliminary data which indicates that polyphenol oxidase which also forms free radical (and quinones) will polymerize some soluble proteins. Thus, it appears that several cross-linking amino acids may be formed by the oxidation of some lysyl residues on the surface of a protein molecule by the peroxidase system which then reacts with amino, sulfhydryl or methionyl groups on a second protein molecule, to form new cross-links and to lower the nutritive value of the protein.

ACKNOWLEDGEMENTS

This study was made in collaboration with several former students. The late Dr. Guy Honold did the first cross-linking experiments with peroxidase and showed altered protein bands on gel electrophoresis. David Demerost chromatographically characterized α-amino adipic acid. Dr. Allen Spencer completed the characterization and measured the molecular weights of the protein polymers. Douglas Held studied the enzymatic hydrolysis of peroxidase treated lysozyme. The author is grateful for all this assistance.

This work was supported in part by grants from the Herman Frasch Foundation, a National Institutes of Health Training Grant No. GM 00236 BCH from the National Institute of General Medical Sciences and a grant from the National Science Foundation.

REFERENCES

Akeson, W. R. and Stahmann, M. A. (1964). A pepsin pancreatin digest index of protein quality evaluation. J. Nutrition, 83, 257-261.

Corey, E. J. and Achiwa, K. (1969). A new method for the oxidation of primary amines to ketones. J. Am. Chem. Soc., 91, 1429-1432.

Dunker, A. K. and Rueckert, R. R. (1969). Observations on molecular weight determinations on polyacrylamide gel. J. Biol. Chem., 244, 5074-5080.

Franzblau, C., Sinex, F. M. and Faris, B. (1965). Identification of a new cross-linking amino acid in elastin. Biochem. Biophys. Res. Commun., 21, 575-581.

Hendrick, J. L. and Smith, A. J. (1968). Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. Arch. Biochem. Biophys., 126, 155-164.

Horigome, T. and Kandatsu, M. (1968). Biological value of proteins allowed to react with phenolic compounds in the presence of o-diphenol oxidase. Agr. Biol. Chem., 32, 1093-1102.

Matacic, A. and Loewy, A. G. (1968). The identification of isopeptide cross-links in insoluble fibrin. Biochem. Biophys. Res. Commun., 30, 356-367.

Pierpoint, W. S. (1970). Formation and behavior of o-quinones in some processes of agricultural importance. Rothamsted Expt. Stn. Rep., part 2, 199-218.

Roubal, W. T. and Tappel, A. L. (1966). Damage to proteins, enzymes and amino acids by peroxidizing lipids. Arch. Biochem. Biophys. 133, 150-155.

Siegel, R. C., Sheldon, R. P. and Martin, G. P. (1970). Cross-linking of collagen and elastin. Properties of lysyl oxidase. Biochemistry 9, 4486-4492.

Stahmann, M. A. and Spencer, A. K. (1976). Deamination of protein lysyl ε-amino groups by peroxidase in vitro. Biopolymers, (in press).

Stahmann, M. A., Spencer, A. K. and Honold, G. R. (1976). Cross-linking of proteins in vitro by peroxidase. Biopolymers, (in press).

Stahmann, M. A. and Woldegiorgis, G. (1975). Enzymatic methods for protein quality determination in "Protein Nutritional Quality of Foods and Feeds," Friedman M., editor, Marcel Dekker Inc., New York, 211-234.

Vitnayathil, P. J. and Murthy, G. S. (1972). New Reaction of o-benzoquinone at the thioether group of methionine. Nature New Biol., 236, 101-103.

21

A MATHEMATICAL ANALYSIS OF KINETICS OF CONSECUTIVE, COMPETITIVE REACTIONS OF PROTEIN AMINO GROUPS

Mendel Friedman and L. David Williams*

Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California 94710

ABSTRACT

A general mathematical analysis of consecutive, competitive reactions of amino groups in amino acids, peptides, and proteins was developed. The analysis is of more than theoretical interest, and should prove useful for interpretation of the reactivity and utility of reagents that modify lysine in proteins.

INTRODUCTION

Amino acids, peptides, proteins and related natural products frequently contain two or more functional groups which may react concurrently with vinyl derivatives such as acrylonitrile, methyl acrylate, ethyl vinyl sulfone, and dehehydroalanine (1-12, 14). The relative rates of reaction of these groups determine the pathways of reaction and the nature of the products. The kinetic behaviour of the ε-NH_2 group in proteins may influence the design of optimum conditions to be used for modifying protein NH_2 groups with mono- and bi-functional reagents. We therefore, offer a general mathematical analysis of the kinetics of consecutive, competitive reactions of an amino group with a vinyl compound (e.g. of an ε-NH_2 amino group with ethyl vinyl sulfone; dehydroalanine, etc.). The described kinetic analysis is applicable not only to such Michael reactions but is also valid for the alkylation of amino or other protein functional groups with other reagents (e.g. fluorodinitrobenzene, methyl iodide, divinyl sulfone), which may give mono- and bis-adducts.

MONOSUBSTITUTION

Rates of cyanoethylation of amino acids as a function of pH in the range 8.4-11.6 were studied by Friedman et al. (1-3, 9-11). Rates increased rapidly as the pH approached the pKa of the amino group and approached an asymptotic value with further increase in pH. The dependence of rates on pH was ascribed to the effect of pH on the concentrations of the various ionized forms of the amino acids and their relative reactivities. The changes in reaction rate with pH may be expressed in terms of the ionization constants of the amino acids. Generally the rate of reaction in aqueous solution increases in proportion to the concentration of unprotonated amino groups, both free and protein-bound. However, nonaqueous solvents and the electronic and steric microenvironments near protein amino groups profoundly alter their reactivities (3-5, 8, 12). For an amino acid in solution,

$$H_2A^+ \rightleftharpoons (H^+) + (HA^{\pm}); \; K_1 = (H^+)(HA^{\pm})/(H_2A^+) \qquad (1)$$

$$HA \rightleftharpoons HA^{\pm}; \; K_D = (HA^{\pm})/(HA) \qquad (2)$$

$$HA^{\pm} \rightleftharpoons (H^+) + (A^-); \; K_2 = (H^+)(A^-)/(HA^{\pm}) \qquad (3)$$

where H_2A^+ is the amino acid cation; HA, the neutral form; $HA^{\pm}$, the zwitterion; and A^-, the anion.

The following terms are defined:

V_1 = velocity due to the reaction of acrylonitrile with HA

V_2 = velocity due to the reaction of acrylonitrile with A^-

k_2 = observed overall second order rate constant

k_{HA} = second-order rate constant associated with species HA

k_{A^-} = second-order anion rate constant associated with species A^-

(B) = concentration of acrylonitrile

Since $H_2A^+ = (H^+)^2(A^-)/K_1K_2$, $HA = (H^+)(A^-)/K_2K_D$, and $HA^{\pm} = (H^-)(A^-)/K_2$, the concentration of all unreacted amino acid

species $(AA)_T$ equals

$$(AA)_T = (H_2A^+) + (HA) + (HA^{\pm}) + (A^-)$$

$$= \left[(H^+)^2/K_1K_2 + (H^+)/K_2K_D + (H^+)/K_2 + 1\right](A^-)$$

$$= \frac{\left[(H^+)^2K_D + K_1(1 + K_D)(H^+) + K_1K_2K_D\right]}{K_1K_2K_D}(A^-)$$

Solving for (A^-)

$$(A^-) = \frac{K_1K_2K_D(AA)_T}{\left[K_1K_2K_D + K_1(1 + K_D)(H^+) + K_D(H^+)^2\right]}$$

$$V_1 = k_{HA}(HA)(B);\ V_2 = \left(k_{A^-}\right)(A^-)(B)$$

$$V_T = V_1 + V_2 = \left[k_{HA}(H^+) + \left(k_{A^-}\right)K_2K_D\right](A^-)(B)/K_2K_D$$

By substituting for (A^-) and equating theoretical and experimental velocities, we developed equation 4, which relates the observed second order rate constant to the second order rate constants associated with the nucleophiles in solution, the neutral amino acid HA and the amino anion A^-, the hydrogen ion concentration, and the three equilibrium constants of the amino acid.

We can simplify equation 4 by making the following approximations: since $K_D = 10^5$, $1 + K_D \approx K_D$; and since H^+ is small relative to both K_1 and K_2K_D at pH's above neutrality, $(H^+)^2$ may be neglected; $(H^+)^1/K_D \approx 0$, and the first term drops out so that we have equation 5.

$$k_2 = \frac{k_{HA}(H^+)K_1}{\left[K_1K_2K_D + (H^+)K_1(1 + K_D) + K_D(H^+)^2\right]} + \frac{k_{A^-}K_1K_2K_D}{\left[K_1K_2K_D + (H^+)K_1(1 + K_D) + K_D(H^+)^2\right]} \quad (4)$$

$$k_{A^-} = k_2\left[1 + \frac{(H^+)}{K_2}\right] \qquad (5)$$

Our results (10, 11) showed that the second order anion (inherent) rate constants (k_{A^-}) were essentially invariant over the pH range studied, and demonstrated that equation 5 agreed with experimental observations.

To calculate predicted rate ratios of two functional groups as a function of hydrogen ion concentration, as we did for the rate ratio for reaction of the SH group in cysteine and the ε-NH_2 group of α-N-acetyl-L-lysine with dehydroalanine methyl ester (Finley et al., this volume), the following version of eq. 5 may be used. In this, k_2, k_{A^-}, and K are associated with one functional group (e.g. SH), and k_2', k_{A^-}', and K' with the second (e.g. NH_2):

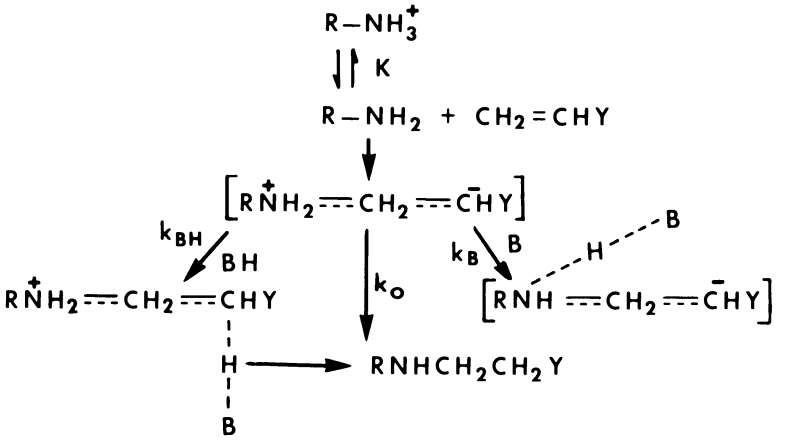
$$\frac{k_2}{k_2'} = \left(\frac{k_{A^-}}{k_{A^-}'}\right)\left(\frac{K}{K'}\right)\left[\frac{K' + (H^+)}{K + (H^+)}\right] \qquad (6)$$

Thus, all that is necessary to calculate the ratio of second-order rate constants at any hydrogen-ion concentration is to determine the k_2 values for the two functional groups at one hydrogen-ion concentration, calculate the k_{A^-} values by means of eq. 5, and then use eq. 6 to calculate the rate ratio at any other hydrogen-ion concentration.

CONSECUTIVE COMPETITIVE DISUBSTITUTIONS

Since amino groups have two hydrogen atoms that may be substituted both simultaneously and consecutively, we derived the following more general mathematical analysis to describe the kinetics of consecutive, competitive reactions of amino groups in amino acids and proteins. The analysis should also be applicable to bifunctional reagents whose two reactive sites react at different rates.

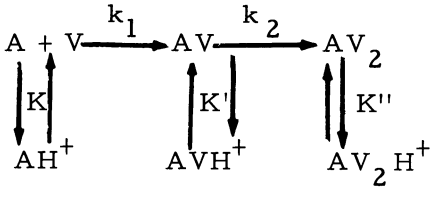
A mechanism for a single substitution reaction of an amino group with a vinyl (or any other) group is shown below:



Since proton transfers are vital to the reaction, general acid or general base catalysis is possible. The rate expression would then be of the form:

$$\frac{d\left[RNH_2 + RNH_3^+\right]}{dt} = -\left\{k_o + k_B(B) + k_{BH}(BH)\right\}(RNH_2)(CH_2 = CHY),$$

The following expressions were derived with the assumptions that the reaction is first order in A and, overall second order, and that the back reaction is negligible:



Specifically, at constant pH

$$A_T = A + AH^+ = A\left(1 + \frac{(H^+)}{K}\right)$$

$$\frac{dA_T}{dt} = -k_1(A)(V)$$

$$AV_T = AV + AVH^+ = AV\left(1 + \frac{(H^+)}{K'}\right)$$

$$\frac{dAV_T}{dt} = k_1(A)(V) - k_2(AV)(V)$$

$$AV_{2T} = AV_2 + AV_2H^+ = AV_2\left(1 + \frac{(H^+)}{K''}\right)$$

$$V = V_o - AV_T - 2AV_{2T}$$

$$\alpha = k_2''/k_1'' \; ; \; k_2'' = \frac{k_2}{1 + \frac{(H^+)}{K'}} \; ; \; k_1'' = \frac{k_1}{1 + \frac{(H^+)}{K}}$$

and for starting conditions A_{To}, V_o:

$$AV_T = \left(\frac{A_{To}}{(1-\alpha)}\right)\left\{\left(\frac{A_T}{A_{To}}\right)^{\alpha} - \left(\frac{A_T}{A_{To}}\right)\right\}, \text{ if } \alpha \neq 1$$

$$= A_T \ln\left(\frac{A_{To}}{A_T}\right); \text{ if } \alpha = 1$$

$$AV_{2T} = A_{To} - AV_T - A_T$$

$$V = V_o - 2A_{To} + \frac{(1-2\alpha)}{(1-\alpha)} A_T + \frac{A_T^{\alpha}}{(1-\alpha) A_{To}^{\alpha-1}} \; ; \text{ if } \alpha \neq 1$$

$$= V_o - 2A_{To} + 2A_T + A_T \ln \frac{A_{To}}{A_T} \; ; \text{ if } \alpha = 1$$

The only restriction on A_T, other than $A_{To} \geq A_T \geq O$, is $V \geq O$.

The time course of the reaction (i.e. A_T and AV_T as functions of time rather than as functions of A_T) has not been derived for the general case, but under pseudo first order conditions ($V_o >> A_{To}$),

$$k_1' = \frac{k_1(V_o)}{1 + \frac{(H^+)}{K}} \; ; \; k_2' = \frac{k_2(V_o)}{1 + \frac{(H^+)}{K'}} \; ; \; \alpha = \frac{k_2'}{k_1'} = \frac{k_2''}{k_1''} \; ; \; t = \text{time}$$

$$A_T = A_{To} e^{-k_1' t}$$

$$AV_T = \frac{A_{To}}{1-\alpha}\left\{e^{-k_2' t} - e^{-k_1' t}\right\}; \text{if } \alpha \neq 1 \quad \text{for } V_o >> A_{To} \text{ (Pseudo-1st order)}$$

$$= A_{To} \; k_1' t \; e^{-k_1' t} \; ; \text{ if } \alpha = 1$$

The maximum attainable concentration of AV_T (assuming V_o is large enough to convert most of A to AV) is expressed as:

$$(AV_T)_{max} = (A_{To}) \left(\frac{\alpha}{\alpha}\right)^{\frac{\alpha}{1-\alpha}} ; \text{ if } \alpha \neq 1$$

$$= (A_{To})\, e^{-1} ; \text{ if } \alpha = 1$$

as illustrated in Table 1. The maximum possible concentration of AV_T is dependent only on A_{To} and on α, i. e. on the relative rate constants for the reaction and on the pH and pK's.

$$\alpha = \frac{k''_2}{k''_1} = \frac{k_2}{k_1} \times \frac{1 + \frac{(H^+)}{K}}{1 + \frac{(H^+)}{K'}}$$

The time required for AV_T to become maximal, however, depends on the magnitude of the rate constants and on the relative concentration of V_o to A_{To}. For first order conditions,

$$V_o \gg A_{To} \quad ; \quad t_{\text{for } (AV_T)_{max}} = \frac{\ln k'_1 - \ln k'_2}{k'_1 - k'_2} \quad ; \quad \text{if } \alpha \neq 1$$

$$= \frac{1}{k'_1} \quad ; \quad \text{if } \alpha = 1$$

For smaller V_o, t for $(AV_T)_{max}$ becomes longer than predicted by this equation.

Application of theoretical predictions to experimental data from protein modifications, e. g. with ethyl vinyl sulfone (Tables 2, 3 and Figures 1, 2) is approximate at best, because the protein lysines have a range of reactivities. The data for bovine serum albumin (BSA) plus ethyl vinyl sulfone show that the lysine reactivities range widely in water, but are more closely bunched, although not identical, in 50% DMSO. The 50% DMSO probably denatures the BSA and makes the lysines comparably accessible; if so, other protein denaturants should have a similar effect.

Table 1

α	$(AV_T)_{max}/(A_{To})$
10^{-4}	0.999
10^{-3}	0.993
10^{-2}	0.955
3×10^{-2}	0.897
10^{-1}	0.774
3×10^{-1}	0.597
5×10^{-1}	0.500
1	0.368
2	0.250
3	0.192
10_2	0.0774
10^3	0.00955
	0.000993

Table 2

Amino acid composition (ratios to alanine) of BSA hydrolyzates. BSA was treated with ethyl vinyl sulfone at pH 9.5

Amino acid	Reaction time (hours)					
	0	0.5	1.5	2.5	21	26
LYS (A)	1.28	0.80	0.51	0.32	0.10	0.06
ε , ε - N,N-bis (ethylsulfonylethyl) lysine (AV_2)		0.14	0.32	0.65	0.97	1.07
ε - N(ethylsulfoneylethyl) lysine (AV)		0.40	0.36	0.24	0.05	0.02

Ref. 6, 7.

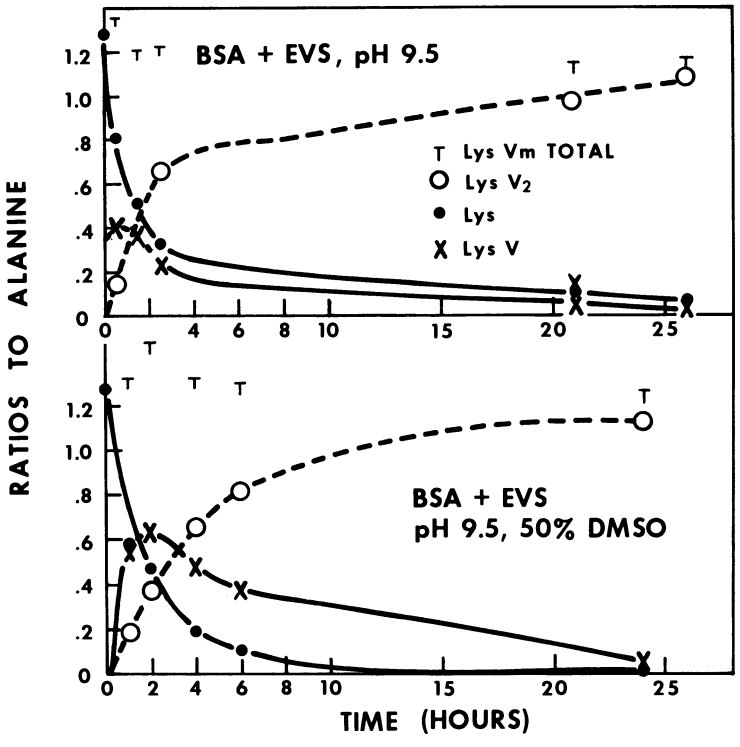


Fig. 1. Reactions of bovine serum albumin with ethyl vinyl sulfone. Data from Tables 2 and 3. Application of the kinetic analysis for consecutive first order reactions is not completely justified since the data describe <u>average</u> reaction of a great many different lysines. Treating all the lysines as equally reactive, however,

for $\frac{AV_{max}}{A_o} = \frac{0.42}{1.2} = 0.35$; $\alpha = \frac{k''_2}{k'_1} \approx 1.1$ in pH 9.5 buffer (upper plot)

for $\frac{AV_{max}}{A_o} = \frac{0.63}{1.3} = 0.48$; $\alpha = \frac{k''_2}{k''_1} \approx 0.6$ in 50% pH 9.5-50% DMSO (lower plot)

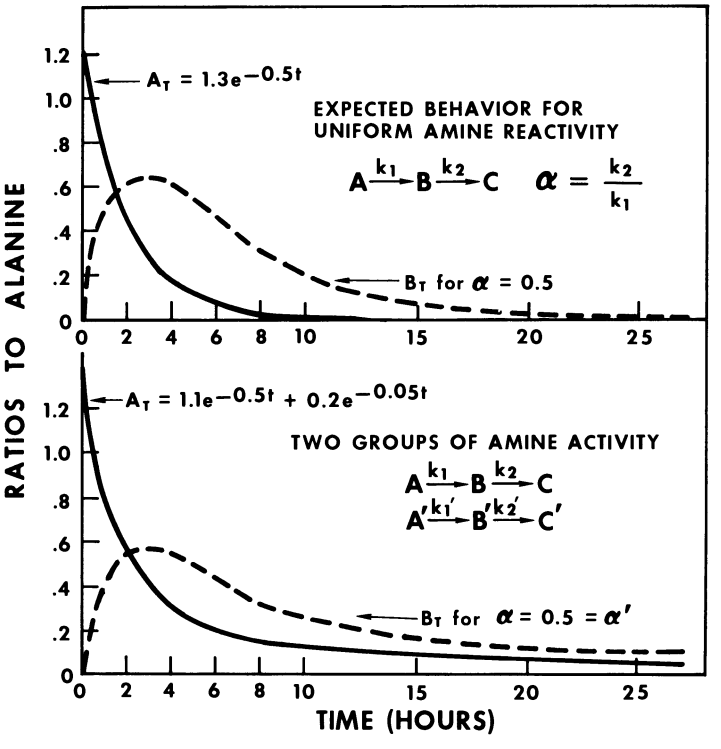


Fig. 2. Schematic representation of the expected behavior for the reaction of a protein amino group with a vinyl compound (e. g., ethyl vinyl sulfone).

If there is no interaction between the reactive lysines; reactivities for all the lysines can be totaled:

$$A = \sum_{\text{Lysines}} A_{io} \, e^{-k_i'' t}$$

$$V + (\text{Lys})_i \xrightarrow{k_i''} (\text{VLys})_i \xrightarrow{\alpha_i k_i''} (V_2 \text{ Lys})_i$$

$$AV = \sum_{\text{Lysines}} \left\{ \frac{A_{io}}{1 - \alpha_i} \left(e^{-\alpha_i k_i'' t} - e^{-k_i'' t} \right) \right\}$$

Table 3

Lysine and its derivatives hydrolyzed from BSA that had been treated with ethyl vinyl sulfone in a 50% pH 9.5 borate buffer-50% DMSO medium

Amino Acid	Reaction time (hours) 0	1	2	4	6	24
LYS (A)	1.28	0.58	0.47	0.19	0.10	0.00
ε, ε -N,N-bis (ethylsuflonylethy) lysine (AV_2)		0.18	0.37	0.65	0.81	1.12
ε,N-(ethylsulfonylethyl) lysine (AV)		0.55	0.63	0.47	0.37	0.04

Ref. 6, 7.

In practice, curve fitting by computer is probably the only reasonable use of this form.

MATHEMATICAL DERIVATIONS

Proof that reaction is only first order in amine is necessary, therefore, before the following derivation can be applied in any particular case. (This is indeed the case (Friedman and Wall, 1964)).

I. Assuming that general acid or base catalysis is negligible and that reaction is first order in amine (A) and first order in vinyl compound (V) and assuming that back reaction is negligible,

as before:

$$A_T = A + AH^+ = (1 + \frac{(H^+)}{K})$$

$$AV_T = AV + AVH^+ = AV(1 + \frac{(H^+)}{K'})$$

$$AV_{2T} = AV_2 + AV_2H^+ = AV_2(1 + \frac{(H^+)}{K''})$$

$$\frac{dA_T}{dt} = -k_1(A)(V) = -\frac{k_1(A_T)(V)}{1 + \frac{(H^+)}{K}}$$

$$\frac{d(AV_T)}{dt} = k_1(A)(V)\ k_2(AV)(V) = \frac{k_1(A_T)(V)}{1 + \frac{(H^+)}{K}} - \frac{k_2(AV_T)(V)}{1 + \frac{(H^+)}{K'}}$$

(1) Pseudo first order, constant pH·

i.e., $V \gg (A_T)_o$; $V \simeq$ constant ; $H^+ \simeq$ constant

define $k'_1 = \frac{k_1(V)}{1 + \frac{(H^+)}{K}}$; $k'_2 = \frac{k_2(V)}{1 + \frac{(H^+)}{K'}}$

then,

(a) $\frac{dA_T}{dt} = -k'_1 A_T$ which has the solution $A_T = (A_T)_o e^{-k'_1 t}$

$(A_T)_o$ = value of A_T at time zero

and $\frac{dAV_T}{dt} = k'_1(A_T) - k'_2(AV_T) = k'_1(A_T)_o\ e^{-k'_1 t} - k'_2(AV_T)$

(b) which has the solutions

$$(AV_T) = \frac{(A_T)_o}{1 - k'_2/k'_1}\left\{e^{-k'_2 t} - e^{-k'_1 t}\right\} \text{ ; if } k'_1 \neq k'_2$$

(c) $(AV_T) = k'_1 (A_T)_o \, t \, e^{-k'_1 t}$; if $k'_1 = k'_2$

(AV_T) reaches its maximum value at $\frac{d(AV_T)}{dt} = O$ which occurs at

(d) $t = \frac{\ln k'_1 - \ln k'_2}{k'_1 - k'_2}$; $(AV_T)_{max} = (A_T)_o \alpha^{\frac{\alpha}{1-\alpha}}$; $\alpha = \frac{k'_2}{k'_1}$

(e) or $t = \frac{1}{k'_1}$; $(AV_T)_{max} = (A_T)_o e^{-1}$; $\alpha = 1$

(a) The solution to $\frac{dA_T}{dt} = - k'_1 A_T$, which is of the usual first order rate form, is a simple exponential $A_T = (A_T)_o e^{-k'_1 t}$ as can be proven by differentiation.

(b) (c). The solutions for AV_T can likewise be proven by differentiation. They are most easily derived by setting $AV_T = ye^{-k'_1 t}$, y some function of t,

then $\frac{dy}{dt} e^{-k'_1 t} - k'_1 y e^{-k'_1 t} = \frac{d(AV_T)}{dt} = k'_1 (A_T)_o e^{-k'_1 t} - k'_1 y e^{-k'_1 t}$

dividing by $e^{-k'_1 t}$; $\frac{dy}{dt} = k'_1 y - k'_2 y + k'_1 (A_T)_o$

which can be rearranged and integrated to give

$$\frac{1}{k'_1 - k'_2} \ln(k'_1 y - k'_2 y + k'_1 (A_T)_o \Big|_o^t = t \; ; \text{ if } k'_1 \neq k'_2$$

$$y \Big|_o^t = k'_1 (A_T)_o t \; ; \text{ if } k'_1 = k'_2$$

On further rearrangement to solve for y, and substitution into $AV_T = ye^{-k'_1 t}$ we get $(AV_T) = \left\{ \frac{(A_T)_o}{1 - k'_2/k'_1} \; e^{-k'_2 t} - e^{-k'_1 t} \right\}$; if $k'_1 \neq k'_2$

$$\text{or } (AV_T) = k'_1 (A_T)_o t e^{-k'_1 t}; \text{ if } k'_1 = k'_2$$

There is no physical discontinuity at $k'_1 = k'_2$, and no real mathematical one since

$$\lim_{k'_2 \to k'_1} (AV_T) = \lim_{k'_2 \to k'_1} \frac{(A_T)_o \, k_1}{k'_1 - k'_2} \left\{ e^{-k'_2 t} - e^{-k'_1 t} \right\} =$$

$$(A_T)_o k'_1 t \lim_{k'_2 \to k'_2} \left\{ \frac{e^{-k'_2 t} - e^{-k'_1 t}}{-k'_2 t + k'_1 t} \right\}$$

$$= (A_T)_o k'_1 t \left[\frac{d}{d(-k'_1 t)} \left(e^{-k' t} \right) \right]_{k' = k'_1} = (A_T)_o k'_1 t e^{-k'_1 t}$$

(d)

$$(AV)_T = \text{maximum value at } \frac{d(AV)_T}{dt} = O$$

$$O = \frac{d}{dt} \left[\left\{ \frac{(A_T)_o}{1 - k'_1/k'_1} \right\} \left\{ e^{-k'_2 t} - e^{-k'_1 t} \right\} \right]$$

$$O = k'_2 \, e^{-k'_2 t} + k'_1 \, e^{-k'_1 t}$$

$$1 = \frac{k'_1}{k'_2} \, e^{(k'_2 - k'_1)t}$$

$$O = \ln k'_1 - \ln k'_2 + (k'_2 - k'_1)t$$

$$t = \frac{\ln k'_1 - \ln k'_2}{k' - k'}$$

and substituting for t

$$(AV_T)_{max} = \frac{(A_{To})}{1 - k'_2/k'_1} \left\{ e^{-k'_2 \frac{\ln k'_1 - \ln k'_2}{k'_1 - k'_2}} - e^{-k'_1 \left[\frac{\ln k'_1 - \ln k'_2}{k'_1 - k'_2} \right]} \right\}$$

let $\alpha = \frac{k'_2}{k'_1}$

$$(AV_T)_{max} = \frac{(A_T)_o}{1 - \alpha} \left\{ e^{\frac{\ln k'_1}{1 - 1/\alpha}} e^{\frac{\ln k'_2}{1/\alpha - 1}} - e^{\frac{-\ln k'_1}{1 - \alpha}} e^{\frac{\ln k'_2}{1-\alpha}} \right\}$$

$$= \frac{(A_T)_o}{1 - \alpha} \left\{ \left(\frac{k'_2}{k'_1} \right)^{\frac{1}{1/\alpha - 1}} - \left(\frac{k'_2}{k'_1} \right)^{\frac{1}{1 - \alpha}} \right\}$$

$$= \frac{(A_T)_o}{1 - \alpha} \left\{ \alpha^{\frac{\alpha}{1 - \alpha}} \right\} \left\{ 1 - \alpha^{\frac{1 - \alpha}{1 - \alpha}} \right\} = (A_T)_o \alpha^{\frac{\alpha}{1 - \alpha}}$$

(e) if $k'_1 = k'_2$, $\alpha = 1$

$$O = \frac{d(AV_T)}{dt} = k'_1 (A_T)_o e^{-k'_1 t} - k'_1 (A_T)_o t\, k'_1 e^{-k'_1 t} \quad ; \quad t = \frac{1}{k'_1}$$

$$(AV_T)_{max} = k'_1 (A_T)_o \frac{1}{k_j} e^{-k'_1 \frac{1}{k'_1}} = (A_T)_o e^{-1}$$

(2) 2nd order, constant pH:

as before:

$$A_T = A + AH^+ = A(1 + \frac{(H^+)}{K})$$

$$AV_T = AV + AVH^+ = AV(1 + \frac{(H^+)}{K'})$$

$$AV_{2T} = AV_2 + AV_2H^+ = AV_2(1 + \frac{(H^+)}{K''})$$

$$\frac{dA_T}{dt} = -k_1(A)(V) = -\frac{k_1}{1 + \frac{(H^+)}{K}}(A_T)(V)$$

$$\frac{d(AV_T)}{dt} = k_1(A)(V) - k_2(AV)(V) = \frac{k_1}{1 + \frac{(H^+)}{K}}(A_T)(V)$$

$$- \frac{k_2}{1 + \frac{(H^+)}{K'}}(AV_T)(V)$$

define $k_1'' = \frac{k_1}{1 + \frac{(H^+)}{K}}$; $k_2'' = \frac{k_2}{1 + \frac{(H^+)}{K'}}$

then $$\frac{dA_T}{dt} = -k_1''(A_T)(V)$$

$$\frac{d(AV)_T}{dt} = k_1''(A_T)(V) - k_2''(AV_T)(V)$$

$$\frac{d(AV_2)_T}{dt} = k_2''(AV_T)(V)$$

These equations cannot be readily solved to give A_T, AV_T, and AV_{2T} as functions of time when V is also changing. However, we already have the solution when $V \gg A_{To}$ (Pseudo first order), and this gives us a certain amount of information about the general solution. Specifically, since all the reactions involved are the same order in V (first order), the relative changes in A_T, AV_T, and AV_{2T} are independent of the concentration of V (so long as it is non-zero).

$$\frac{dAV_T}{dA_T} = \frac{k_1''(A_T)(V) - k_2''(AV_T)(V)}{-k_1''(A_T)(V)} = \frac{k_2''(AV_T)}{k_1''(A_T)} - 1$$

$$\frac{dAV_{2T}}{dA_T} = \frac{k_2''(AV_T)(V)}{-k_1''(AT)(V)} = -\frac{k_2''(AV_T)}{k_1''(A_T)}$$

Thus, as the initial concentration of V is changed, the relative concentrations of A_T, AV_T, and AV_{2T} at any % reaction remain the same. Only the time needed to reach that % reaction changes.

For any $(V_o) < 2(A_T)_o$ there is insufficient V to convert all A to AV_2, so a certain range of conversions will be unattainable no matter how long the reaction runs, but all lesser conversions have their pseudo first order counterparts.

Using the results from the pseudo first order derivation, we can calculate the general second order solutions in terms of A_T by eliminating t via $t = \dfrac{\ln \dfrac{A_{To}}{A_T}}{k_1'}$

(a) $$AV_T = \frac{(A_{To})}{(1-\alpha)}\left\{\left(\frac{A_T}{A_{To}}\right)^{\alpha} - \left(\frac{A_T}{A_{To}}\right)\right\}, \quad \alpha \neq 1; \quad = A_T \ln \frac{A_{To}}{A_T}, \quad \alpha = 1$$

$$AV_{2T} = A_{To} - A_T - AV_T$$

$$(b)\quad V = V_o - AV_T - 2AV_{2T} = V_o - 2A_{To} + \frac{A_T^{\alpha}}{(1-\alpha)A_{To}^{\alpha-1}} + \frac{A_T}{(1-\alpha)}(1-2\alpha),\ \alpha \neq 1$$

$$= V_o - 2A_{To} + 2A_T + A_T \ln \frac{A_{To}}{A_T},\ \alpha = 1$$

These expressions are, in fact solutions to the differential equations for $\frac{dAV_T}{dA_T}$ or $\frac{dAV_{2T}}{dA_T}$, as can be proven by substitution as well.

The first order solutions are $A_T = A_{To}\, e^{-k'_1 t}$,

$$AV_T = \frac{A_{To}}{1-\alpha}\left\{e^{-k'_2 t} - e^{-k'_1 t}\right\}$$

$\alpha = k'_2/k'_1 = k''_2/k''_1$ or $AV_T = k'_1 A_{To} t e^{-k'_1 t}$; if $\alpha = 1$

Eliminating t,

$$(a)\quad \ln\left(\frac{A_T}{A_{To}}\right) = k'_1 t,\quad t = \frac{\ln(A_{To}/A_T)}{k'_1}$$

$$AV_T = \frac{A_{To}}{1-\alpha}\left\{e^{\frac{-k'_2}{k'_1}\ln\frac{A_{To}}{A_T}} - e^{\frac{-k'_1}{k'_1}\ln\left(\frac{A_{To}}{A_T}\right)}\right\} =$$

$$\frac{A_{To}}{1-\alpha}\left\{e^{\ln\left(\frac{A_T}{A_{To}}\right)^{\alpha}} - e^{\ln\left(\frac{A_T}{A_{To}}\right)}\right\}$$

$$= \frac{A_{To}}{(1-\alpha)} \left\{ \left(\frac{A_T}{A_{To}}\right)^{\alpha} - \left(\frac{A_T}{A_{To}}\right) \right\} \quad \text{if } \alpha \neq 1$$

or

$$AV_T = k_1' A_{To} \frac{\ln(A_{To}/A_T)}{k_1'} e^{\frac{-k_1'}{k_1} \ln \frac{A_{To}}{A_T}} = A_{To} \left\{ \ln\left(\frac{A_{To}}{A_T}\right) \right\} \left(\frac{A_T}{A_{To}}\right)$$

$$= A_T \ln\left(\frac{A_{To}}{A_T}\right) \; ; \text{ if } \alpha = 1$$

(b) $V = V_o A V_T - 2AV_{2T}$

$$= V_o - AV_T - 2\left\{A_{To} - A_T - AV_T\right\} = V_o - 2A_{To} + 2A_T + AV_T$$

$$= V_o - 2A_{To} + 2A_T + \frac{A_{To}}{(1-\alpha)} \left\{ \left(\frac{A_T}{A_{To}}\right)^{\alpha} - \left(\frac{A_T}{A_{To}}\right) \right\}$$

$$= V_o - 2A_{To} + \frac{A_T^{\alpha}}{(1-\alpha) A_{To}^{\alpha-1}} + \frac{A_T}{(1-\alpha)} (1-2\alpha) \; ; \text{ if } \alpha \neq 1$$

$$V = V_o - 2A_{To} + 2A_T + A_T \ln\left(\frac{A_{To}}{A_T}\right) \; ; \text{ if } \alpha = 1$$

In order for the solution to be physically meaningful, we must have V, A_T, AV_T, etc., $\geq O$. If $V_o \geq 2A_{To}$, then inspection of the above equations shows that $A_T = O$ is possible (and in fact is the value at infinite time) since it keeps $V \geq O$. If $V_o < 2A_{To}$, however, we must have $A_T > O$, even at infinite time, in order to keep $V \geq O$. The minimum (infinite time) value of A_T can be determined by solving

$$V = O = V_o - 2A_{To} + 2A_{T_{min}} + \frac{A_{To}}{(1-\alpha)} \left\{ \left(\frac{A_{T_{min}}}{A_{To}}\right)^{\alpha} - \left(\frac{A_{T_{min}}}{A_{To}}\right) \right\}$$

for A_T min.

Unfortunately, the general solution for this equation (which has the form $A + Bx + Cx^{\alpha} = O$) is unavailable.

Similarly, although substitution of the values of AV_T and AV_{2T} and V (in terms of A_T) into the rate equations gives a differential equation with two separable variables (A_T and t)

$$\frac{dA_T}{dt} = -k_1''(A_T)(V)$$

$$= -k_1''(A_T)\left\{V_o - 2A_{To} + 2A_T + A_T \ln\left(\frac{A_{To}}{A_T}\right)\right\}, \quad \text{if } \alpha = 1$$

$$= -k_1''(A_T)\quad V_o - 2A_{To} + \frac{A_T^{\alpha}}{(1-\alpha)A_{To}^{\alpha-1}} + \frac{A_T}{(1-\alpha)}(1-2\alpha)\ ; \quad \text{if } \alpha \neq 1$$

thus,

$$-k_1''t = \int_o^t \frac{dA_T}{V_o\ 2A_{To} + A_T^{\alpha}\Big/(1-\alpha)A_{To}^{\alpha-1} + A_T(1-2\alpha)/(1-\alpha)\quad A_T}\ ; \quad \alpha = 1$$

the resulting integral $\int \frac{dx}{A + Be^{x} + Ce^{dx}}$ equivalent to $x = \ln A_T$

is at best difficult to evaluate in general form.

Essentially, the same problem has been treated by Szabo (13) and by Riggs (14), but without taking account of the effects of protonation.

REFERENCES

*Present address: Diagnostic Data, Mountain View, CA.

1. Cavins, J. F., and Friedman, M. (1967). New amino acids derived from reactions of ε-amino groups in proteins with α,β-unsaturated compounds. Biochemistry, 6, 3766-3770.
2. Finley, J. W., and Friedman, M. (1973). Chemical methods for available lysine. Cereal Chem., 50, 101-105.
3. Friedman, M. (1967). Solvent effects in reactions of amino groups in amino acids, peptides, and proteins with α,β-unsaturated compounds. J. Am. Chem. Soc., 89, 4709-4713.

4. Friedman, M. (1968). Solvent effects in reactions of protein functional groups. Quarterly Reports on Sulfur Chemistry, 3, 125-144.
5. Friedman, M. (1977). Effects of lysine modification on chemical, physical, nutritive, and functional properties of proteins. In "Fundamental Aspects of Protein Chemistry Basic to Foods," edited by J. R. Whitaker and S. R. Tannenbaum. Avi, Westport, Connecticut.
6. Friedman, M., and Finley, J. W. (1975a). Reactions of proteins with ethyl vinyl sulfone. Int. J. Peptide Protein Res., 7, 481-486.
7. Friedman M., and Finley, J. W. (1975b). Vinyl compounds as reagents for available lysine. In "Protein Nutritional Quality of Foods and Feeds," Part 1, M. Friedman (Editor), Dekker, New York, pp. 503-520.
8. Friedman, M., and Koenig, N. H. (1971). Effect of dimethyl sulfoxide on chemical and physical properties of wool. Textile Res. J., 41, 605-609.
9. Friedman, M., and Romersberger, J. S. (1968). Relative influences of electron-withdrawing functional groups on basicities of amino acid derivatives. J. Org. Chem., 33, 154-157.
10. Friedman, M., and Wall, J. W. (1964). Application of a Hammett-Taft relation to kinetics of alkylation of amino acid and peptide model compounds with acrylonitrile. J. Am. Chem. Soc., 86, 3735-3741.
11. Friedman, M., Cavins, J. F., and Wall, J. S. (1965). Relative nucleophilic reactivities of amino groups and mercaptide ions in addition reactions with α,β-unsaturated compounds. J. Am. Chem. Soc., 87, 3572-3682.
12. Koenig, N. H., Muir, M. W., and Friedman, M. (1973). Properties of wool modified with vinyl compounds. Textile Res. J., 43, 682-688.
13. Riggs, N. V. (1958). Kinetic treatment of two-stage second-order consecutive reactions with a common factor. Aust. J. Chem., 11, 86-92.
14. Snow, J. T., Finley, J. W., and Friedman, M. (1975). Relative reactivities of sulfhydryl groups with N-acetyldehydroalanine and N-acetyldehydroalanine methyl ester. Int. J. Peptide Protein Res., 7, 461-466.
15. Szabo, Z. G. (1969). Kinetic characterization of complex reaction systems. In "Chemical Kinetics," Chapter 1, C. H. Bamford and C. F. H. Tipper (Editors), Elsevier, Amsterdam, Holland.

22

EFFECT OF MAILLARD BROWNING REACTION ON NUTRITIONAL QUALITY OF PROTEIN

M. Tanaka, M. Kimiagar, Tung-Ching Lee,
and C. O. Chichester

Department of Food Science and Technology
University of Rhode Island
Kingston, Rhode Island 02881

The Maillard reaction, or non-enzymatic browning reaction, between reducing sugars and proteins (amino acids), is known to cause serious deterioration of food quality during processing and storage. Increasing evidence shows that these compounds formed under mild conditions substantially reduce the availability of amino acids and proteins. Data obtained by ourselves and others has shown there is a significant decrease in the nutritional value of foods which undergo the Maillard reaction beyond that accounted for in the loss of biologically available lysine. In the present investigation, a mixture of egg albumin and glucose was used as a model system. The nutritional quality of egg albumin as a function of the extent of Maillard browning with periods of less than 10 days of storage was evaluated by *in vivo* and *in vitro* methods. A substantial decrease in nutritional quality of protein was observed even at the initial period of storage (less than three days) and most available *in vitro* methods could not reveal this change. The result of a three-month rat feeding experiment indicated that there were physiological and biochemical changes in rats fed with browned protein diet. It is extremely inportant at the present time to have information on the nutritional value and aspects of food safety of browned food products with respect to nutritional labelling policy. Therefore, in addition to the development of new food products with high protein quality, the practical applications of this study are also discussed.

INTRODUCTION

The loss of nutritional value in foods due to the non-enzymatic browning reaction during processing and storage is a serious problem in food industries (Clinger *et al.*, 1951; Henry *et al.*, 1948;

Mauron et al., 1955; Rand, 1960; Neishiem and Carpenter, 1967; Sgarbieri, 1971; Carpenter and Booth, 1973; Lee, 1974; Tanaka, 1975; Lee et al., 1976; Amaya et al., 1976). No work has been done on the real cuases responsible for the decrease in nutritional value of foods containing sugars and nitrogenous substances beyond that accounted for by the loss of biologically available lysine. A review of the literature on nutritional studies concerned with the browning reactions seems to point out that the severe loss of nutritional value is attributed to some or all of the following factors:

(a) a decrease in availability of some amino acids when heated or stored in the presence of reducing sugars;
(b) a decrease in digestibility of proteins heated in the presence of carbohydrates;
(c) the possible production of toxic substances or metabolic inhibitors during the reaction.

These effects, both nutritional and toxicological, are not completely explainable on the basis of the data which has been available thus far (Tanaka, 1974). By using a model system with egg albumin and glucose, it has been demonstrated that the biological value of egg albumin decreased 50% within 10 days of storage at 37°C and 68% relative humidity (Tanaka et al., 1976). Furthermore, some correlations between the loss of nutritional value and the changes in chemical properties of egg albumin have been found (Tanaka et al., 1975). Considering this fact, it seemed interesting and important to investigate these changes with periods of less than 10 days. There is no literature available on the effects of browned proteins for less than 10 days at temperatures below 40°C. Most of the literature deals with browning of proteins by heat (Bjarnason and Carpenter, 1969; Bjarnason and Carpenter, 1970).

From a nutritional point of view, it has always been assumed that thermally processed foods are equivalent nutritionally to their components. In many foods this is indeed the case; in others, however, where there are both free amino acids and carbohydrates coexisting, there is a substantial reduction in protein quality due to the non-enzymatic browning reaction during thermal processing and storage (Nesheim and Carpenter, 1967; Boctor and Harper, 1968; Erbersdobler, 1969; Valle-Riestra and Barnes, 1970; Ford and Shorrock, 1971; Sgarbieri, 1971; Carpenter and Booth, 1973; Adrian and Flangne, 1973; Sgarbieri et al., 1973; Chichester, 1973; Lee, 1974; Tanaka et al., 1976; Lee et al., 1976; Amaya et al., 1976). Additionally, we and others have shown that in order to get a true evaluation of protein quality in foods which have undergone heat treatment, biological testing is the only reliable methodology available at this time (Tanaka, 1974; Boctor and Harper, 1968).

There are only scattered reports concerning possible adverse effects of feeding browned food to experimental animals (Fink et al., 1958; Adrian, 1974; Lee, 1974). Therefore, it seemed important to

carry out a systematic long-term feeding experiment on rats to further investigate the cumulative effects of feeding browned food products.

MATERIALS AND METHODS

A) Preparation of browned egg albumin: A mixture of egg albumin (3 parts) (Nutritional Biochemical Company, Cleveland, Ohio) and glucose powder (d+ dextrose anhydrous) (2 parts) (NBCo.) was prepared with a moisture content of 15% and stored at 37°C in a sealed glass chamber for 1/2(1/2D), 1(1D), 2(2D), 3(3D), 6(6D) and 10(10D) days. Relative humidity inside the chamber was kept constant at 68% using 40% sulfuric acid solution. The browned mixture was stored in the freezer (-20°C) until used.

B) Determination of the amount of glucose bound to egg albumin: The browned mixture was dissolved in distilled water and filtered through Whatman No. 1 filter paper. Free glucose in the filtrate was determined by the Glucostat reagent, according to the directions of the manufacturer (Worthington Biochemical Corp., Freehold, New Jersey). The glucose bound to protein was calculated by the difference in free glucose at the beginning and after different periods of storage at 37°C and 68% relative humidity.

C) Determination of flourescence development: Flourescence of the filtrate prepared for the glucose determination was measured with an AMINCO fluorocolorimeter (American Instrument Co. Inc., Silver Springs, Maryland), calibrated with quinine sulfate after a 100-fold dilution.

D) Amino acid composition of egg albumin before and after storage: Egg albumin was hydroylzed at 110°C for 20 hours in 6N Hydrochloric acid. The total amount of each amino acid was determined by ion-exchange chromatography (Technicon Autoanalyzer).

The method of Kakade and Liener (1969) was used to determine the available lysine in egg albumin before and after its storage with glucose.

E) Electrophoresis analyses: Electrophoresis analysis of browned egg albumin was conducted with 7% Polyacrylamide gels at pH 8.9. The solutions for the determination of free glucose were used for the analysis. Forty µl of each sample, having an equal amount of nitrogen, was applied on the top of the gels. Electrophoresis was performed for about two hours at 150V. At the completion of the run, the gels were removed from the cells and the protein bands were located by staining with Amido black. Gel solution and electrode buffer were prepared by the method described by Coduri and Rand (1972). Electrophoresis apparatus used in this study was

purchased from Buchler Instruments (Fort Lee, New Jersey).

F) Determination of the Protein Efficiency Ratio (PER): The change in protein quality of egg albumin during storage with glucose was measured by the PER method (AOAC, 1970). Egg albumin stored for 0, 1/2, 1, 2, 3, 6 and 10 days was used as the source of protein in the diets. The protein content of the diets was 10%.

Male weanling Sprague-Dawley rats (Charles River Breeding Lab., Wilmington, Massachusetts), with initial body weights ranging from 50 to 60 g were employed for the PER determination. They were fed ad libitum from a powder feeder which had a stainless steel slip as a lid to minimize spilling of the diet. Food intake was recorded although water intake was unrestricted. Body weights were measured once a week. Recovery experimentation was further conducted at the end of the PER determination period. Four rats from each group were kept on Purina Rat Chow diet for five additional weeks.

G) Three-month feeding experiment: In order to investigate the effects of long-term feeding of brown protein, a one-year feeding experiment was designed with one experimental group and three control groups. Rats were scheduled to be sacrificed at the end of three months, six months and one year feeding periods respectively. Weight gain, relative organ weight, blood chemistry and enzyme activity of liver and small intestinal homogenates were determined. The results of the three-month feeding study are presented in this paper.

Male and female Sprague-Dawley rats (Charles River Breeding Labs.) were used for the three-month feeding experiment. Initial body weights were 50 to 60 g. The animals were put on Rat Chow (Purina Laboratory Chow, Ralston Purina Co.) for 48 hours to adjust to the animal room conditions. They were then caged individually and fed for three months. Except for group I, which was fed ad libitum, groups II, III and IV were pair-fed. Groups III and IV were fed the average amount of food consumed by rats in group II. The room was air-conditioned and equipped with automatic light and temperature control systems. The animals were weighed at weekly intervals to monitor their growth. At the end of three months, 25 animals (12 males, 13 females) were sacrificed by decapitation. Blood was collected in heparinized capillaries and test tubes. Hematocrit (Packed Cell Volume) was determined on the spot and blood was immediately centrifuged in an automatic refrigerated centrifuge (Sorvall Superspeed RC2-B, Sorvall Inc., Norwalk, Connecticut) at 5000 rpm for 15 minutes. Serum was collected and frozen at -20°C until used. Liver, heart, kidney, spleen and testes were kept in 0.9% saline for approximately one hour before being weighed. The organs were blotted on paper towels and trimmed of excess fat; the kidneys were peeled before weighing.

H) Preparation of the diet: Four different diets were prepared as follows:

1) a control diet containing 10% untreated egg albumin;
2) a brown diet containing 10% egg albumin which was browned with glucose for 10 days as described previously;
3) a diet containing 5% untreated egg albumin, plus 5% non-essential amino acids in a proportion recommended by Sauberlich (1961) as shown here:

Composition of non-essential amino acids added to diet #3

Alanine	54 g
Asparagine	54 g
Aspartic acid	54 g
Glutamic acid	355 g
Glycine	36 g
Proline	45 g
Serine	45 g

This diet was used to provide an iso-nitrogenous, iso-caloric control diet for diet #2.

4) a diet containing 4.5% untreated egg albumin, in which the difference of protein content was made up by the addition of dextrine.

This diet was included as an additional control diet. The reason for including it was to provide a control for diet #3. That is, to observe possible effects of supplemented non-essential amino acids in diet #3. Diet #4 also served as a control for diet #2, since it was observed in the preliminary study that diets #2 and #4 yielded similar initial weight gain; they were iso-caloric but not iso-nitrogenous. Even though diet #4 is not iso-nitrogenous with diet #2 (brown diet), it has the advantage of its protein balance not being altered with the addition of non-essential amino acids.

The other components of all the diets were as follows:

Dextrine	54%
Corn oil	8%
Salt mixture(1)	5%
Vitamin mixture(2)	1%
Sucrose	16%
Dextrose	6%

(1) Jones Foster Salt Mixture, ICN Pharmaceutical Inc. Cleveland, Ohio.
(2) Vitamin Fortification Mixture, Nutritional Biochemical Company, Cleveland, Ohio.

I) Preparation of tissue homogenates: Liver and whole small intestine were cut into small pieces and mixed with approximately two parts ice-cold saline and homogenized by hand in a glass tissue homogenizer with teflon pestle (Arthur H. Thomas Co., Philadelphia, Pennsylvania). The homogenate was then centrifuged at 12,000 rpm for 20 minutes in an automatic refrigerated centrifuge (Sorval Inc.). The supernatant was collected and kept frozen at -20°C for future analysis.

J) Blood chemistry: Serum analysis was done within two weeks after sample collection according to the following procedures.

Blood urea nitrogen (BUN) was determined according to the Sigma Chemical Company Technical Bulletin No. 535, St. Louis, Missouri (1975). Five ml of BUN reagent was added to 20 µl serum and the tubes were put into boiling water for 10 minutes. After cooling, the tubes were read for color in a Bausch & Lomb Spectronic 20 Spectraphotometer (Bausch & Lomb Company, Rochester, New York) at 520 nm. The results were calculated by establishing a standard curve, using different concentrations of a standard serum (Sigma Chemical Company).

Serum glucose was assayed according to Richterich (1969) by incubating 20 µl of serum with 1 ml mixture of Tris buffer and glucose reagent (Worthington Biochemical Company) for 30 minutes at room temperature. One ml of sulfuric acid was then added and absorbance was read against water at 546 nm on Beckman Spectrophotometer Type DBG (Beckman Instruments, Fullerton, California).

Serum and small intestinal alkaline phosphotase assays were conducted according to the method of Newman and Vreedendaal (1967). A mixture of 0.5 ml buffer (pH 10) and 0.5 ml para-nitrophenyl phosphate (1 mg/ml, Nutritional Biochemical Company) was tempered for 5 minutes at 37°C followed by the addition of 0.5 ml diluted homogenate. After exactly 30 minutes the reaction was stopped by adding 0.5 ml of 1N NaOH. Each mixture was brought to a volume of 10 ml with water and color was read at 400 nm in a Beckman Spectrophotometer Type DBG. Protein in the enzyme solution was measured by the Lowry method (1951).

Serum glutamate oxalate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) were determined by following the procedure outlined by Richterich (1969). 0.1 ml substrate solution (L-aspartate and α-ketoglutarate) was added to 20 µl serum and incubated at 37°C for 60 minutes in a water bath. 0.1 ml phenylhydrazine solution was then added to each sample. After standing at room temperature for 10 minutes, 1 ml sodium hydroxide (0.4 N) was added and color intensity was measured in the Beckman Spectrophotometer Type DBG at 546 nm. Serum GPT was assayed by the same method as SGOT using alanine instead of aspartic acid in substrate solution.

Dissacharidase activities in small intestinal homogenate were determined by the method of Dahlquist (1964) using maltose as substrate in a sodium maleate buffer at pH 6.2. 0.1 ml of an appropriate dilution of small intestine was mixed with 0.1 ml substrate. The mixture was incubated at 37°C for 60 minutes. The amount of glucose released was measured using glucostat reagent (Worthington Biochemical Corp.).

Serum total protein was determined by the Biuret method as described by Richterich (1969).

Dipeptidase activity of intestinal mucosa was determined using the method of Josefsson and Lindberg (1965). A mixture of dipeptide solution and borate buffer (pH 7.9) was placed in a water bath at 40°C and equilibrated. At zero time, 20 µl of intestinal homogenate was added; after 30 minutes, the reaction was stopped by precipitating the protein with ethanol. The solution was clarified by centrifugation and the absorbancy of the unhydrolyzed dipeptide bond was read at 220 nm in the Beckman DBG Spectrophotometer.

RESULTS AND DISCUSSION

Effect of Short-Term (under 10 days) Browning Reaction on Protein Quality

After 10 days of storage there was no significant development of color, however, fluorescence developed with an induction period during the first three days as shown in Figure 1. The development of fluorescence was almost linear with the storage time after 3 days. Figure 2 illustrates the amount of glucose bound to egg albumin during storage. The binding of glucose to protein took place after one day of storage. It seems that fluorescence develops only after a significant amount of glucose is bound to protein.

Table 1 compares the amino acid composition of the control and stored egg albumins. Lysine had the largest loss among them. This is probably due to ε-amino group in the molecule which is very reactive to glucose. The loss of arginine, serine and threonine was next. This loss is not surprising since they have their own reactive side groups toward the reducing sugars. Isoleucine and leucine also showed significant losses during storage although they are known to be nonreactive in the browning reaction.

Figure 3 presents the electrophoretic pattern of browned egg albumin with changes in available lysine during storage. The sample was applied at the minus end of the gel and the control egg albumin had six main protein bands (bands A to F). Bands A and C disappeared after 1 or 2 days of storage while new bands started forming after

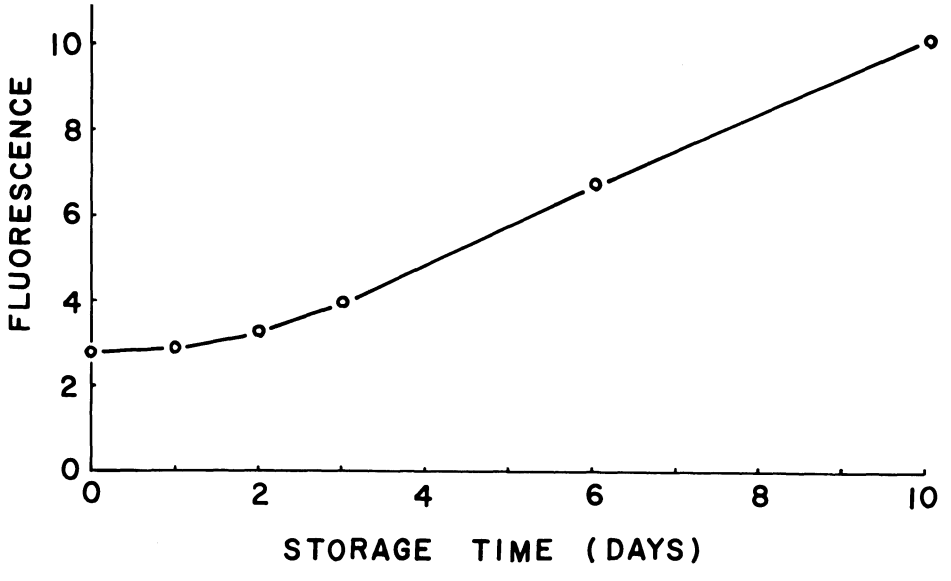


Figure 1. Fluorescence development in a mixture of egg albumin (3 parts) and glucose (2 parts) with 15% moisture stored at 37°C and 68% relative humidity.

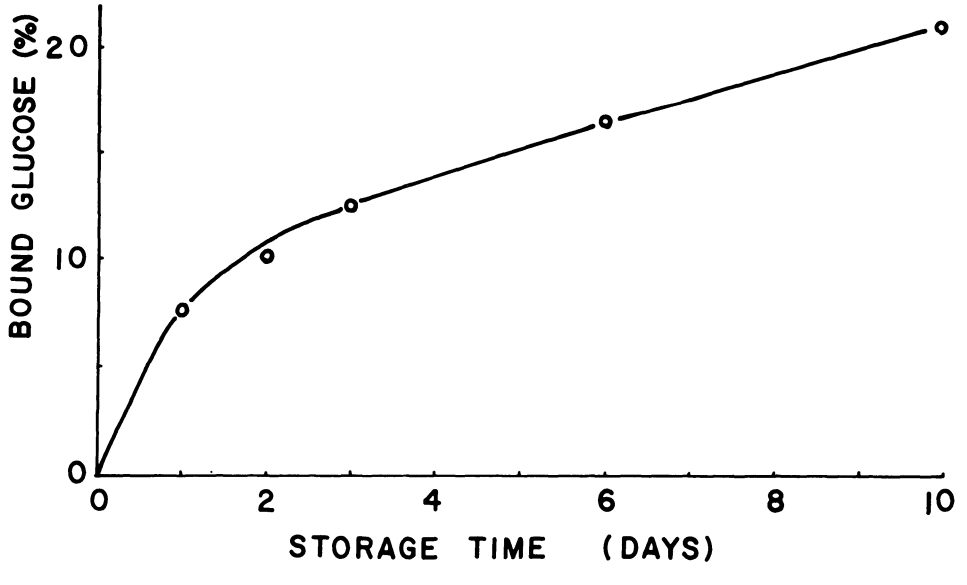


Figure 2. Binding of glucose to protein in a mixture of egg albumin (3 parts) and glucose (2 parts) with 15% moisture stroed at 37°C and 68% relative humidity.

2 or 3 days of storage at the minus side of the gel. The relative mobilities of bands D and E did not change in spite of the decreasing concentrations during the storage. The most interesting observation was made for band B which moved further toward the positive end of the gel with increasing storage time, suggesting that band B tends to have more negative charge on the molecule with longer periods of storage. Band B protein may have a higher content of lysine compared to the rest of the proteins, so that the attaching of glucose to the lysine ε-amino group of band B protein would make the total net charge of the molecule more negative. Figure 4 shows that there is a linear correlation between the relative mobility of band B and the amount of available lysine of browned egg albumin.

Table 1. Results of amino acid analysis of egg albumin browned with glucose for different periods of time.

Days of browning / Amino acid	0 D	3 D	6 D	10 D
	mg amino acid / 16 gm nitrogen			
1. Aspartic acid	11.98	10.49	9.58	9.18
2. Threonine	5.37	4.54	4.15	4.00
3. Serine	7.72	7.05	6.28	5.93
4. Glutamic acid	11.26	11.20	10.26	10.47
5. Proline	2.68	3.06	2.87	2.87
6. Glycine	2.65	2.82	2.69	2.56
7. Alanine	7.06	6.02	5.70	5.11
8. Valine	6.47	6.15	6.10	5.65
9. ½ Cystine	2.66	2.47	2.38	2.08
10. Methionine	3.82	4.10	3.58	2.97
11. Isoleucine	5.31	4.40	4.21	3.13
12. Leucine	8.23	5.48	4.84	5.06
13. Norleucine	-	-	-	-
14. Tyrosine	3.22	3.19	2.84	3.07
15. Pheylalanine	5.08	5.48	4.84	5.06
16. Ammonia	-	-	-	-
17. Lysine	7.38	5.31	4.47	3.57
18. Histidine	1.86	1.91	1.81	1.75
19. Arginine	5.12	4.91	4.71	3.78

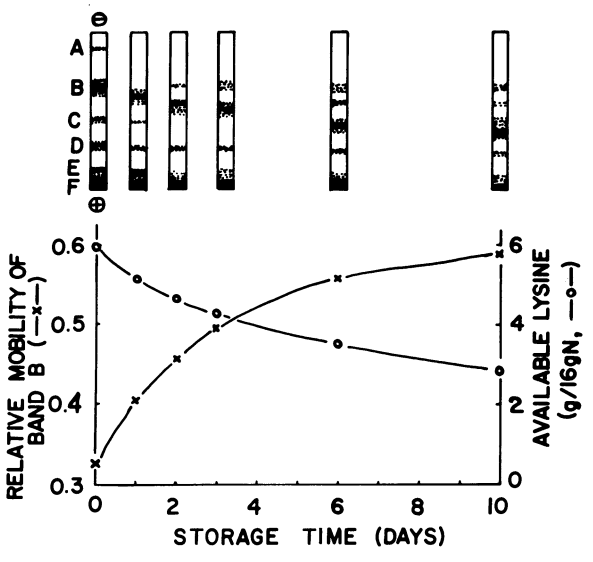


Figure 3. The relationship of electrophoretic pattern of browned egg albumin with available lysine.

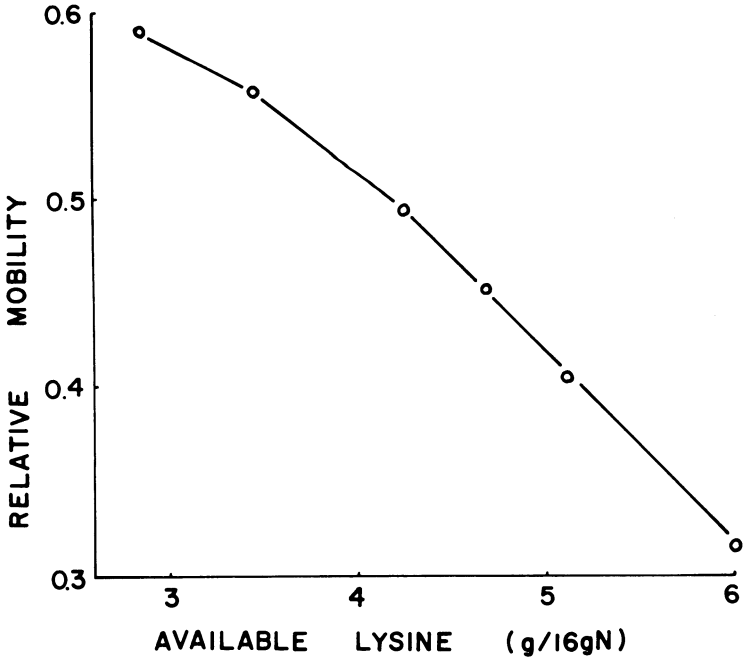


Figure 4. Linear correlation between the relative mobility of band B (Fig.3) and amount of available lysine of browned egg albumin.

If band B is more susceptible to non-enzymatic browning, it may explain our previous finding (Amaya et al., 1974) that some oligopeptides are not digestible in rats. Based on the preliminary data available here, it is thus possible to develop an electrophoretic method to estimate the extent of the browning reaction in food products. Such a method could be used as an alternative to the cumbersome biological assays. It may also provide a better estimation than measuring the available lysine as an index of protein quality in browned food. It has been shown by Bocter and Harper (1968) that some lysine measured as available by FDNB method (Carpenter, 1960) may be excreted as part of the undigestible residue in feces.

The correlation between the PER value of browned egg albumin and the storage time is illustrated in Figure 5. It indicates that the nutritional value of stored egg albumin decreased proportionally with the time of storage. It also clearly demonstrates that the determinations of fluorescence development, the amount of glucose bound and the available lysine were not accurate enough to assess the change in the nutritional value of protein during the initial period of storage.

As can be observed, there is a substantial loss of nutritional quality of protein during storage of egg-albumin with glucose for periods of less than 10 days. There is more than 33 percent loss

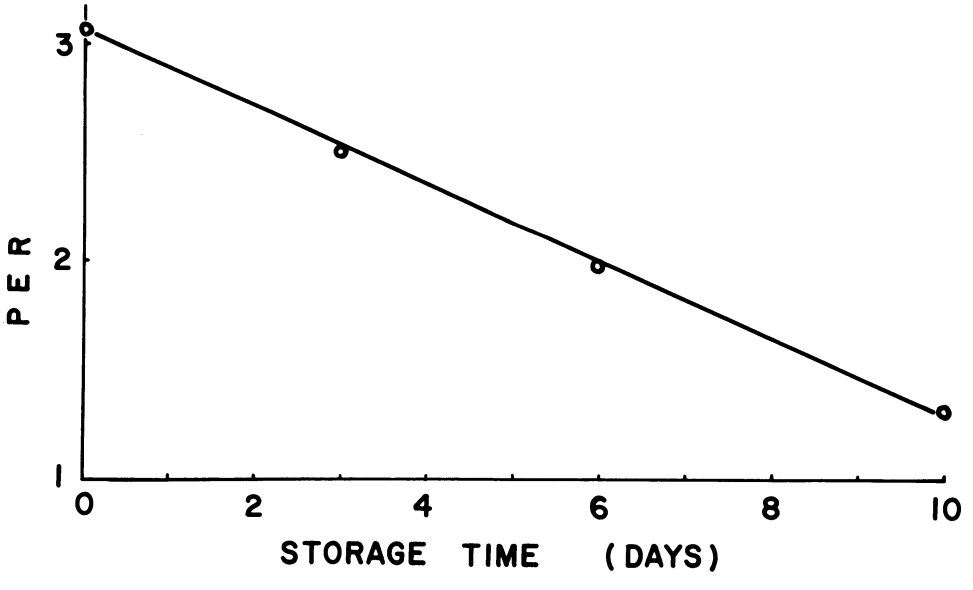


Figure 5. The correlation of PER value of browned egg albumin and the storage time.

in PER value after 6 days of browning and 17 percent loss after only 3 days of browning at 37°C. This reveals that the real nutritional value of some processed foods may differ considerably from the protein content assayed chemically.

Rat Feeding Experiments

Figure 6 presents the growth curves of rats during the short-term feeding experiment and five-week recovery period. Growth curves of each group during the recovery period (Rat Chow diet) were almost parallel to each other, suggesting that the damage inflicted upon growth by five-week feeding of brown protein is reparable.

Experiments with longer periods of feeding are presently being carried out in our laboratory to clarify the effects of long-term feeding.

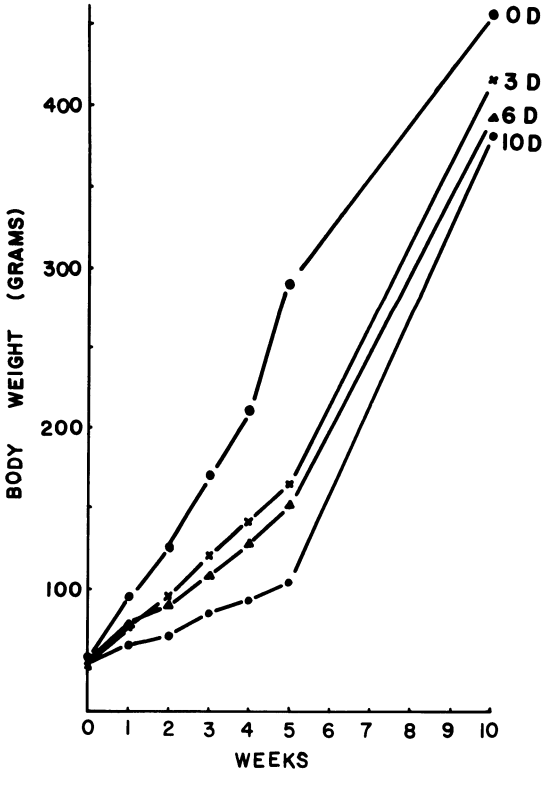


Figure 6. Body weight gain of rats fed browned and control diets during experimental and recovery periods.

In the three-month feeding study in which animals in groups II, III and IV were pair-fed, group II had significantly less weight gain than groups III and IV. Since the diets were iso-caloric and their initial feed efficiency was similar, it is suggested that there may have been factors other than protein quality existing in the brown protein which caused growth retardation.

Table 2 shows that the relative liver and kidney size in male rats fed the brown diet is significantly different from other groups. Such a difference is not observed in female rats. The comparison of group I with the other three groups does not seem appropriate because rats in group I had unlimited access to food while the others were pair-fed. However, pair-feeding did not cause any significant changes in the relative organ weight of group IV. This may provide sufficient justification to conclude that the differences observed in kidney weights for group II, along with the protruded penis, may be the result of a number of related effects on the urinary tract in male rats. Higher blood urea values may be another indication of the difficulty of passing nitrogen-containing metabolites.

Table 2. Relative organ weights of rats fed brown and control diets for three months.

Group	Terminal Wt. (gm) Ave. ± S.D.		Kidneys (% body wt.) Ave. ± S.D.		Liver (% body wt.) Ave. ± S.D.		Spleen (% body wt.) Ave. ± S.D.		Heart (% body wt.) Ave. ± S.D.		Testes (% body wt.) Ave. ± S.D.
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
I Control	358± 27	296± 10	0.61± 0.038	0.83± 0.04	2.41± 0.22	3.16± 0.12	0.137± 0.006	0.170± 0.021	0.31± 0.056	0.32± 0.018	0.95± 0
II Brown	A,B,C 110± 23	A,B,C 132± 10	A,B,C, 0.95± 0.10	A 0.725± 0.05	A,B,C 4.16± 0.73	3.40± 1.0	0.189± 0.058	0.153± 0.036	0.46± 0.13	0.37± 0.54	1.83± 0.34
III 5% Prot.+ 5% NEAA	140± 12	182± 12	0.80± 0.06	0.71± 0.03	3.08± 0.60	3.95± 0.26	0.154± 0.02	0.189± 0.004	0.43± 0.026	0.363± 0.023	1.50± 0.29
IV 4.5% Prot.	161± 19	165± 15	0.69± 0.16	0.74± 0.11	2.54± 0.11	3.65± 0.54	0.169± 0.01	0.155± 0.043	0.35± 0.019	0.365± 0.029	1.50± 0.028

A - SIGNIFICANTLY DIFFERENT ($P < .05$) FROM GROUP I.
B - SIGNIFICANTLY DIFFERENT ($P < .05$) FROM GROUP III.
C - SIGNIFICANTLY DIFFERENT ($P < .05$) FROM GROUP IV.

The values for serum glucose, BUN, alkaline phosphatase, SGOT, SGPT and hematocrit are shown in Table 3. As can be observed, serum glucose, BUN, alkaline phosphatase, SGOT and SGPT are higher in male rats fed brown protein. SGOT was also higher in female rats on brown diet (Diet #2) when compared to diet #3. Hematocrit values were lower in male rats fed brown diet when compared to all other groups. Higher serum glucose could be an indication of physiological stress (Root and Bailey, 1964).

Alkaline phosphatase activity has been shown to change during physiological abnormalities. Its elevation in serum is a diagnostic test for liver damage (Boyd, 1965). Thus, the increase in its activity in the present experiment could suggest a possible toxic effect with brown protein feeding of the rat for a three month period. The increased activities of serum transaminases are other indications of possible toxic effects of brown compounds (Chinsky et al., 1956; Karmen et al., 1955).

Table 3. Blood chemistry of rats fed brown and control diet for three months.

Group	Serum glucose (mg %) Ave. ± S.D.		Blood Urea Nitrogen, (BUN) (mg %) Ave. ± S.D.		SGOT (IU) Ave. ± S.D.		SGPT (IU) Ave. ± S.D.		% PCV Ave. ± S.D.		Serum Alkaline Phosphatase (IU) Ave. ± S.D.	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
I Control	63± 14.8	69.3± 5.3	14.9± 2.8	11.0± 2.1	395± 46	288± 27	121± 33	72± 4	46.5± 2.1	45.3± 1.5	44.5± 6.4	127± 2.3
II Brown	A,B,C 110± 28.5	64.6± 23	B 19.6± 5.25	C 11.1± 3.36	B 513± 14	B 340± 76	C 210± 89	87± 21	37.7± 3.3	45± 1.4	A,B,C 259.5± ±75.5	178.7± 85
III 5% Prot.+ 5% NEAA	74± 13.4	84± 3.4	12.2± 6.3	7.8± 2.9	379± 55	211± 51	118± 11	75± 9	44.7± 4.5	44.0± 2	133± 29.7	159± 21.7
IV 4.5% Prot.	61± 18.5	82.6± 6.5	14.1± 4.8	6.7± 1.6	547± 94	257± 26	68± 4	84± 26	49.5± 0.7	47.7± 0.58	119± 4.9	142.7± 15.5

A - SIGNIFICANTLY DIFFERENT FROM GROUP I.
B - SIGNIFICANTLY DIFFERENT FROM GROUP III.
C - SIGNIFICANTLY DIFFERENT FROM GROUP IV.

Serum protein and albumin-globulin ratios are presented in Table 4. As can be observed, serum protein values in females were lower in group II. In males, the ratio of albumin-globulin was lower in group II fed brown protein as compared to group I. Since there is no significant difference between groups II, III and IV with respect to serum protein and globulin-albumin ratios, the lower values seem to be due to a lesser food intake rather than the browned diet.

Table 5 shows the enzyme activities in the small intestine and liver of rats. Among the male rats fed brown protein, the activity of liver GOT was higher in group II. Since the activity of this enzyme was higher in serum as well, it could be another indication of possible toxicity of brown protein.

There is evidence that the volume and enzyme content of digestive juices varies with the quality and quantity of ingested food (Bollman, 1964). In the present study, diets #3 and #4 were

Table 4. Serum "protein" and "Albumin-globulin ratio" in rats fed brown and control diets for three months.

Group	Serum Protein (GM %) Ave. ± S.D.		Albumin-Globulin Ratio Ave. ± S.D.	
	Male	Female	Male	Female
I	7.87± 0.45	8.53± 0.52	2.1± 0.14	2.16± 0.25
II	7.06± 0.77	6.7± A 1.1	1.06± A 0.37	1.85± 0.31
III	6.57± 0.68	5.9± A 0.44	1.47+ A 0.16	1.32± A 0.54
IV	7.26± 0.67	6.45± A 0.33	1.04± A 0.38	1.38± A 0.45

A - SIGNIFICANTLY DIFFERENT FROM GROUP I.

Table 5. Enzyme activity of rats fed brown and control diets for three months.

Enzyme Activity / Group	Small Intestine Alkaline Phosphatase(1) Ave. ± S.D.		Small Intestine Maltase(2) Ave. ± S.D.		Liver GOT IU/mg Prot. Ave. ± S.D.		Liver GPT IU/mg Prot. Ave. ± S.D.		Small Intestine Dipeptidase (3) (Glycylvaline) Ave. ± S.D.	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
I	159± 14	85± 41	--- ---	--- ---	55± 0.63	83± 10	109± 4.2	118± 11	3.1± 0.42	3.9± 1.0
II	223± 43	64± 5.6	11.6± 3.6	B 5.8± 1.08	A,B,C 130± 15	A 110± 18.7	A,B,C 41± 30	111± 29	A,B 0.57± 0.39	1.19± 0.97
III	162± 7	52± 31	12.7± 5.92	14.1± 5.0	94± 27	99± 18	110± 42	121± 31	0.28± 0.09	1.4± 1.0
IV	132± 69	112± 8.5	--- ---	--- ---	100± 21	89± 13	127± 47	153± 28	0.35± 0.10	2.3± 2.5

1 - Micromoles substrate hydrolyzed per minute per mg protein.
2 - Micromoles substrate hydrolyzed per 60 minutes per mg protein.
3 - Micrograms dipeptide hydroylzed per mg protein per 60 minutes.

A - Significantly different from Group I.
B - Significantly different from Group III.
C - Significantly different from Group IV.

formulated and included to eliminate protein deficiency as a variable factor of browned diet.

The maltase activity was lower in female rats fed brown protein (Table 5). Decreased dissacharidase activity in rats fed brown compounds has been observed in our laboratory in previous studies (Lee, 1975). The protein fed to rats in that investigation was casein with browned apricot added at the expense of carbohydrates. This, along with the findings of the present study, clearly indicates that the lower activity of dissacharidases is not due to protein deficiency, but rather to the brown diet itself. The lowered maltase activity in rats fed brown diet may indicate a change in the intestinal microvilli or some other inhibitory effects. The differences observed between female and male rats could not be reconciled at this stage and should be clarified as data for longer periods of feeding (six months, one year, etc.) becomes available. However, it is certain that the present study has indicated that some physiological disturbance does indeed exist after feeding rats a browned diet for three months. The differences shown in Tables 2, 3, 4 and 5 for

diet #2 (brown diet) could not be solely attributed to the protein quality of the diet since diets having the same protein quality (diets #3 and #4) did not manifest such effects. Whether these physiological disturbances are caused by the accumulation of biologically active or toxic compounds or merely by imposing a physiological barrier on the absorption mechanism of rats is not known at this time. However, changes in some of the toxicity indices such as alkaline phosphatase and transaminases, and relative organ weights, do imply such a possibility. Fink et al. (1958) have also found some hepatic disorders by feeding Maillard products to rats. Adrian (1973) reported a slight hypertrophy of liver and a more pronounced hypertrophy of kidney in rats fed premelanoidins.

In this investigation, browned egg albumin was prepared under relatively mild conditions without excessive heat treatment or long periods of storage. Protein was kept for only 10 days at 37°C and 68% R.H. Diets were formulated with only 10 percent of such protein. However, the results from the feeding experiments have obvious practical implications.

Although research on the Maillard reaction is in relatively good standing today, continuing efforts to emphasize its basic chemistry and implications with regard to nutritional and physiological effects, as well as food safety and technology, are eminently needed. On the basis of the data available at the present time, these effects are not completely explainable. It is therefore extremely important to continue our long-term feeding experiments to determine whether the continued and increased consumption of thermally processed food materials containing non-enzymatic browned compounds may have an adverse effect on the nutriture of the public. These effects may be physiological as well as toxicological. The concern is even more significant when one considers that almost all foods, including frozen foods, are thermally processed. As the public continues to modify its eating habits, there is an increasing possibility of sufficiently altering the intake of these physiologically active materials. If the effects of non-enzymatic browned food, when consumed in large quantities, is injurious to good health, modifications in processing procedures can and should be instituted. It is imperative, in the face of data accumulated over the past decade, to further elucidate the magnitude of the problem. Furthermore, it is essential to isolate and identify those physiologically active compounds derived from the browning reaction.

From a nutritional point of view, there is a substantial reduction in protein quality due to the non-enzymatic browning reaction during thermal processing and storage. If this can be eliminated or controlled, it would of course result in a substantial nutritional benefit to the consumer of these products. Additionally, we and others have shown that in order to get a true evaluation of protein quality in foods which have undergone browning, biological testing

is the only reliable methodology available at the present time. This adds to the problem of attempting to develop a quality assurance program by the food processor since the biological method is both time consuming and expensive. The consumer ultimately pays the price of such a quality assurance program and therefore the development of a reliable and low-cost method is desirable. Moreover, the influence of protein quality information on nutritional labelling policies is obvious.

A better understanding of the Maillard browning reaction will of course allow methodology in food preservation to be developed which will avoid some of the disadvantages of this reaction while retaining the advantages. The food processor has at the moment a large number of options available for the preservation of food. If the economic analysis of alternate strategies in preservation must take into account numerous biological values, with a comparative lack of knowledge about consequences of the browning reaction, the input to such analysis will be lacking. It is therefore necessary to develop a clearer and more fundamental knowledge of this ubiquitous reaction.

From a technology standpoint, there is clear indication that an increased awareness of and interest in the nutritional qualities of the food supply does indeed exist. Alternately, from the consumer's standpoint, the same awareness and interest exists. This industrial and consumer interest in potential toxicants in foods thus creates an overwhelming national concern with the quality of our food supply. Although it is difficult to state specifically that there is an interest in the general nature of the Maillard reaction, per se, it is not unfair to say in the context of the overall interest, that there must and should be. There is some indication that from a commercial standpoint the analysis of thermally processed products has shown, on occasion, an unexpectedly low nutritional value thereby implicating the browning reaction as a point of concern.

ACKNOWLEDGEMENT

This investigation was made possible by research grant 5R01-FD-00433-05 TOX from the Department of Health, Education and Welfare/Public Health Service.

Rhode Island Agricultural Experiment Station Contribution Number 1706. University of Rhode Island, Kingston, Rhode Island, 02881

REFERENCES

Adrian, J. 1974. Nutritional and physiological consequences of the Maillard reaction. World Rev. of Nutr. and Diet. 19:71.

Amaya, J. F. 1974. The Maillard reaction of proteins. Ph.D. dissertation. University of Rhode Island, Kingston, R.I. 02881.

Amaya, J. F., Tung-Ching Lee and C. O. Chichester. 1976. Biological inactivation of proteins by the Maillard reaction. Effect of mild heat on the tertiary structure of insulin. J. Agric. Food Chem. 24:465.

Bjarnason, J. and K. J. Carpenter. 1969. Mechanisms of heat damage in proteins, I. Models with acylated lysine units. Brit. J. Nutr. 23:859.

Bjarnason, J. and K. J. Carpenter. 1970. Mechanisms of heat damage in proteins, II. Chemical changes in pure proteins. Brit. J. Nutr. 24:313.

Boctor, A. M. and A. E. Harper. 1968. Measurement of available lysine in heated and unheated foodstuffs by chemical and biological methods. J. Nutr. 94:289.

Boyd, W. 1965. Textbook of pathology. Seventh edition. Lea and Febiger, Philadelphia, Pennsylvania. p. 552.

Carpenter, K. J. 1960. The estimation of available lysine in animal protein foods. Biochem. J. 77:604.

Carpenter, K. J. and V. H. Booth. 1973. Damage to lysine in food processing: Its measurement and significance. Nutr. Abs. Rev. 43:424.

Chichester, C. O. 1973. Nutrition in food processing. World Rev. Nutr. and Diet. 16:318.

Chinsky, M., G. L. Shmagranoff and S. Sherry. 1956. Serum transaminase activity. J. Lab. and Clin. Med. 47:108.

Clinger, C., A. Yound, I. Prudent and A. R. Winter. 1951. The influence of pasteurization, freezing and storage on the functional properties of egg white. Food Technol. 5:166.

Coduri, R. J. and A. G. Rand, Jr. 1972. Vertical plate gel electrophoresis for the differentiation of meat species. J. A. O. A. C. 55:461.

Dahlquist, A. 1964. Method for assay of intestinal disaccharidases. Anal. Biochem. 7:18.

Erbersdobler, H. 1969. Aminosäurenanflutung im pfortaderplasma nach fütterung hitzegeschädigten proteins und hocheiweissadaptierter ratten. Z. Tierphysiol. Tierernähr. Futtermittelk. 25:119.

Fink, H., I. Schlie and U. Ruge. 1958. Über ernährung-sphysiologische veränderungen der milch beim technischen trocknen. Z. Naturforsch. 13B:610.

Ford, J. E. and C. Shorrock. 1971. Influence of heat damaged proteins in the rat. Brit. J. Nutr. 26:311.

Henry, K. M., S. K. Kon, C. H. Lea and J. C. D. White. 1948. Deterioration on storage of dried milk. J. Dairy Res. 15:292.

Josefsson, L. and T. Lindberg. 1965. Intestinal dipeptidases. Biochem. Biophys. Acta. 105:149.

Kakade, M. L. and I. E. Liener. 1969. Determination of available lysine in proteins. Anal. Biochem. 27:273.

Karmen, A., F. Wroblewski and J. S. LaDue. 1955. Transaminase activity in human blood serum. J. Clin. Invest. 34:126.

Lee, C. M. 1974. Physiological consequences of non-enzymatic browning. Ph.D. dissertation. University of Rhode Island, Kingston, Rhode Island 02881.

Lee, C. M., Tung-Ching Lee and C. O. Chichester. 1976. Physiological consequences of browned food products. Proceedings of the IV International Congress of Food Science and Technology (in press).

Lowry, O. H., N. J. Rosebrough, A. L. Parr and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265.

Mauron, J., F. Mottu, E. Bujard and R. H. Egli. 1955. The availability of lysine, methionine and tryptophan in condensed milk and milk powder "in vivo" digestion studies. Arch. Biochem. Biophys. 59:433.

Nesheim, M. C. and K. J. Carpenter. 1967. The digestion of heat-damaged protein. Brit. J. Nutr. 21:399.

Newmann, H. and M. Van Vreedendaal. 1967. An improved alkaline phosphatase determination with p-nitrophenyl phosphate. Clin. Chim. Acta. 17:183.

Patton, S. 1955. Browning and associated changes in milk and its products. A review. J. Dairy Sci. 38:457.

Rand, N. T., V. K. Collins, D. S. Varner and J. D. Masser. 1960. Biological evaluation of the factors affecting the protein quality of fish meals. Poultry Sci. 39:45.

Root, H. F. and C. C. Bailey. 1964. In: Modern Nutrition in Health and Disease. Ed. by Whol and Goodhart. Lee and Febiger, Philadelphia, Pennsylvania. p. 796.

Sauberlich, H. E. 1961. Growth of rats fed protein-free diets supplemented with purified amino acid mixtures. J. Nutr. 74:298.

Sgarbieri, V. C., M. Tanaka, C. O. Chichester and J. Amaya. 1971. Some nutritional consequences of the Maillard reaction. Proceedings of III Western Hemisphere Nutrition Congress. Futura Publishing Co., Mount Kisco, New York. p. 330.

Sigma Chemical Company Technical Bulletin. 1975. No. 535. The colorimetric determination of urea nitrogen in blood, plasma or serum. Sigma Chemical Company, St. Louis, Missouri.

Tanaka, M. 1974. Effect of the browning reaction on quality, digestion and absorption of protein. Ph.D. dissertation. University of Rhode Island, Kingston, Rhode Island 02881

Tanaka, M., J. Amaya, Tung-Ching Lee and C. O. Chichester. 1976. Proceedings of the IV International Congress of Food Science and Technology (in press).

Tanaka, M., Tung-Ching Lee and C. O. Chichester. 1975. Nutritional consequences of the Maillard reaction. The absorption of fructose-L-tryptophan in the large intestine of the rat. J. Nutr. 105:989.

Tanaka, M., Tung-Ching Lee and C. O. Chichester. 1975. Effect of browning on chemical properties of egg albumin. Agric. Biol. Chem. 39:863.

23

AVAILABILITY OF THE TRUE SCHIFF'S BASES OF LYSINE. CHEMICAL EVALUATION OF THE SCHIFF'S BASE BETWEEN LYSINE AND LACTOSE IN MILK

Paul-André Finot, Eliane Bujard, Françoise Mottu

and Jean Mauron

Nestlé Products Technical Assistance Co. Ltd.

CH-1814 La Tour-de-Peilz - Switzerland

ABSTRACT

During the heat-treatment of milk, the Maillard reaction which takes place between the epsilon-amino group of lysine and lactose leads to the formation of two well-defined chemical types : the Schiff's base in equilibrium with its aldosylamine form and the deoxyketose (Amadori product). Rat growth assays showed that the synthetic ε-N-deoxyketosyl-L-lysine was not utilized as a source of lysine and that the true Schiff's bases resulting from the reaction of lysine with aromatic aldehydes were 100% utilized indicating that the Schiff's base ⇄ aldosylamine are also 100% utilized.

Taking the "in vitro" enzymatic method as references for the determination of available lysine, the classic acid hydrolysis and the reduction method by $NaBH_4$ were used to evaluate lysine bound to lactose as Schiff's base ⇄ aldosylamine or deoxyketose forms. It was demonstrated that a) the deoxyketose is the only form of unavailable lysine in milk, b) the furosine content in the acid hydrolysates multiplied by the conversion factor 3.1 gives the quantity of the deoxyketose form present, c) the reduction by $NaBH_4$ permits one to evaluate totally the two types of binding; the difference between the values obtained by both methods corresponds to the Schiff's base ⇄ aldosylamine form.

INTRODUCTION

The Maillard reaction which occurs in food proteins leads principally to the blockage of lysine, which becomes biologically unavailable. This type of reaction appears during sterilization or drying of industrial milk, which is thus an excellent model for the specific study of the relations between the chemical structure and biological availability of the lysine-sugar compounds in proteins.

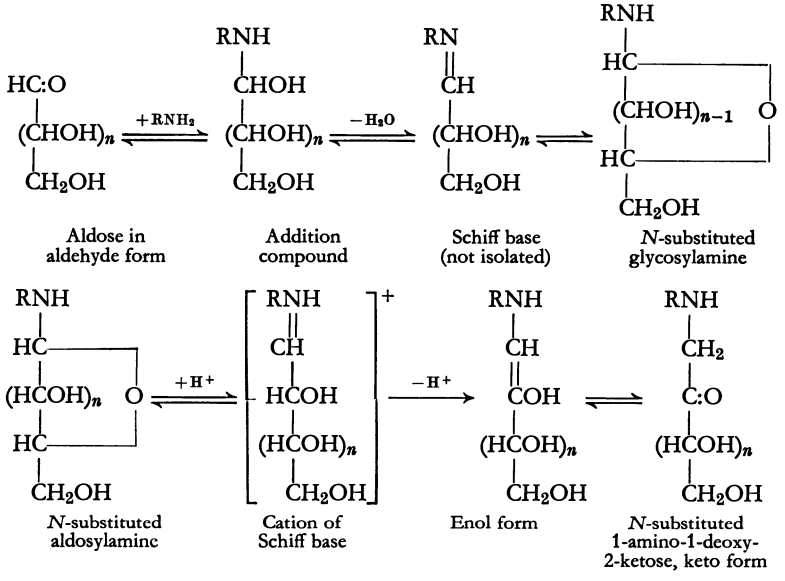


Fig. 1. The initial steps of the Maillard reaction

The initial steps of the Maillard reaction have been described by Hodge (1953) (Fig. 1). Certain derivatives correspond to non-isolatable intermediate forms, such as the addition compound or the Schiff base cation, whereas others correspond to well-defined chemical types which may be either unstable and difficult to isolate (e.g. the Schiff base in equilibrium with N-substituted aldosylamine, [Schiff's base ⇄ aldosylamine]) or stable (e.g. the deoxyketose form [Amadori product]) and hence more likely to be isolated and identified.

Two questions may be asked about these lysine-sugar derivatives (the Schiff's base ⇄ aldosylamine form and the deoxyketose form)

found in food proteins, chiefly milk.
1) Are these derivatives biologically available?
2) Can these derivatives be determined quantitatively?
Well-defined chemical models have been used to answer these questions, and the results obtained have been compared to results obtained with heat-treated milk.

BIOLOGICAL AVAILABILITY OF LYSINE-SUGAR DERIVATIVES AND LYSINE-ALDEHYDE

The biological availability of the following compounds has been measured :
a) certain Amadori products : ε-N-deoxyfructosyl-L-lysine, α-N-deoxyfructosyl-L-lysine and α-N,ε-N-dideoxyfructosyl-L-lysine, which were synthesized as described by Finot and Mauron (1969).
b) certain Schiff's bases : ε-N-benzylidene-L-lysine, ε-N-salicylidene-L-lysine and ε-N-(5-OH-methyl)-furfurylidene-L-lysine, which were synthesized as described by Polyanovskii (1963).
c) lysine fixed on polyaldehyde polymers, peroxidized starch and polyacroleine. The peroxidized starch was prepared by oxidation ($NaIO_4$) of soluble starch by Bobbitt's method (1956). The acroleine was polymerized by the method of Kern et al. (1960). The immobilization of lysine on these polymers was carried out at pH 7.0 and ambient temperature, and the reaction was followed by measuring NaOH utilisation on a pH-stat. After dialysis for one night, the dark brown peroxidised lysine-starch complex was concentrated to a small volume and freeze-dried, and the yellow lysine-polyacrolein was washed with water and freeze-dried. The amount of immobilized lysine (34.3% on peroxidized starch and 17.5% on polyacrolein) was calculated from the amount of nitrogen in these products.

A) Rat Assays for Lysine Availability

The biological availability of these lysine derivatives was determined by a growth assay on rats as described by Mottu and Mauron (1967). Four groups of 6 rats were used each time. The first group received a lysine-free basal diet, groups 2 and 3 received the same diet enriched with different known levels of L-lysine-HCl, and the fourth group received the basal diet enriched with the lysine derivative under study. The percentage availability of the different derivatives was estimated by the growth response produced by the appropriate diet compared to the control diets, correcting for differences in consumption. The 90% confidence limits for the percentage utilisation were calculated by Finney's method (1952).

B) Biological Availability of the Deoxyketosyl-L-lysines

The deoxyketosyl-L-lysine derivatives are not used as lysine source by the rat (Table 1) confirming earlier results (Mottu et al., 1970). The experimentally measured values do not differ significantly from zero, with a probability of 90%. This result has also been confirmed elsewhere by a metabolic study with ε-N-deoxyfructosyl-L-[U-^{14}C]-lysine, synthesized exactly as the unlabelled product, but starting from [U-^{14}C]-L-lysine. The ε-N-fructosyl-L-lysine, which is partly absorbed by the intestine (20-60%), is excreted in the urine. The non-absorbed fraction passes into the caecum where it is rapidly transformed by the intestinal microflora, with production of $^{14}CO_2$ which is eliminated by respiration. When radioactive ε-N-deoxyfructosyl-L-lysine is given by stomach tube to starved rats, $^{14}CO_2$ is respired 2 to 2½ hrs after administration (Fig. 2), i.e. after the time required for the product to reach the caecum. The flora of the caecum is thus responsible for the production of $^{14}CO_2$.

To verify this, ε-N-deoxyfructosyl-[U-^{14}C]-L-lysine was incubated aerobically at 37°C with the contents of a rat caecum suspended in 20 ml of water. [U-^{14}C]-L-lysine was quickly (30 min) and quantitatively regenerated, without, however, formation of $^{14}CO_2$.

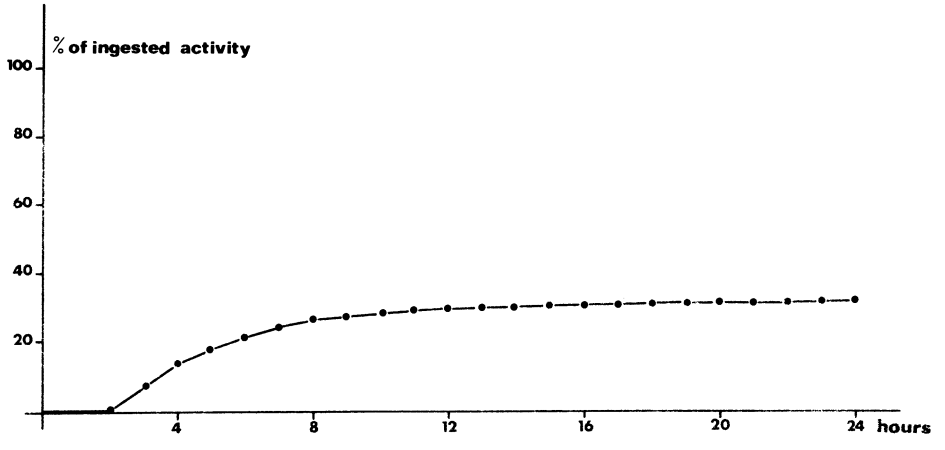


Fig. 2. Production of exhaled $^{14}CO_2$ after oral administration of ε-N-deoxy-fructosyl-L-(U-^{14}C)-lysine

This experiment shows that the microflora of the caecum is capable of breaking the lysine-sugar bond of Amadori product. If the lysine were actually liberated by the caecal flora, it would probably be utilized by the flora much more rapidly than it could be absorbed into the bloodstream for utilisation by the organism. The fact that the measured biological availability to the rat was always a little greater than 0% permits the conclusion that a very small part of ε-N-deoxyfructosyl-L-lysine could be utilized by the organism. The same phenomenon was observed by Sgarbieri et al. (1973) with deoxyfructosyl-L-tryptophan, which is not incorporated into the liver of rats treated with antibiotics, and slightly incorporated into the liver of untreated rats, but not until at least 8 hrs after oral administration.

C) Biological Availability of the Schiff's Bases of Lysine

The Schiff's bases in equilibrium with their aldosylamine, formed between the reducing sugars and the ε-amino group of lysine, have so far not been isolated because of their instability. The aldosylamines resulting from the reaction of hexoses with α-amino acids have been synthesized in anhydrous methanol by Weitzel et al. (1957) in the form of sodium salts or metal complexes (Ca, Mg, Zn, Co). The sodium salts are extremely unstable in water, reverting to hexose and the corresponding amino acid, whereas the metal complexes, especially those of the heavy metals such as Zn, although more stable in water, are completely hydrolysed after a few hours.

The glucosylamines resulting from the reaction of glucose with the aliphatic amines (butylamine, hexylamine, etc.) have been isolated in the anhydrous state by Pigman et al. (1951), but they are rapidly hydrolysed in water or in slightly acid conditions.

The presence of a Schiff's base $\rightleftarrows$ aldosylamine in an aqueous system containing an amine and a reducing sugar results in the following equilibrium :

$$R - NH_2 + \text{glucose} \overset{1}{\underset{2}{\rightleftarrows}} \text{(Schiff's base} \rightleftarrows \text{aldosylamine)}$$

depending on the pH, the temperature and the concentration of each of the reagents. The equilibrium is very favourable to reaction 2.

Because of the impossiblity of isolating the Schiff's bases resulting from the combination of lysine and a reducing sugar, a study has been made of the biological availability of the true Schiff's bases resulting from the reaction of lysine with the following aromatic aldehydes : benzaldehyde, salicylaldehyde and

(5-OH-methyl)-furfuraldehyde. These derivatives are easily isolated in crystalline form and are stable in neutral and alkaline conditions.

The biological availability of these three Schiff's bases of lysine has been determined by growth trials on rats. The results (Table 1) show almost 100% utilization. If the most stable Schiff's bases of lysine are 100% utilized as lysine sources, then it follows that the Schiff's base $\rightleftarrows$ aldosylamine derivatives of lysine which are very easily hydrolyzed will also be 100% utilized. This result seems now evident, given the known instability of Schiff's bases, but for a long time the opposite was thought to be the case based on the hypothesis of a greater stability of protein-bound glycosylamines (Mauron, 1956; Finot, 1973). The confirmation of their utilization was provided by more stable model molecules (true Schiff's bases made from aromatic amines) than the Schiff's base $\rightleftarrows$ aldosylamine combination found in milks.

D) Biological Availability of Lysine Linked to Polyaldehyde Polymers

The idea of enriching lysine-deficient proteins with a biologically available lysine derivative incapable of reacting with reducing sugars and thus chemically stable under the conditions of the technological treatment of food, led us to bind lysine onto polymers containing free aldehyde groups. Peroxidized starch and polyacrolein were chosen for this study.

The biological availability of lysine bound on these aldehyde polymers is very weak (Table 1): practically zero for lysine-polyacrolein and about 25% for lysine-peroxidized starch.

These results can be interpreted in the way that bound lysine, in the form of Schiff's base linkages, is probably eliminated to a large extent during washing and that the remaining lysine can no longer be released because other chemical reactions have bound it irreversibly. The dark brown colour in particular of the lysine-peroxidized starch complex is an argument in favour of this possibility.

CHEMICAL EVALUATION OF THE DEOXY-KETOSYL AND SCHIFF'S BASE $\rightleftarrows$ ALDOSYLAMINE DERIVATIVES OF LYSINE

Because the ε-N-deoxyketosyl-L-lysines are biologically unavailable, it is important to be able to determine them chemically, especially in industrial milks, and to evaluate what proportion

Table 1

Biological availability (in % utilization) of deoxy-fructosyl-L-lysines, Schiff's bases of lysine and lysine-polyaldehyde complexes

N-substituted lysines	lysine equivalent added to the diet (mg/100 g diet)	available lysine measured (mg/100 g diet)	% utilization (confidence limits at 90% probability)
a) Deoxyfructosyl-L-lysines			
ε-N-deoxyfructosyl-L-lysine	240	6	5 (-26; 30)
		22	15 (- 2; 31)
α-N-deoxyfructosyl-L-lysine	150	11	7 (-12; 24)
α-N,ε-N-dideoxyfructosyl-L-lysine	180	16	9 (- 6; 22)
b) Schiff's Bases			
ε-N-benzylidene-L-lysine	108	122	113 (92; 140)
	111	101	91 (73; 118)
ε-N-salicylidene-L-lysine	111	113	102 (88; 118)
ε-N-(5-OH-methyl)-furfurylidène-L-lysine	114	97	85 (73; 100)
c) Lysine-polyaldehyde complexes			
Lysine fixed on peroxydized starch	120	31	26 (11; 39)
Lysine fixed on polyacrolein	95	6	6,3 (-14; 23)

they represent of the total unavailable lysine. On the other hand, although the Schiff's base $\rightleftarrows$ aldosylamine derivatives of lysine are available, it is still of interest to know if they can be assessed quantitatively in milks under given conditions of pH and temperature, and to establish whether they interfere with the chemical methods of evaluation of available lysine.

A) Acid Hydrolysis of Deoxyketose-L-Lysines

Brüggemann and Erbersdobler (1968) have shown that the acid hydrolysates of superheated milk contained a new amino acid, eluted after arginine and arising from the transformation of deoxy-ketosyl-lysine during hydrolysis.

This compound was isolated starting from an acid hydrolysate of ε-deoxyfructosyl-L-lysine, and its structure was determined by UV, IR, NMR and mass spectrometry (Heyns et al., 1968 and Finot et al., 1968). It corresponds to ε-N-(2-furoylmethyl)-L-lysine or furosine (Fig. 3). Another compound, pyridosine, is also produced under the same hydrolysis conditions, but its separation on an amino acid analyzer is more difficult (Finot et al., 1969). The rate of formation of furosine during acid hydrolysis has been evaluated for ε-N-deoxyfructosyl-L-lysine, α-N-formyl-ε-deoxyfructosyl-L-lysine and α-N-formyl-ε-N-deoxylactulosyl-L-lysine (Finot and Mauron, 1972), and it corresponds to 20.3% of the blocked lysine (taking for furosine the same colour factor as for lysine). However, lysine is also released during the acid hydrolysis (49.5%) (Fig. 4).

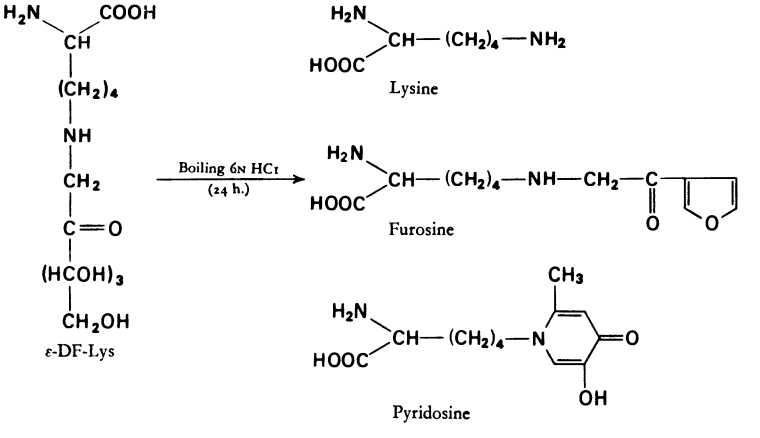


Fig. 3. Compounds obtained by acid hydrolysis of ε-N-deoxyfructosyl-lysine

These values are different when the hydrolysis is done in 7.75 N HCl (Erbersdobler, 1970), (Table 2).

Table 2

Regenerated lysine and furosine content found in the acid hydrolysates of the ε-N-deoxyketosyl-L-lysines derivatives of glucose and lactose

		hydrolysis in 6 N HCl	hydrolysis in 7.75 N HCl
Regenerated lysine	%	49.5 ± 2.6	43.8 ± 0.20
Furosine	%	20.3	29.3

Since only the ε-N-deoxyketosyl-L-lysines give furosine during acid hydrolysis, the evaluation of this form in a milk may be made by measuring the amount of furosine present in the acid hydrolysate.

Now, the furosine (FUR) corresponds to 20.3% of the ε-N-deoxyketosyl-L-lysines (DOK), and so

$$(1) \quad DOK = \frac{FUR}{20.3} \times 100 \simeq 5 \times FUR$$

Furthermore, as it has been shown that the form Schiff's base $\rightleftarrows$ aldosylamine of lysine is biologically available, contrary to previously held opinion (Finot, 1973), it follows that inactivated lysine is found only in the deoxyketosyl form (DOK). Thus, a value for available lysine may be calculated from the furosine content (ALV_{fur}) with the help of the equation

$$ALV_{fur} = ThLV - DOK$$

$$(2) \quad ALV_{fur} = ThLV - 5\ FUR$$

ThLV (theoretical lysine value) is equal to 8.2 g/16 g N in milks, hence

$$(3) \quad ALV_{fur} = 8.2 - 5\ FUR$$

This formula has been used to evaluate the available lysine in the milk samples.

For this purpose, a series of milk samples were submitted to various heat treatments. These milks (Table 3) were hydrolyzed and

Table 3

Values obtained (g/16 g N) after acid hydrolysis, reduction and enzymatic digestion

Milks	Acid Hydrolysis				Reduction		Enzymatic Digestion
	TLV	FUR	ALV_{fur}	ALV'_{fur}	LYS_{red}	Σpeaks	ALV_{enz}
untreated							
RM Raw Milk	8.21	0	8.2	8.2	7.89	0.23	8.20
LM Lyoph. "	8.21	0	8.2	8.2	7.78	0.29	8.12
SD Spray-dried M.	8.34	0	8.2	8.2	7.38	0.79	8.30
SM Stand. Milk	8.18	0	8.2	8.2	7.40	0.69	8.04
sterilized							
SM_1 115° 10'	7.70	0.30	6.70	7.27	7.05	1.04	7.64
SM_2 115° 20'	7.64	0.31	6.55	7.24	6.91	1.09	6.81
SM_3 115° 30'	7.71	0.34	6.50	7.15	6.79	1.21	6.44
SM_4 115° 40'	7.64	0.34	6.50	7.15	6.55	1.46	5.93
roller-dried							
RD_A 165° 11 rpm	5.67	1.24	2.00	4.36	3.60	4.44	4.57
RD_B 165° 6.5 rpm	5.22	1.50	0.70	3.55	2.78	5.00	2.99
RD_C 165° 2 rpm	4.49	1.95	-1.55	2.15	1.61	6.05	2.11

TLV = total lysine value; ALV_{fur} = 8.2 - 5 FUR; ALV'_{fur} = 8.2 - 3.09 FUR; LYS_{red} = after reduction; ALV_{enz} = available lysine after enzymatic digestion.

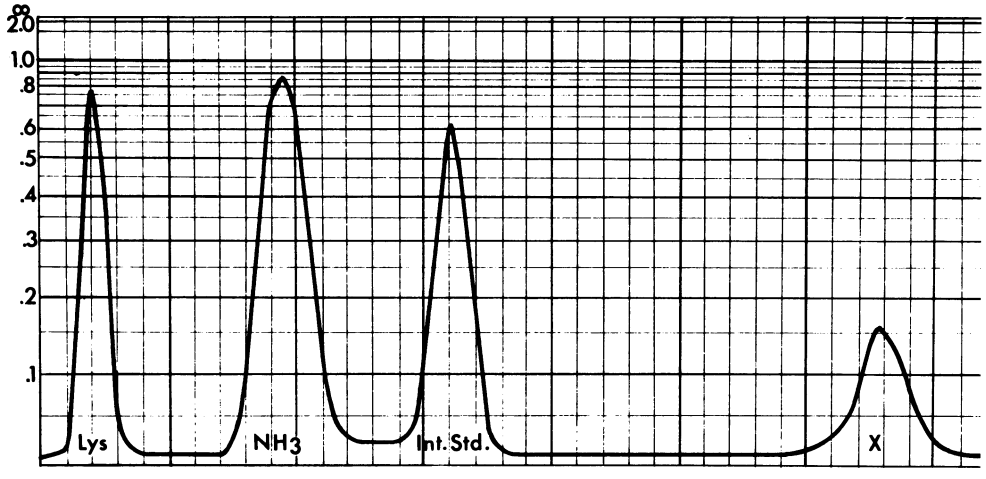


Fig. 4. Separation by ion exchange chromatography of the acid hydrolysate of the ε-N-deoxyketosyl-L-lysines

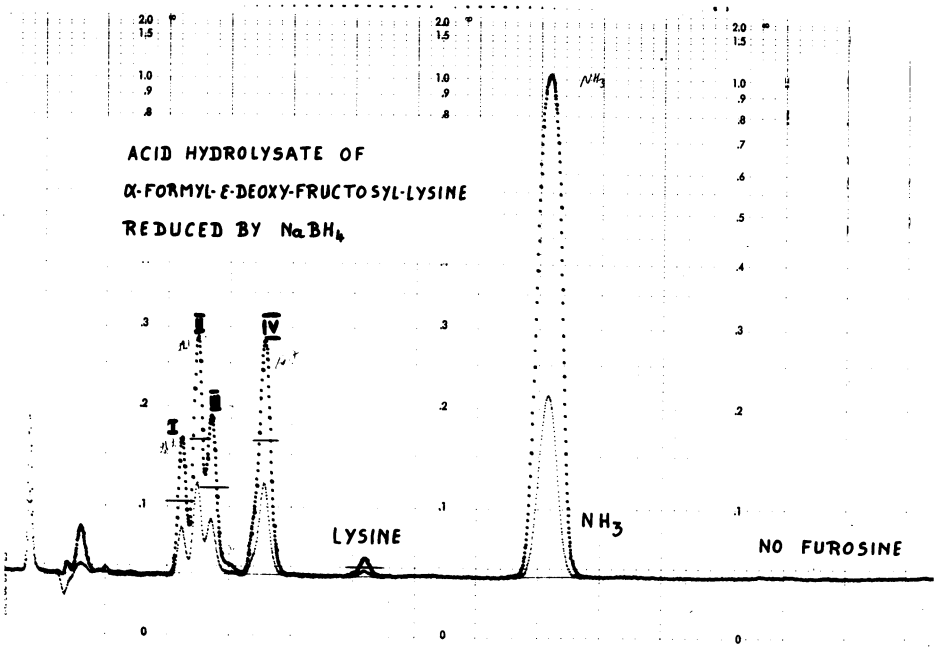


Fig. 5. Separation by ion exchange chromatography of the acid hydrolysate of α-N-formyl-ε-N-deoxyfructosyl-L-lysine reduced by $NaBH_4$.

their lysine availability evaluated by equation 3.

The regression line calculated between the available lysine value after enzymatic digestion (ALV_{enz}) taken as a reference (see p. 17) and ALV_{fur} gives the following equation

(4) $$ALV_{enz} = 0.62\ ALV_{fur} + 2.90 \quad (r = 0.976)$$

which does not pass through the origin; its slope is less than 1 (Fig. 6).

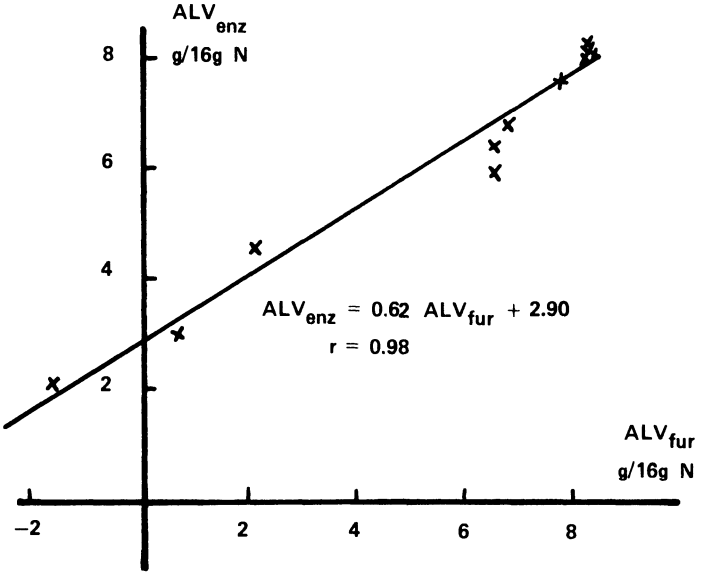


Fig. 6. Correlation between the available lysine values measured by the enzymatic method (ALV_{enz}) and by the furosine method (ALV_{fur} = 8.2-5 FUR).

This method of estimating the available lysine from the furosine content overestimates the blockage : protein-linked deoxyketosyl lysines thus produce more furosine during acid hydrolysis than when free. It was decided to try to evaluate the rate of transformation to furosine of the linked deoxyketosyl lysines by using the method of reduction by $NaBH_4$, proposed by Hurrell and Carpenter (1974).

B) Reduction of Synthesized Deoxyketosyl-Lysines

It has been shown by Hurrell and Carpenter (1974) that α-formyl-ε-N-deoxyfructosyl-L-lysine, reduced by $NaBH_4$ does not regenerate lysine, nor does it give furosine during acid hydrolysis. We have confirmed this property for the same molecule and also for the deoxylactulosyl derivative (Fig. 7).

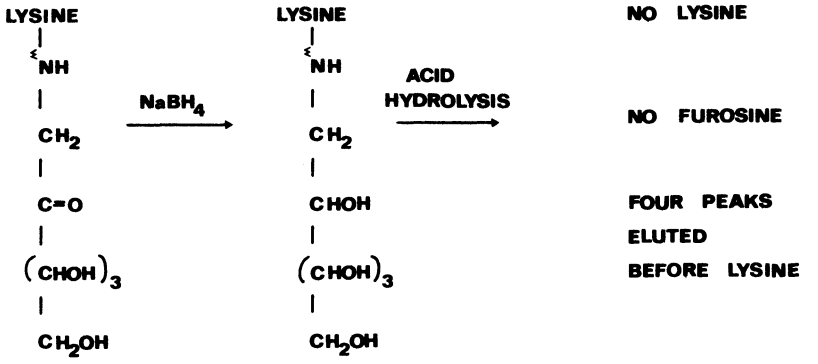


Fig. 7. Reduction and acid hydrolysis of ε-N-deoxyfructosyl-L-lysine.

On the other hand, four new peaks (peaks I, II, III and IV) appear, which are eluted before lysine on a 16 cm-column (resin PA-35, Beckman) at 54°C with a citrate buffer, 0.35 N in Na, pH 5.28 (Fig. 5).

C) Reduction of Heat-Treated Milks : Reduction Balance

These peaks are also present in the hydrolysates of milks which are heat-treated and then reduced by $NaBH_4$, and their relative proportions are the same as those found with the deoxylactulosyl derivative (Table 4). This observation clearly shows that these peaks have the same origin, i.e. the reduced form of ε-N-deoxylactulosyl-L-lysine. Furthermore, if one makes the correlation between the sum of these peaks (ξ peaks) calculated with the colour factor of lysine, and the lysine not recovered after reduction, i.e. the difference between the theoretical lysine and the measured lysine after reduction (8.2-Lys_{red}), one finds a perfect linear relation (equ. 5) (Fig. 8).

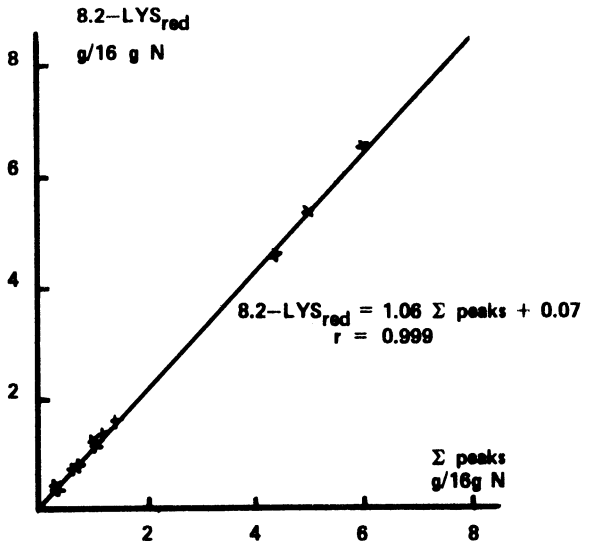


Fig. 8. Correlation between lysine which is not recovered after reduction (8.2-LYS$_{red}$) and the sum of the peaks I, II, III, IV ($\sum$ peaks).

(5) $8.2\text{-Lys}_{red} = 1.06 \sum \text{peaks} + 0.07 \quad (r = 0.999)$

This regression line passes through the origin and its slope of 1.06 corresponds to the factor by which the $\sum$ peaks must be multiplied in order to obtain the lysine not recovered after reduction. This linear relation also shows that the form of blockage does not differ from that demonstrated by the reduction method : all the lysine unrecovered after reduction is held in the form of peaks I, II, III and IV.

D) Interference by Schiff's base $\rightleftarrows$ Aldosylamine during Reduction of the Milk Samples

As the peaks which appear after reduction and furosine derive from the same precursor, the Amadori product, there must exist a linear relation between the values of these two parameters. If one calculates the regression line between the sum of peaks I, II, III and IV ($\sum$ peaks) and the furosine content for each of the milks, one obtains equation (6) (Fig. 9).

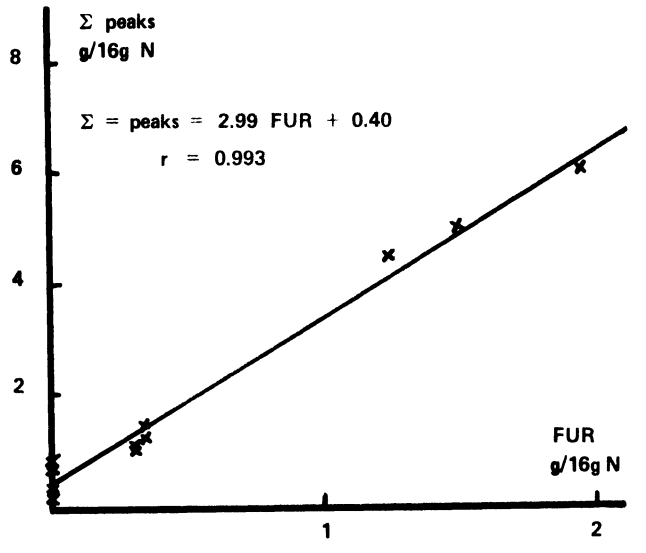


Fig. 9. Correlation between the sum of the peaks I, II, III and IV ($\sum$ peaks) in the reduced milks and the furosin content (FUR)

(6) $\sum$ peaks = 2.99 FUR + 0.40, r = 0.993

This line does not pass through the origin. In fact, untreated milks, giving no furosine on hydrolysis, also produce peaks I, II, III and IV after reduction. This means that when there is no deoxyketosyl-lysine in the milk, reduction by $NaBH_4$ forms a product which, after hydrolysis, gives the same peaks as the deoxyketosyl-lysines. This product can only be the reduced form Schiff's base $\rightleftarrows$ aldosylamine, which has the same chemical structure as reduced ε-deoxyketosyl-L-lysine, except that carbon atom 2 of the sugar residue is asymmetric in one of them (Fig. 10).

This hypothesis could be verified on a simple model, by reducing and then hydrolyzing under the same conditions as for the milk, a freshly prepared solution of polylysine and glucose or lactose at pH 7.0, and thus incapable of containing deoxyketosyl-lysine. The same peaks I, II, III and IV were detected (Table 4).

Table 4

Respective proportion of the peaks I, II, III and IV (in % of the sum of the four peaks) in the milk samples, in the deoxyketosyl-lysines and in solutions of poly-L-lysine plus reducing sugars

Peaks	I %	II %	III %	IV %
Untreated milks				
RM	0	traces	22.4	77.6
LM	0	12.5	27.7	59.9
SD	5.4	18.1	26.0	50.5
SM	4.5	18.2	27.3	50.0
Sterilized milks				
SM_1	7.1	21.8	25.3	45.8
SM_2	7.3	20.0	24.6	47.9
SM_3	7.6	21.6	27.1	43.7
SM_4	11.7	21.5	26.0	40.8
Roller-dried milks				
RD_A	9.2	30.2	23.1	37.6
RD_B	8.0	23.4	26.4	42.2
RD_C	9.2	25.2	28.5	37.1
ε-N-deoxyfructosyl-L-lysine	14.5	31.8	19.7	34.1
α-formyl-ε-deoxy-lactulosyl-L-lysine	5.2	24.1	25.2	45.5
Poly-L-lysine + glucose	traces	36.5	21.5	41.9
Poly-L-lysine + lactose		++	+	+++

Because of the interference of the Schiff's base ≠ aldosyl-amine, the method of reduction by $NaBH_4$ does not allow evaluation of the rate of formation of furosine from the linked deoxyketosyl-lysines. However, it does confirm that the inactivated lysine in milks is found only in the deoxyketosyl form.

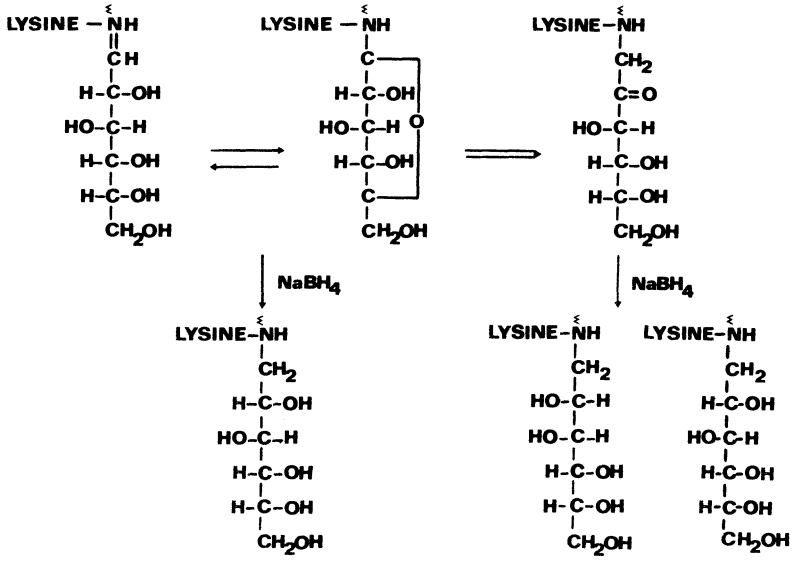


Fig. 10. Reduction by $NaBH_4$ of the Schiff's base ⇄ aldosylamine and of the deoxyketosyl derivatives of lysine.

E) The Furosine → Deoxyketose Conversion Factor

In order to evaluate the factor of conversion of furosine to deoxyketose, we refer to the method of biological dosage of available lysine by "in vitro" enzymatic digestion, described by Mauron et al. (1955). By this method, the protein is first treated by pepsin and then digested by pancreatin in a dialysis bag which allows passage of free amino acids. These are collected outside the bag at regular intervals for 24 hrs, to be chromatographed just as they are. The availability of the lysine in the heat-treated samples is calculated with reference to the amount of lysine released by an untreated milk sample having 100% availability. This method gives lysine availability values (ALV_{enz}) comparable to the values obtained by growth trials on animals (ALV_{rat}) (Mottu and Mauron, 1967).

(7) $ALV_{rat} = 1.02\ ALV_{enz} - 0.23, \quad r = 0.98$

As the unavailable lysine is in the form of deoxyketose-lysine (DOK), it may be calculated for each milk by the difference between the theoretical lysine and the available lysine (ALV_{enz}) :

(8) $$DOK = 8.2 - ALV_{enz}$$

The regression line (Fig. 11) between DOK and FUR is the following:

(9) $$DOK = 3.09\ FUR + 0.23 \quad (r = 0.977)$$

To find the amount of DOK, one needs only multiply the amount of FUR by 3.09.

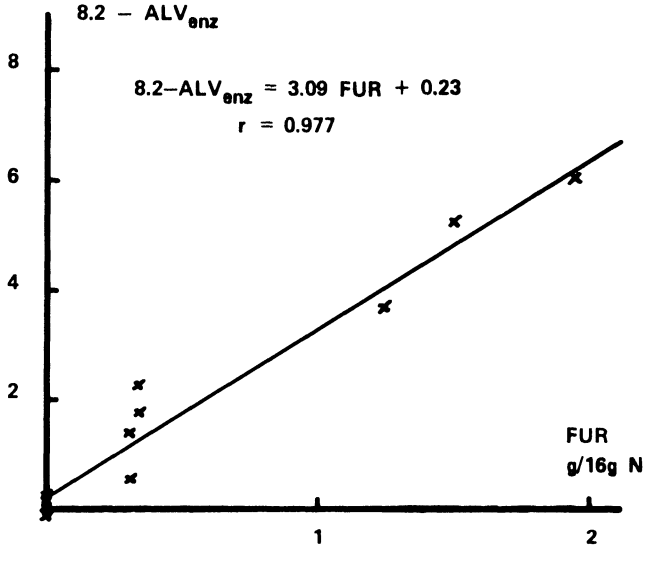


Fig. 11. Correlation between the inactivated lysine ($DOK = 8.2-ALV_{enz}$) and the furosine content (FUR)

Protein-linked deoxyketosyl-lysine thus produces during acid hydrolysis 1/3.09, or 32.4%, of furosine instead of the 20.3% found with synthesized deoxyketosyl-lysines. This new conversion factor permits the calculation of available lysine, ALV'_{fur}, by the equation 10 derived from equation 3

(10) $$ALV'_{fur} = 8.2 - 3.09\ FUR$$

These values are in good agreement with the enzymatically measured availability (ALV_{enz}). The regression line (Fig. 12) is as follows:

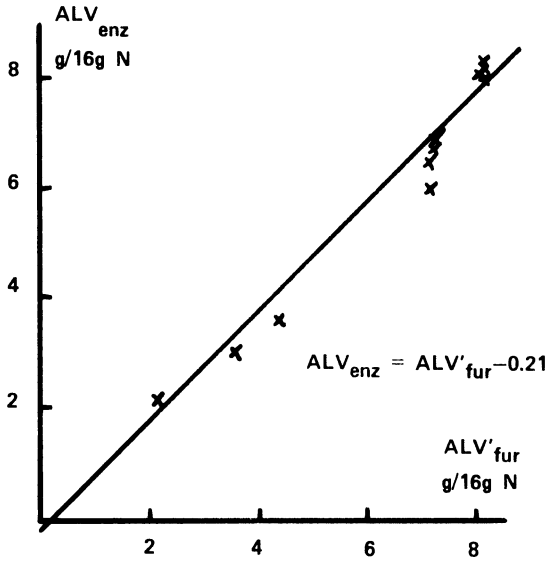


Fig. 12. Correlation between the available lysine values measured by the enzymatic method (ALV_{enz}) and calculated from the furosine content (ALV'_{fur}) = 8.2-3.09 FUR)

(11) $ALV'_{fur} = 1.00\ ALV_{enz} - 0.21 \quad (r = 0.98)$

F) Quantitative Evaluation of Schiff's Base ⇄ Aldosylamine

As the ∑ peaks after reduction corresponds to lysine in the form Schiff's base ⇄ aldosylamine plus the deoxyketosyl form, and as furosine is derived only from deoxyketosyl-lysine, it is possible to calculate the form Schiff's base ⇄ aldosylamine as the difference between the equivalent of ∑ peaks (1.06 ∑ peaks) and the deoxyketose (3.09 FUR) (equ. 12).

(12) Schiff's base ⇄ aldosylamine = 1.06 ∑ peaks - 3.09 FUR

It appears that in the group of samples tested, this form varies between 0.2 and 0.9 g/16 g N, that is to say between 2.5 and 10% of the theoretical lysine (Table 5). Since the formation of the

Table 5

Quantitative evaluation (g/16 g N) of Schiff base ⇄ aldosylamine of lysine in milk according to the acid hydrolysis and reduction methods

	Furosine content FUR	Deoxy-Keto-syl-Lysine = 3.09 FUR (A)	∑ peaks x 1.06 (B)	Schiff base ⇄ aldosyl-amine (B) - (A)
Untreated Milks				
RM	0	0	0.24	0.24
LM	0	0	0.31	0.31
SD	0	0	0.84	0.84
SM	0	0	0.73	0.73
Sterilized Milks				
SM_1	0.30	0.93	1.10	0.17
SM_2	0.31	0.96	1.15	0.19
SM_3	0.34	1.05	1.28	0.23
SM_4	0.34	1.05	1.55	0.50
Roller-dried Milks				
RD_A	1.24	3.84	4.71	0.87
RD_B	1.50	4.65	5.30	0.65
RD_C	1.95	6.05	6.41	0.36

Schiff's base ⇄ aldosylamine results from an equilibrium, the calculated values correspond only to the quantity stabilized by reduction in our conditions.

CONCLUSIONS

By using two chemical methods (acid hydrolysis and reduction by $NaBH_4$), model molecules and the method of enzymatic digestion as a reference for lysine availability, it has been shown that :

1. There exists in milk an available lysine-sugar linkage, probably the Schiff's base ⇄ aldosylamine form, varying between 2.5

and 10% of the theoretical lysine.

2. The deoxyketose form is the only blocked form of lysine in milks, and it may be estimated quantitatively from the amount of furosine of which it represents 32%.

ACKNOWLEDGMENTS

Sincere thanks are due to Dr. E. van Leemputten for preparing the complexes of lysine-polyaldehydes and Dr. L. Vuataz for the statistical analyses. The skilful technical assistance of Mrs. L. Dey and Mrs. R. Madelaine, Miss E. Magnenat, Mr. J.P. Corbaz, Mr. R. Deutsch and Mr. Ch. Dormond is gratefully acknowledged.

REFERENCES

Bobbitt, J.J. (1956). Periodate oxydation of Carbohydrates. In Advances in Carbohydrate Chemistry. M.L. Wolfrom and R.S. Tipson (Editors), Academic Press Inc., Publishers, New York, p. 1-41.

Brüggemann, J. und Erbersdobler, H. (1968). Fructoselysin als wichtigstes Reaktionsprodukt von Lysin mit Glucose bei Hitzeschädigung von Lebens- und Futtermitteln. Z. Lebensmittel-Untersuchung 137, 137-143.

Erbersdobler, H. (1970). Zur Schädigung des Lysins bei der Herstellung und Lagerung von Trockenmilch. Milchwissenschaft 25, 280-284.

Finney, D.J. (1952). Fieller's method cited p. 27 in Statistical Methods in Biological Assay. Griffin.

Finot, P.A., Bricout, J., Viani, R., and Mauron, J. (1968). Identification of a New Lysine Derivative obtained upon Acid Hydrolysis of Heated Milk. Experientia 24, 1097-1099.

Finot, P.A., Viani, R., Bricout, J., and Mauron, J. (1969). Detection and Identification of Pyridosine, a Second Lysine Derivative obtained upon Acid Hydrolysis of Heated Milk. Experientia 25, 134-135.

Finot, P.A., and Mauron, J. (1969). Le blocage de la lysine par la réaction de Maillard. I. Synthèse de N-(desoxy-1-D-fructosyl-1) et N-(desoxy-1-D-lactulosyl-1)-L-lysines. Helv. Chim. Acta 52, 1488-1495 /(1972) , II. Propriétés chimiques, id. 55, 1153.

Finot, P.A. (1973). Non-enzymic Browning. In Proteins in Human Nutrition: Ed. by Porter J.W.G. Rolls B.A., Academic Press London, p. 501-514.

Heyns, K., Heukeshoven, J., and Brose, K.H. (1968). Der Abbau von Fructose-Aminosäuren zu N-(2-Furosylmethyl) Aminosäuren : Zwischenprodukte von Bräunungsreaktionen. Angew. Chem. 80, 627.

Hodge, J.F. (1953). Chemistry of Browning. Reactions in Model Systems. Agr. Food Chem. 1, 928-943.

Hurrell, R.F., and Carpenter, K.J. (1974). Mechanisms of heat damage in proteins. 4. The reactive lysine content of heat-damaged material as measured in different ways. Br. J. Nutr. 32, 589-604.

Kern, W., Schulz, R.C., and Cherdron, H. (1960). Macromolecular polyacroleins. German Patent 1.082.054 (Cl. 39 c).

Mauron, J., Mottu, F., Bujard, E., and Egli, R.H. (1955). The availability of Lysine, Methionine and Tryptophan in condensed and Milk Powder. "In Vitro" Digestion Studies. Arch. Biochem. and Biophys. 59, 433-451.

Mottu, F., and Mauron, J. (1967). The differential determination of lysine in heated milk. II. Comparison of the "in vitro" methods with biological evaluation. J. Sci. Fd. Agric. 18, 57-62.

Mottu, F., Finot, P.A., Mauron, J., cited by Mauron (1970). Le comportement chimique des protéines lors de la préparation des aliments et ses incidences biologiques. J. Internat. Vitaminol. 40, 209-227.

Pigman, W., Cleveland, E.A., Couch, D.H., and Cleveland, J.H. (1951). Reactions of Carbohydrates with Nitrogenous Substances. I Mutarotations of some Glycosylamines. J. Am. Chem. Soc. 73, 1976-1979.

Polyanovskii, O.L. (1963). The synthesis and some properties of ε-N-Pyridoxyl Lysine. Biochemistry (URSS) 28, 751-754.

Sgarbieri, V.C., Amaya, J., Tanaka, M., and Chichester, C.O. (1973). Nutritional Consequences of the Maillard Reaction. Amino Acid Availability from Fructose-Leucine and Fructose-Tryptophan in the Rat. J. Nutr. 103, 657-663.

Weitzel, G., Geyer, H.U., and Fretzdorff, A.M. (1957). Darstellung und Stabilität der Salze von Aminosäure-N-Glykosiden. Chem. Ber. 90, 1153-1161.

24

THE BIOLOGICAL SIGNIFICANCE OF CARBOHYDRATE-LYSINE CROSSLINKING DURING HEAT-TREATMENT OF FOOD PROTEINS

Helmut F. Erbersdobler

Institute of Physiology, Physiological Chemistry and Physiology of Nutrition. Faculty of Veterinary Medicine, University of Munich, Veterinärstr. 13, D-8000 München 22

I. ABSTRACT

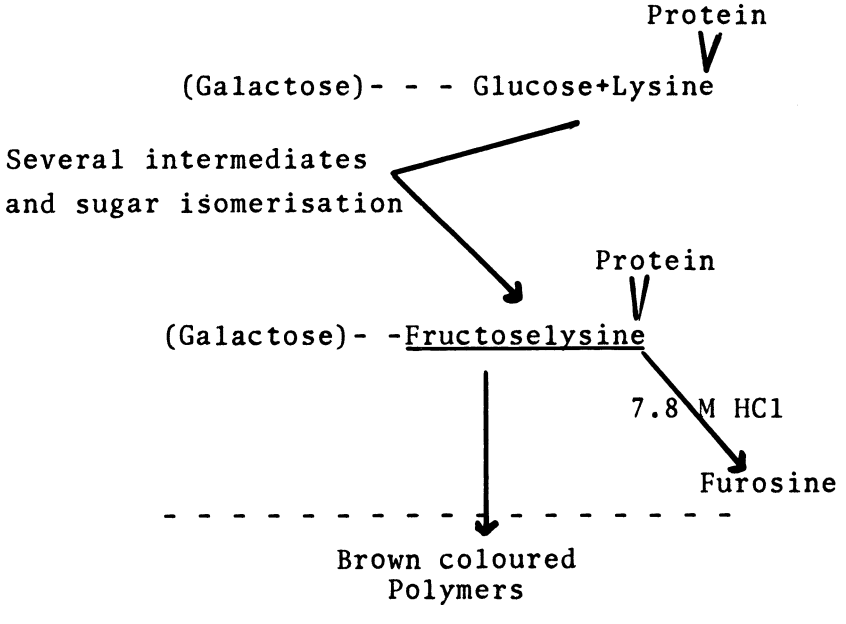
The heating of proteins in the presence of glucose or lactose results in the formation of fructoselysine or lactuloselysine (galactose-fructoselysine), in which the sugars are linked at the ε-amino group of lysine. Both compounds, which are very unstable to acid hydrolysis, can be estimated by analysing furosine, which is formed during the hydrolysis with strong hydrochloric acid. With this useful indicator it could be shown that the fructoselysine group occurs in considerable amounts in many heat-damaged foods.

Results of balance trials with casein containing radioactive labeled fructoselysine indicate a 30-40 % release of fructoselysine by digestion. Fructoselysine or lactuloselysine escaping the digestion and absorption are destroyed by the microorganisms in the hind gut. Free fructoselysine is not actively transported out of the intestine but absorbed by diffusion. Experiments with pregnant guinea pigs have shown a rapid penetration in large amounts through the placental wall. Our results indicate that fructoselysine does not interfere with the physiological and metabolic functions of the organism. The intestinal absorption of short-chain amino

acids seems to be affected by the presence of fructoselysine. Fructoselysine is rapidly excreted by the kidneys as an intact molecule

II. INTRODUCTION

The heating of proteins in the presence of reducing sugars results in the formation of lysine-sugar-complexes in the initial stage of the Maillard reaction. During storage or heat treatment of proteins with glucose or lactose under mild or moderate conditions firstly fructoselysine or lactuloselysine (galactose-fructoselysine) are formed. In these intermediate products the sugars are linked at the ε-amino-group of lysine, while the α-amino-group is bound in the protein structure. The fructoselysine-group itself is very unstable during the hydrolysis of proteins. But it can be estimated by analysing furosine (ε-N-(2-furosyl-methyl-)-L-lysine) which is formed at a constant rate during the hydrolysis with 7.75 M hydrochloric acid (Erbersdobler, 1970). We detected this compound ten years ago (Erbersdobler and Zucker, 1966) and identified it as a secondary product of fructoselysine or lactuloselysine. Heyns et al. (1968) and Finot et al. (1968) determined the structure of the compound and named it furosine. The following scheme shows the initial steps of the Maillard reaction as well as the formation of furosine during the hydrolysis.



III. FRUCTOSELYSINE IN FOOD PROTEINS

In our calculations we have found (Erbersdobler, 1970) that during the hydrolysis of milk products 50 % of the fructoselysine-group are split to lysine, 40 % are transformed to furosine, whereas the rest passes into some other intermediates (see also Erbersdobler, 1976). Using these relations, a calculation of the amount of fructoselysine in heat-damaged proteins can be made. These calculations were applied to a number of furosine values from different foods. The results are given in table 1.

Table 1

The lysine and furosine contents in several food proteins (g/16 g N) and the calculated amounts of available lysine and lysine-sugar complexes (given as fructoselysine) in g/100 g of the dry matter

	No. of samples	in g/16 g N		calc.mean values, g in 100 g dry matter	
		lysine	furosine	available lysine	Fructoselysine
Milk	11	9.1±0.2	0	2.4	0
Sterilized milk	7	8.1±0.6	0.1±0.1	2.1	0.1
Condensed milk	11	7.7±0.3	0.8±0.2	1.9	0.6
Spray dried skim milk	35	8.5±0.5	0.4±0.3	3.0	0.4
Roller dried skim milk	42	8.2±0.5	0.6±0.4	2.8	0.7
Spray dried whey	33	8.3±0.3	0.3±0.3	1.2	0.2
Roller dried whey	16	5.9±1.2	2.7±0.8	0.5	1.1
Whey concentrates	47	7.4±1.2	1.1±1.2	1.6	0.8
Baby food	80	8.0±0.6	0.5±0.4	1.1	0.2
Chocolate	7	5.8±0.9	1.3±0.6	0.4	0.3

As can be seen from table 1, some foods contained considerable amounts of inactivated lysine. Thus this new analytical possibility has shown that fructoselysine is the most important reaction product during the heat damage of foods under mild or moderate conditions. These surprising results led us to the decision to examine whether this product of lysine crosslinking was biologically active or not.

IV. STUDIES WITH PROTEIN BOUND FRUCTOSELYSINE

In order to measure the utilisation of protein-bound fructoselysine, we made balance trials with male rats weighing about 300 g (Erbersdobler, 1975). A protein containing labeled fructoselysine was obtained by heating casein with 1-^{14}C-glucose and an intensive washing procedure afterwards. After feeding the labeled casein in a single meal the urine and the respiratory gases were collected for 48 hours and analysed. The results are given in figure 1.

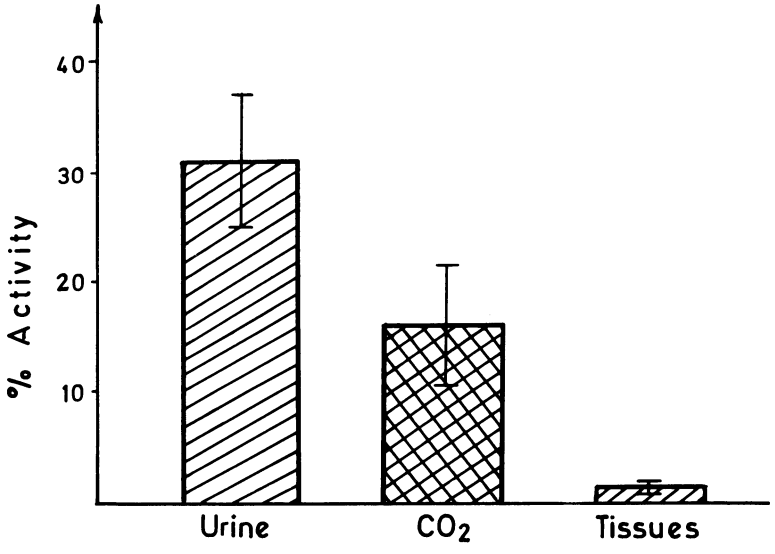


Fig. 1. Metabolism of protein bound fructoselysine in the rat. ^{14}C-activity in urine, in respiratory CO_2 and in the tissues after a test meal containing labeled fructoselysine. Activity in the test meal = 100% (n = 8 rats).

Generally the substitution of the ε-amino group of lysine with fructose seems to hinder the action of trypsin and possibly some other enzymes (e.g. Folk, 1956). The results of our balance trials given in figure 1 indicate a 30 - 40 % release by digestion and subsequent absorption of the protein-bound fructoselysine, which was excreted in an unchanged form in the urine. These results are in good agreement with the data of Mori (B. Mori, 1976) who found 34 % of the ingested radioactivity from unavailable lysine 22 hours after a test meal of ^{14}C-lysine labeled casein browned by reaction with glucose. In the experiments of Mori (1975 and 1976) 50 - 60 % of the radioactivity in the urine could be identified as fructoselysine while the rest were other ninhydrine positive or negative fractions. In our results nearly all of the radioactivity in the urine could be attributed to fructoselysine. These differences may be due to our different labeling methods of casein.

The relatively high amounts of ^{14}C-carbon dioxide found in the experiments given in figure 1 may result from the degradation of undigested fructoselysine by the microorganisms in the intestinal tract. Mori also found a higher rate of respiratory radioactivity than could be expected from the metabolism of lysine, which in his model system had not reacted with glucose. Actually many results indicate that fructoselysine which is not digested reaches the hind gut and can there be destroyed and presumably utilized by the microorganisms. Our in vitro experiments incubating protein containing a great amount of fructoselysine with gut contents of rats and pigs (Erbersdobler et al., 1970) have proved that the microorganisms of the digestive tract are able to deaminate these undigested compounds. The recovery of fructoselysine in the faeces of animals with an intact intestinal flora is generally low (Brüggemann and Erbersdobler, 1968; Finot, 1973; Mori, 1976), while we could find great amounts of fructoselysine in the droppings of germfree chicks (unpublished data).

Lactuloselysine is only poorly digested. The results of Ford and Shorrock (1971) lead to the conclusion that less than 10 % of the lactuloselysine in a sample of heat damaged milk-protein were digested and absorbed. Finot (1973) recovered only 6 - 9 % of the ingested lactuloselysine in the urine, but in the form of fructoselysine, whereas the faeces contained free fructoselysine, lactuloselysine and peptides with lactuloselysine. The total recovery of the blocked lysine was small (12 - 30 %).

V. STUDIES WITH FREE FRUCTOSELYSINES

Our experiments were carried out with radioactive labeled fructoselysine, which was prepared by reaction of glucose together with U-^{14}C-L-lysine. The protection of either the α- or ε -amino group of lysine by previous formylation (Finot and Mauron, 1972) leads to either ε-fructoselysine or α-fructoselysine. α-fructoselysine can be found in heated foods which originally contained free lysine. In both cases we obtained highly purified samples by a preparative ion exchange chromatography with pyridine-acetic acid buffer and subsequent freeze-drying of the fractions.

Fructoselysine is not split in the course of digestion (Finot, 1973). In all our experiments with labeled fructoselysine we could detect free lysine only in traces. An incubation at 37 °C of a heat-damaged protein with pepsin in 0.05 N hydrochloric acid for 24 hours and subsequently with papain at pH 6.5 for another 24 hours showed great amounts of furosine in the TCA-soluble fraction after the digestion. From these data could be estimated that the TCA-soluble peptides contained the greater part of the fructoselysine from the heat-damaged protein.

Fructoselysine is not actively transported out of the intestine. An uptake by diffusion, however, is possible. Compared to lysine, ε -fructoselysine is absorbed at a slower rate, whereas α-fructoselysine is only poorly absorbed (Erbersdobler et al., 1974; see also Erbersdobler, 1976). The distribution of the absorbed fructoselysine also happens by diffusion. In this way fructoslysine also reaches the fetus through the placental wall. Figure 2 shows results of experiments with pregnant guinea pigs, which show similar placental conditions as women. The behaviour of ε-fructoselysine was compared with that of mannitol which is known to be non-metabolisable, exclusively distributed in the extracellular fluid and not reabsorbed in the kidneys. The concentrations of ε -fructoselysine in the fetuses were much higher than those of mannitol. This shows that ε -fructoselysine penetrates the cellular membranes more rapidly than mannitol (Erbersdobler et al., 1974). α -fructoselysine is transported at a low rate through the placental wall (unpublished results).

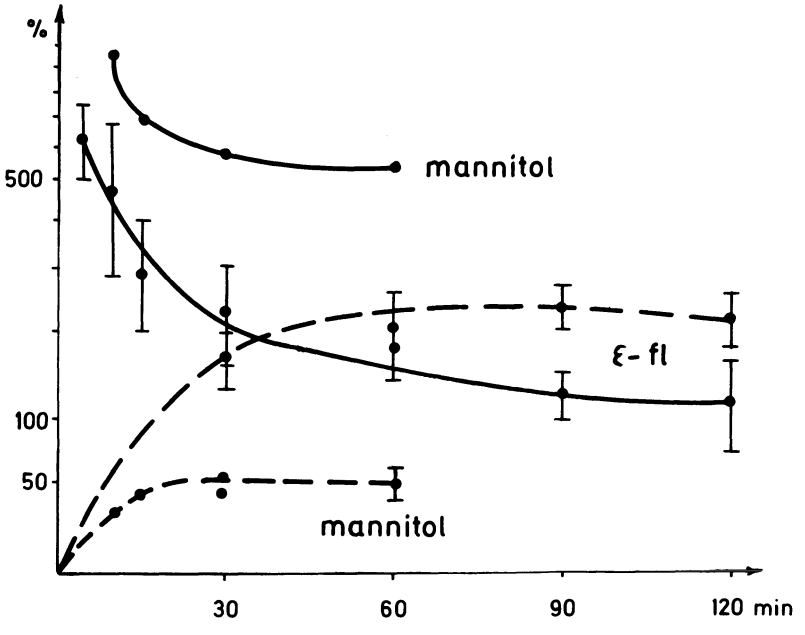


Fig. 2. Transport of ε-fructoselysine through the placental wall in guinea pigs.
Concentrations of fructoselysine and mannitol in 1 ml plasma of the maternal (——) and fetal (- - -) blood. The intravenously injected activity/g of the animal ($6\text{-}9 \times 10^{-9}$ Mol with a specific activity of 25 mCi/mol)=100.

Since an active reabsorption does not take place in the kidneys either, the excretion will occur most rapidly. Table 2 shows results of balance trials with rats, comparing the excretion of ^{14}C-labeled ε-fructoselysine and α-fructoselysine with the excretion of ^{14}C-α-aminoisobutyric acid and ^{14}C-mannitol. The fructoselysines were excreted in a similar manner as mannitol whereas α-aminoisobutyric acid was reabsorbed to a large extent. In the case of the fructoselysines all of the radioactivity in the urine could be attributed to the intact fructoselysine molecule. Less than 5 % could be found as labeled free lysine.

Table 2

Recovery of ^{14}C-activity 6-48 hours after the intravenous injection of labeled fructoselysines compared to mannitol and α-amino isobutyric acid (AIBA). Injected activity (0.6-0.9 μmoles with a specific activity of 25 mCi/mol per 100 g of rat) = 100 per cent.

	ε-fructose-lysine (n=11)	α-fructose-lysine (n=6)	manni-tol (n=6)	AIBA (n=6)
in the urine (cumulative)				
6 hours	75±6	81±2	74±2	4±1
12 hours	79±6	83±2	77±2	9±2
24 hours	81±6	86±2	79±2	15±2
48 hours	82±6	88±2	80±2	25±2
in the faeces (up to 48h)	3±1	3±1	10±2	4±3
gut content	1±1	2±1	5±2	9±1
other organs	1	2	1	14
sum	86	95	96	52

The utilization or metabolism of fructoselysine, if they really exist, tend to be extremely low. Figure 3 shows results of our balance experiments (Erbersdobler and Schlecht, unpublished) with rats. After an intravenous injection of ^{14}C- ε-fructoselysine only traces of ^{14}C-carbon dioxide could be found, which seem to originate mainly from a microbial degradation of fructoselysine secreted into the intestine. These results obtained with the pure and radioactive labeled lysine-carbohydrate compound confirm the data and suggestions of others (e.g. Valle-Riestra and Barnes, 1970).

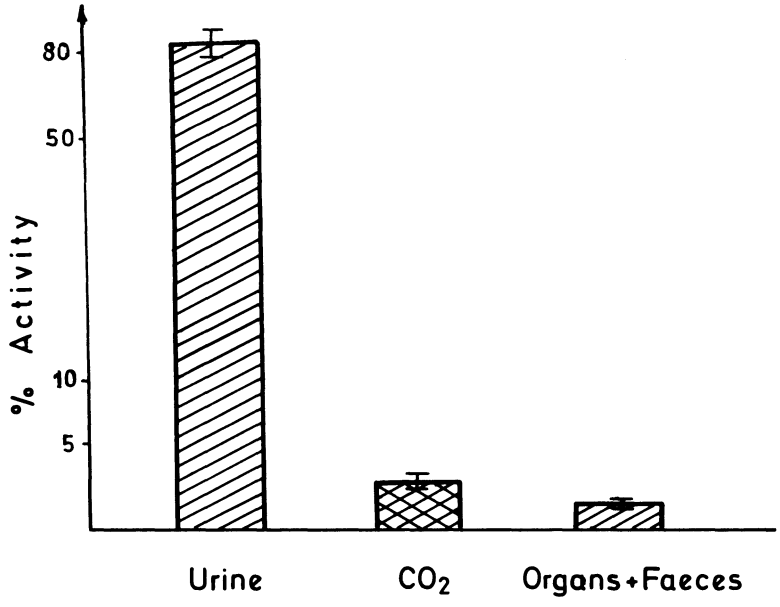


Fig. 3. Metabolism of ε-fructoselysine, ^{14}C-activity in urine, CO_2 and in organs + faeces until 24h after an intravenous injection of ^{14}C- ε-fructoselysine. Injected activity (6.2 µMol with a specific activity of 60 mCi/mol per 100 g of rat) = 100 per cent (n = 4).

Fructoselysine does not seem to be harmful to the organism. As preliminary results (Brandt and Erbersdobler, unpublished) indicate, fructoselysine, although not metabolized, does not interfere with physiological and metabolic functions like gluconeogenesis. Following the suggestions of Buraczewski and Ford (1967) who postulated that some peptides or peptide-like compounds might interfere with amino acid absorption we tested the active transport of some amino acids in the presence of ε-fructoselysine. Some of the results (Summerer, 1976) are given in table 3. Our results show that in the presence of fructoselysine the transport rates of threonine, proline and glycine were decreased while those of lysine, methionine and galactose were not influenced. The mechanism of this effect and the practical importance of these findings must be elucidated by further studies.

Table 3

Influence of ε-fructoselysine on the transport of several amino acids by everted sacs of the upper jejunum of rats

substrate concentration	n	s/m concentration gradients[+] without fructoselysine	s/m concentration gradients[+] with 1mM fructoselysine	significant[++]
0.1 mM Threonine	8/10	4.7±1.3	2.9±0.7	$2\alpha < 0.01$
0.1 mM Proline	15/22	6.5±1.8	4.3±1.2	$2\alpha < 0.01$
0.1 mM Glycine	15/14	2.3±0.5	1.9±0.2	$2\alpha \simeq 0.05$
0.1 mM Lysine	15/15	15.8±5.2	16.6±4.9	n.s.
0.1 mM Methionine	10/10	10.8±3.8	12.3±4.1	n.s.
0.1 mM Galactose	9/8	3.2±0.5	3.1±0.6	n.s.

[+] ratio between concentration of serosal and mucosal medium.

[++] Wilcoxon test

The experiments with fructoselysine show that ε-fructoselysine can be regarded as a perfect model of an unavailable amino acid which is slowly digested and absorbed, not utilized by the organism and soon excreted without undergoing any changes. It can be stated that fructoselysine passes the organism as an indifferent compound. In any case this lysine-carbohydrate crosslinking causes a decrease in the lysine availability and thus to a great extent influences the protein quality of foods. Moreover this lysine-crosslinking can reduce the availability of amino acids in general by disturbing the protein digestion (and possibly the absorption of several amino acids) as a whole. In order to elucidate the behaviour of other lysine carbohydrate complexes and products from later stages of the Maillard Reaction, further experiments are necessary.

REFERENCES

Brüggemann, J. and Erbersdobler, H. (1968). Fructoselysin als wichtigstes Reaktionsprodukt von Lysin mit Glucose bei Hitzeschädigung von Lebens- und Futtermitteln. Z. Lebensmittel-Unters. u. -Forschung 137, 137.

Buraczewski, S., Buraczewska,L. and Ford, J.E. (1967). The influence of heating of fish proteins on the course of their digestion. Acta biochem. polon. 14, 121.

Erbersdobler, H. (1970). Zur Schädigung des Lysins bei der Herstellung und Lagerung von Trockenmilch. Milchwissenschaft 25, 280.

Erbersdobler, H. (1975). The biological significance of fructoselysine. Paper at the Xth Int. Congr. Nutr. , Kyoto, Japan, Abstract No. 5316.

Erbersdobler, H. (1976). Amino Acid Availability. In: Protein Metabolism and Nutrition (D.J.A. Cole et al, ed.), Butterworths, London-Boston.

Erbersdobler, H., Gunsser, I. and G. Weber (1970). Abbau von Fruktoselysin durch die Darmflora. Zbl. Vet.Med. A 17, 573.

Erbersdobler, H., Husstedt, W., Alfke, F., Brandt,A. and Chelius, H.H. (1974). Intestinale Resorption, Verteilung und diaplacentarer Übergang von Fructoselysin. Z. Tierphysiol., Tierern., Futtermittelkde. 33, 202.

Erbersdobler,H. and Zucker, H. (1966). Untersuchungen zum Gehalt an Lysin und verfügbarem Lysin in Trokkenmagermilch. Milchwissenschaft 21, 564.

Finot, P.A. (1973). "Non-enzymic browning". In: "Proteins in human nutrition" (Porter, J.W.G. and Rolls,B.A., eds.), p. 501, Academic Press, London-New York.

Finot, P.A., Bricout, J., Viani, R. and Mauron, J. (1968). Identification of a new lysine derivate obtained upon acid hydrolysis of heated milk. Experientia 24, 1097.

Finot, P.A. and Mauron, J. (1969). Le blocage de la lysine par la reaction de Maillard I. Synthese de N-(desoxy-1-D-fructosyl-1)-et N-(desoxy-1-D-lactulosyl-1)-L-lysines. Helv. Chim. Acta 52, 1488.

Folk, J.E. (1956). The influence of the lysine-glucose reaction on enzymatic digestion. Arch. Biochem. Biophys. 64,6.

Ford, J.E. and Shorrock, C. (1971). Metabolism of heat-damaged protein in the rat. Influence of heat-damage on the excretion of amino acids and peptides in the urine. Br. J. Nutr. 26, 311.

Heyns, K., Heukeshoven, J. and Brose, K.H. (1968). Der Abbau von Fruktoseaminosäuren zu N-(2-Furoylmethyl)-Aminosüuren. Zwischenprodukte von Bräunungsreaktionen. Angewandte Chemie 80, 62.

Mori, B. (1975). On intestinal absorption delay of browned labeled casein by amino-carbonyl reaction. Paper at the Xth Int. Congr. Nutr., Kyoto, Japan. Abstract No. 5228.

Mori, B. (1976). Utilisation in rats of ^{14}C-1-lysine labeled casein browned by amino-carbonyl reaction. Paper at the Faculty of Veterinarian Medicine in Munich, 13.7.1976.

Summerer, F. (1976). Beeinflussung des intestinalen Aminosäuren- und Monosaccharidtransports durch Fruktoselysin. Dissertation, University of Munich, Fac. Vet.Med.

Valle-Riestra, J. and Barnes, R.H. (1970). Digestion of heat-damaged egg albumen by the rat. J. Nutr. 100, 873.

(Supported by: Deutsche Forschungsgemeinschaft).

25

THE PHYSICAL ASPECTS WITH RESPECT TO WATER AND NON-ENZYMATIC BROWNING

Theodore P. Labuza, Rita M. Warren,
and Henry C. Warmbier

University of Minnesota
Department of Food Science and Nutrition
St. Paul, Minnesota 55108

I. ABSTRACT

Non-enzymatic browning is one of the major problems that occurs during the processing and storage of dehydrated and semi-moist foods. One type of browning is the Maillard reaction between reducing sugars and proteins or free amines. This leads to a darkening of color, protein insolubility with subsequent possible nutrition loss and a bitter off-flavor. The reaction has been extensively researched since the early 1940's. From a storage standpoint, browning rate increases as water activity (a_w) increases up to a maximum where reactant dilution causes a subsequent decrease in rate. Use of liquid humectants in high moisture foods increase phase volume and viscosity, thereby lowering the a_w of the rate maximum. These humectants thus act as inhibitors at high a_w. Sorbitol also decreases the rate by a viscosity effect. Kinetic studies at normal storage conditions show browning to occur by a zero order reaction although the sugar and amine initial reaction occurs by first order. Studies with Tetrahymena show that the initial Schiff's base and reaction products may be partially biologically available although chemical assays show up to 50% loss of protein value. For dehydrated foods the zero order kinetics can be used to predict shelf life under a variable time-temperature-humidity condition.

II. INTRODUCTION

One of the most important reactions that can occur in dehydrated or semi-moist foods is the Maillard non-enzymatic reaction. This results from the reaction of reducing sugars with proteins or

free amine groups and can result in changes in the chemical and physiological properties of proteins and consequently, a change in nutritional value as well.

The influence of the water content on the browning reaction is important to the food industry. Product formulation and processing as well as storage conditions in the case of dehydrated and semi-moist foods, may affect appearance, flavor, and more important, the nutritional quality of the protein. The role of water and the use of various ingredients such as liquid humectants, must be considered in the factors affecting non-enzymatic browning rates and kinetics. An understanding of these factors would be useful in controlling the shelf-life of susceptible products.

III. DEFINITION OF THE STATE OF WATER IN FOODS AND ITS EFFECT ON KINETICS

Water not only contributes to the textural characteristics of a food through its physical state but its interaction with food components is directly related to the chemical reactions that can take place (Labuza, 1971, 1975; and Rockland, 1969).

The term by which the interaction is quantified is called the water activity of the food which is a measure of the relative vapor pressure of water above the food. This is given in equation (1):

$$a_w = P/P_o = \frac{\%RH}{100} \qquad (1)$$

a = water activity
P = vapor pressure above food at Temperature T
P_o = vapor pressure of pure water at T
%RH = equilibrium relative humidity at which the food neither gains nor loses water

Water activity or relative humidity is related to the moisture content of the food through the sorption isotherm as seen in Figure 1. Methods to determine isotherms and measure water activity have been reviewed by Labuza, 1968, 1974, 1975; and Gal, 1975. The lower part of the curve in Figure 1 is the region of dehydrated foods (a_w 0 to 0.5) and the upper part (a_w 0.6 to 0.9) applies to semi-moist products. Most natural tissue foods such as meats, fish, vegetables and fruits have an $a_w \approx 1.0$ and moisture contents greater than 60% water.

To understand the effect of a_w on non-enzymatic browning one must examine the degree of boundness of water. At low a_w water is tightly bound to surface polar sites by chemi-sorption

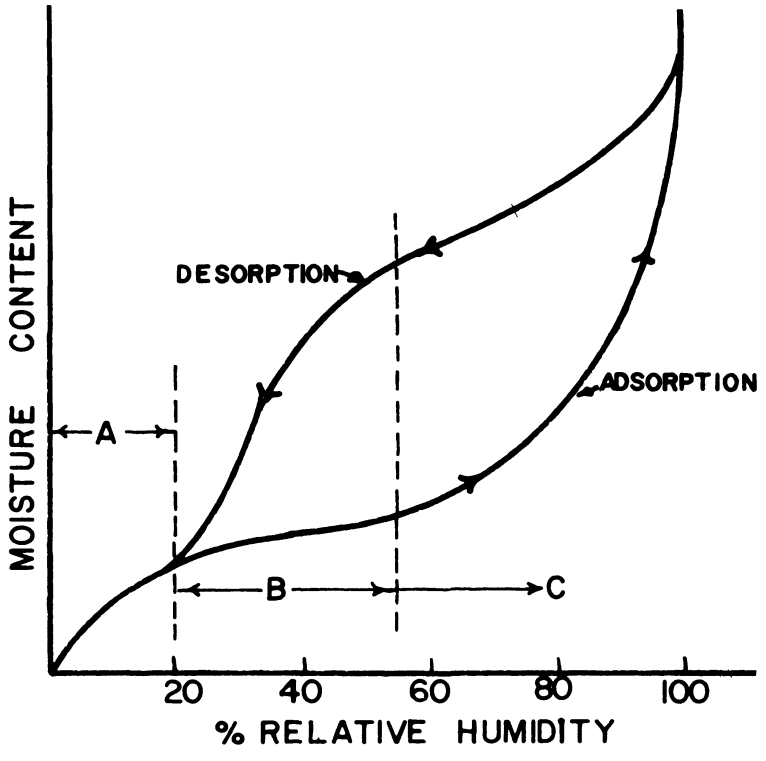


FIGURE 1

Moisture Sorption Isotherm For A Food Product Showing Sorption Hysteresis
(moisture content vs. % equilibrium relative humidity)

and is generally unavailable for reaction and solution. The upper limit of this region is called the BET-monolayer value which occurs at about a_w 0.2 to 0.3 for most foods. This value is the most stable moisture content for most dehydrated foods (Salwin, 1959).

Above the monolayer the water is held to a varying degree in multilayers; in capillaries, and possibly entrapped in various structural components. In addition, dissolved solutes reduce the freedom of evaporation of water due to colligative properties

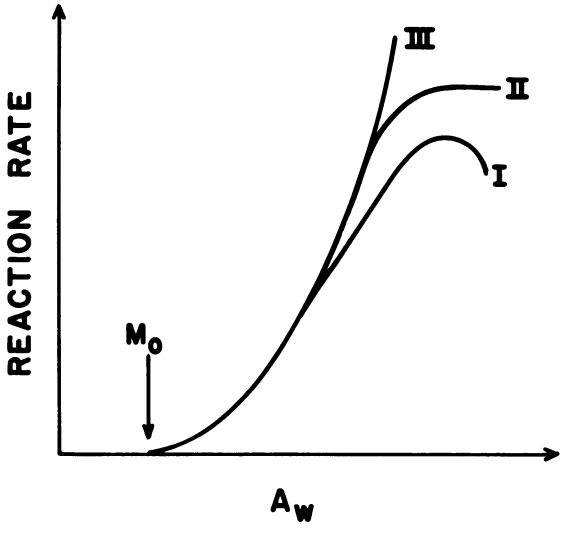


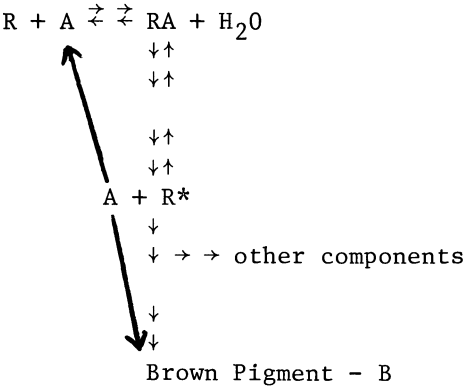
FIGURE 2

Effect of Water Activity on Relative Reaction Rates: I - Continuous Increase; II - Leveling Off; III - Maxima as in Browning (M_o is BET monolayer)

as defined by Raoult's Law. All these factors account for a reduction in the relative vapor pressure of water but do not completely inhibit its ability to act as a solvent and reaction medium as well as a reactant itself (Labuza, 1975). Because of this, many deteriorative reactions increase exponentially in rate as the a_w increases above the monolayer. With some reactions however, the rate may level off at high a_w or even decrease again. This is due to thermodynamic, dilution and possible viscous effects (Lee and Labuza, 1975). These effects of a_w on rate are summarized in Figure 2. Non-enzymatic browning generally follows the pattern shown by condition I i.e., a rate maximum occurs as a_w increases and then the rate falls. This is most likely due to three factors. In browning about three moles of water are produced per mole of carbohydrate used (Eichner and Karel, 1972). Thus water can act through product inhibition by retarding the formation of the initial glycosylamine reaction. However, water may enhance deamination reactions later on in the sequence as shown by Reynolds (1963).

In addition, and probably more importantly, dilution of reactive components as water content increases can occur (Eichner, 1975; and Labuza, 1971). Thirdly, the aqueous phase becomes less viscous allowing faster mobility. The first two factors obviously over-compensate for a decreased medium viscosity so the rate decreases after the maximum.

The initial browning reaction which produces brown pigment precursors can be viewed as an overall second order mechanism such as:



R = reducing sugar concentration (moles/liter)
A = reactive amine group concentration (moles/liter)
RA = intermediates
R* = reactive reducing compound intermediates
B = brown pigment

The rate of brown pigment formation is related to the formation of R* by:

$$\frac{dR^*}{d\Theta} = k_1 (R)^a (A)^b \qquad (2)$$

where: k_1 = overall rate constant, depends on a_w and inversely is proportional to phase viscosity

The exponential factors a and b are usually considered to be equal to one (first order destruction). Thus as a_w increases from the BET value (R) and (A) will initially increase as more reactants can be dissolved above the crystallization point (Sloan and Labuza, 1975).

Once the aqueous phase is saturated and no more reactants can dissolve, concentrations of R and A will decrease continuously as the water content increases. In fact, in the intermediate moisture food (IMF) range for most foods, a change in a_w from 0.7 to 0.9 can mean a doubling to tripling of the water content

and thus a concomitant decrease per gram of solids in reactant concentration. If no change in viscosity occurs (i.e., k_1 remains constant), this can mean a four to nine fold decrease in the rate. As noted however, as water content increases the viscosity of the aqueous phase decreases allowing greater reactant mobility. This would increase the value of k since it is inversely proportinal to viscosity. Lee and Labuza, 1974, using NMR T_1 measurements have shown a two fold decrease in phase viscosity going from the BET monolayer to an a_w of 0.9. This should proportionally double the rate. But as previously noted, the concentration effect is greater and the possible local product inhibition of water occurs. Thus a rate maximum occurs with a large drop in the rate in the higher IMF water activity range following the type I reaction pattern. This has been found for many dry foods humidified to different water activities such as seen for dry pork in Figure 3 (Lee and Hannan, 1949; Loncin et al., 1965; Labuza, 1970, 1971; Sharp, 1957; Sharp and Rolfe, 1958; and Hendel et al., 1955).

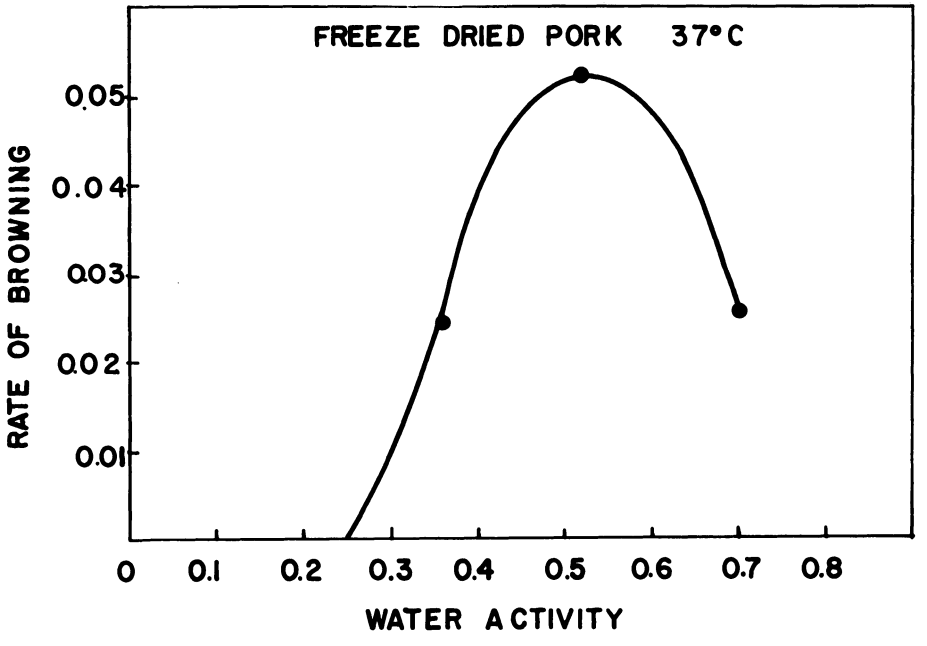


FIGURE 3

Rate of Nonenzymatic Browning as a Function of Water Activity of Pork (Labuza, 1973)

The browning reaction can occur as an enzymatic process (Eskin, 1971; Greenshields and Macgillivary, 1972), such as that which occurs in fresh cut fruits and vegetables. Tissue damage significantly enhances the rate of enzymatic browning. The injured tissue rapidly darkens upon exposure to air due to the conversion of phenolic compounds to melanins. Polyphenol oxidase, phenolase, o-diphenol oxidase, and tyrosinase are all names given to the enzyme systems that catalyze the hydroxylation and oxidation of phenolic compounds. These compounds subsequently polymerize to form the brown melanoidin pigments which do not involve proteins. Enzymatic browning can be controlled by blanching, pH adjustment, sulfiting or oxygen exclusion.

In general, there are three major pathways for non-enzymatic browning: carmelization, ascorbic acid oxidation, and the Maillard reaction.

Carmelization is that browning reaction in which sugars, in the absence of amino acids or proteins, are heated above their melting point at which monosaccharides are converted into the 1,2-enol form. At high temperature and low pH hydroxymethyl furfural (from hexoses) or furfural (from pentoses) are formed. Under basic conditions, three carbon and/or two carbon fragments are formed. Caramel, a complicated family of pigments, is the end-product of the overall carmelization reaction. Commercially, caramels are prepared by heating concentrated solutions of carbohydrates with ammonia or ammonia salts. Carmelization can be prevented by preventing the exposure of sugars to high temperature.

Ascorbic acid (vitamin C) oxidation is another type of browning reaction. The reaction is catalyzed by low pH and elevated temperatures and converts ascorbic acid to dehydroascorbic acid, which quickly oxidizes to 2,3-diketo-gulonic acid. Ultimately, furfural and carbon dioxide are formed. In total, some 17 decomposition products of ascorbic acid have been identified (Hermann and Andrae, 1963). Polymerization of some or all of the decomposition products produces the typical brown discoloration. Ascorbic acid oxidation is important since some animals, including man, cannot synthesize vitamin C. Prevention of oxidation of ascorbic acid in foods is accomplished by avoiding exposure to high temperatures and, to a limited extent, by excluding oxygen and avoiding neutral pH conditions.

The Maillard reaction is the third type of non-enzymatic browning. It occurs in foods which contain certain reducing sugars, and free amino groups, and/or protein. The Maillard reaction is a major cause of browning developed during the heating or prolonged storage of many dehydrated and intermediate moisture foods resulting in loss of protein biological value.

The Maillard browning reaction was first reported in 1912 by the French chemist Maillard (Maillard, 1912). He observed the formation of brown melanoidin pigments during the heating of a solution of the amino acid glycine and the reducing sugar glucose. Since that time, many researchers have studied the mechanism and control of the Maillard browning reaction. A classic series of experiments was conducted by Lea and his co-workers (Lea and Hannan, 1949, 1950a, 1950b; Lea et al., 1951; Hannan, and Lea, 1952; and Lea and Rhodes, 1952). Subsequently, several in-depth reviews covering the subject have been written (Hodge, 1953, 1967; Lea, 1958; Ellis, 1959; Reynolds, 1963, 1965; and Bender, 1972).

IV. MECHANISM OF THE MAILLARD REACTION

There are three phases to the mechanism of the Maillard browning reaction. Figure 4 represents the overall pathway. Maillard browning as elucidated by Hodge (1953). As one can see from the diagram, the first step involves a condensation reaction between the free amino group and the carboxyl group of a reducing sugar. The amino group can be either a free amino acid or may be attached in a protein such as lysine. The initial product, a Shiff's base, undergoes cyclization to the corresponding N-substituted glycosylamine. Subsequently acidic conditions catalyze the isomerization of the parent aldose to a ketone. This isomerization reaction is known as the Amadori rearrangement reaction. The end-product of this rearrangement reaction is a 1-amino-1-deoxy-2-ketose derivative. This derivative and its immediate precursors are colorless. Therefore, this initial step of the Maillard browning reaction contributes no brown discoloration as has been verified by Eichner (1975). This first phase of the Maillard reaction is also reversible.

A reducing sugar is required for glycosyl-amine formation. Both mono- and di-saccharide reducing sugars can react, with order of reactivity being aldopentoses greater than aldohexoses greater than disaccharides (Spark, 1969). As seen in Table 1, if a non-reducing disccharide containing a reducing mono-saccharide moiety undergoes hydrolysis, the reducing moiety can participate with the protein in browning reaction (Karel and Labuza, 1968).

Lea et al., (1951) experimentally showed that once the initial glycosyl-amino condensation and Amadori reactions occur, removal of free reducing sugar will not inhibit the development of the brown pigments. Eichner (1975) has shown that in fact, the Amadori rearrangement products will brown at a lower water activity if removed from the system (Figure 5).

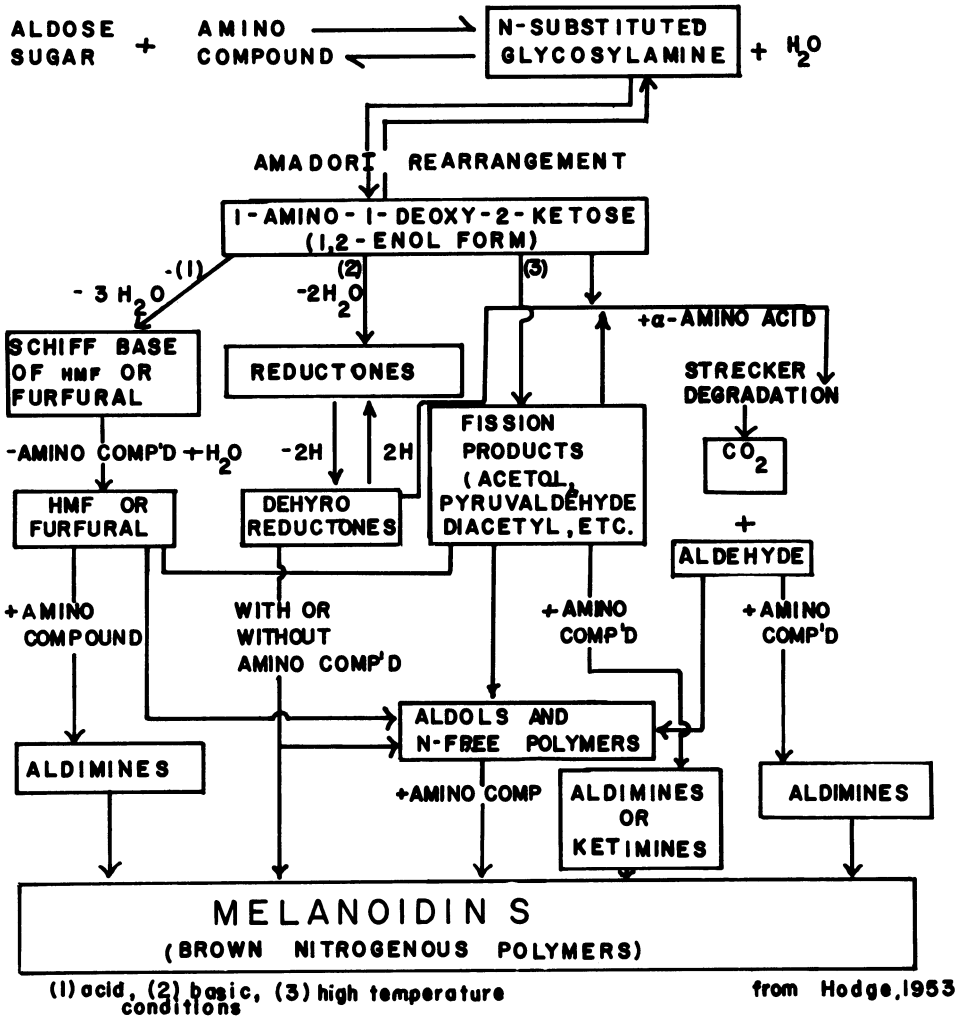


FIGURE 4

Maillard Browning Pathway

Table 1

Sucrose Hydrolysis and Browning *

Time Days	System A ** a_w 0.10 % Hy	OD/g x 100	0.31 % Hy	OD/g x 100	0.75 % Hy	OD/g x 100
0	0.9	0.2	0.9	0.2	2.4	0.2
3	1.1	-	4.8	-	11.9	-
7	0.9	0.2	15.9	0.3	21.7	0.7
10	2.9	0.2	14.4	0.4	16.3	0.8
14	2.8	0.2	15.6	1.8	25.8	2.1
17	2.8	0.2	18.1	4.2	40.4	4.8

* Karel and Labuza, 1968
** sucrose, citric acid, egg albumin, water

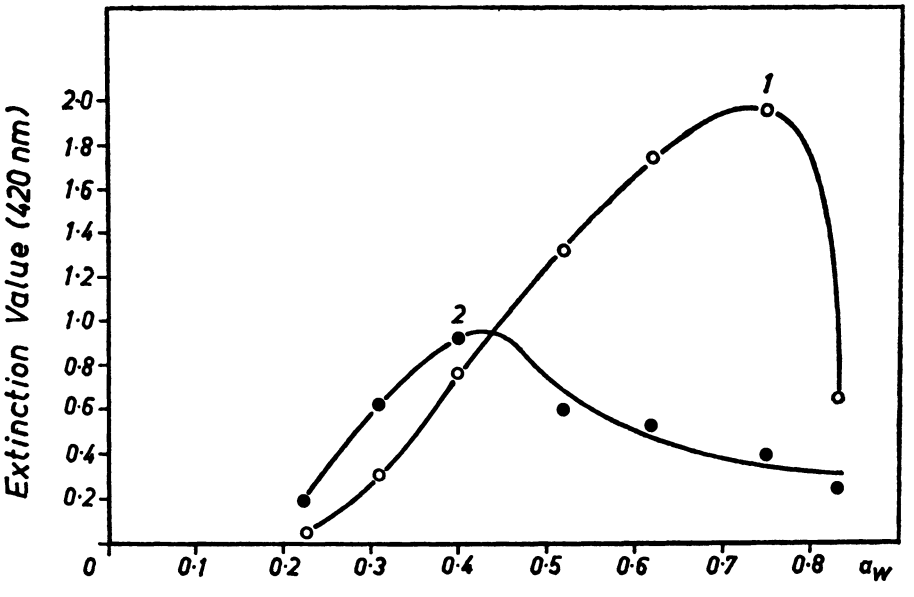


FIGURE 5

Browning rates for a glucose-lysine-cellulose model system (1) as a function of a_w and for the intermediate products extracted from the system humidified to similar a_w's.

Eichner (1975)

Amino groups must be chemically free to participate in the Maillard browning reaction. Primary amines react the most rapidly. Peptide bonded nitrogen and acetylated terminal amino groups do not react. Lea and Rhodes (1952) showed that the rate of the Van Slyke free nitrogen loss, as an indication of initial browning rate, was greater when 2-amino-D-glucose reacted with N-acetylated casein than with regular casein. Color development was approximately the same in both systems. Hence, once the initial glycoslyamine condensation product is formed, excess free amino groups apparently cannot readily enhance the browning rate.

The second or intermediate reaction sequence involves the removal of the amino group(s) from the sugar moiety (except as advocated by Song and Chichester, 1966). This is followed by dehydration and cyclization, fragmentation, or amine condensation. Three general pathways exist for this intermediate reaction sequence. First, under acidic conditions, dehydration and cyclization produces hydroxymethyl furfural (from hexoses) or furfural (from pentoses) with a possible regeneration of the amine. These two products are colorless, but their subsequent oxidation to γ-unsaturated decarboxyl compounds yields yellow colored products (Taher and Cates, 1974). Second, under basic conditions the 2-keto form of the glycosyl parent equilibrates predominantly to the 1,2-enol glycosyl form. Rearrangement to the 2,3-enol form followed by dehydration and oxidation yields reductones and dehydroreductones. The dehydroreductones can combine with α-amino acids yielding carbon dioxide, aldehydes, and amino-keto derivatives. This reaction is commonly referred to as the Strecker degradation (Schonberg and Moubacher, 1952). Third, conditions of elevated temperature can produce fragmentation products from the Amadori product. Three or four carbon aldehydes, alcohols, acids or ketones may be produced. These compounds produce the characteristic flavor and odor associated with heat catalyzed Maillard browning, as, the 2- and 3-methylbutanal and 2-methyl propanal formed in drying of potatoes (Sullivan, et al., 1974).

The third and final phase of the Maillard browning reaction produces the brown melanoidin pigments. Polymerization of the products from the second phase and copolymerization with amino compounds and proteins yields the colored products. The chemistry compounds will react with amines to give polymerized water-soluble brown pigments; furfural compounds will polymerize with amines to give water insoluble brown products (Reynolds, 1965). Heterocyclic amines are thought to be formed to cause the brown color development. These reactions definitely lead to toughening of stored foods as seen in Figure 6 (Labuza, 1973). In this study of an intermediate moisture food product at a_w 0.75 an increase in browning was directly correlated ($R^2 = 0.96$) with an increase

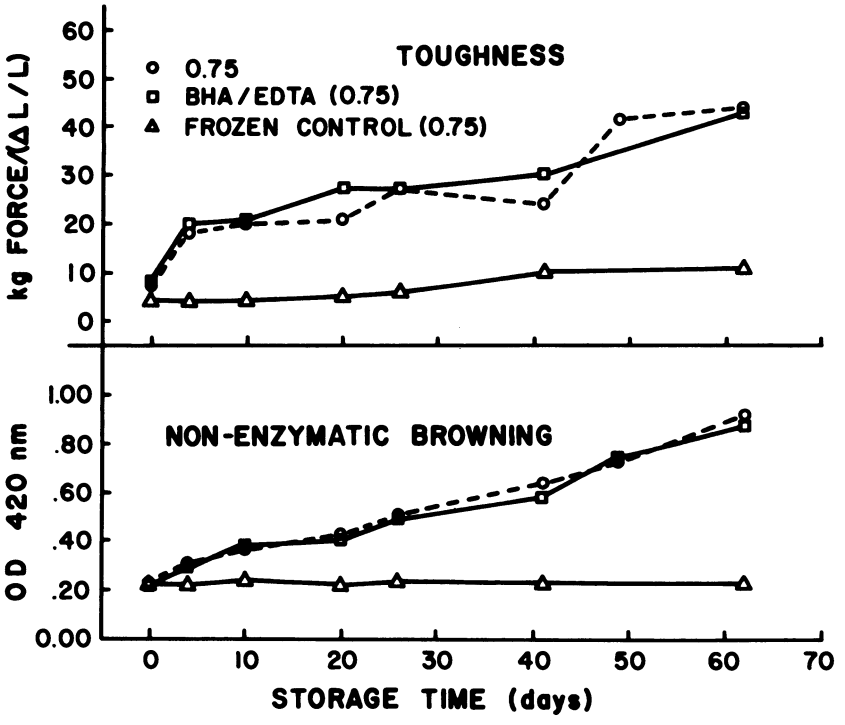


FIGURE 6

Effect of storage at 37°C and a_w 0.75 on browning and toughening of an intermediate moisture food.

in toughness as measured by an Instron. The browning that occurred was not as the result of carbonyl compounds formed from lipid oxidation since the same changes occurred even if protected with antioxidants.

However, in studies with other IMF systems such as chicken die soaked in glycerol to achieve an a_w of 0.7 to 0.8, Labuza (1973) found browning to occur as a result of reactive carbonyls formed during lipid oxidation. In this product no reducing sugars were originally present. As seen in Figure 7, protection of the chicken with either EDTA or BHA reduced the rate of browning. Both act as antioxidants to slow rancidity.

The most obvious indication that Maillard browning has occurred in a food containing both carbohydrate and protein is

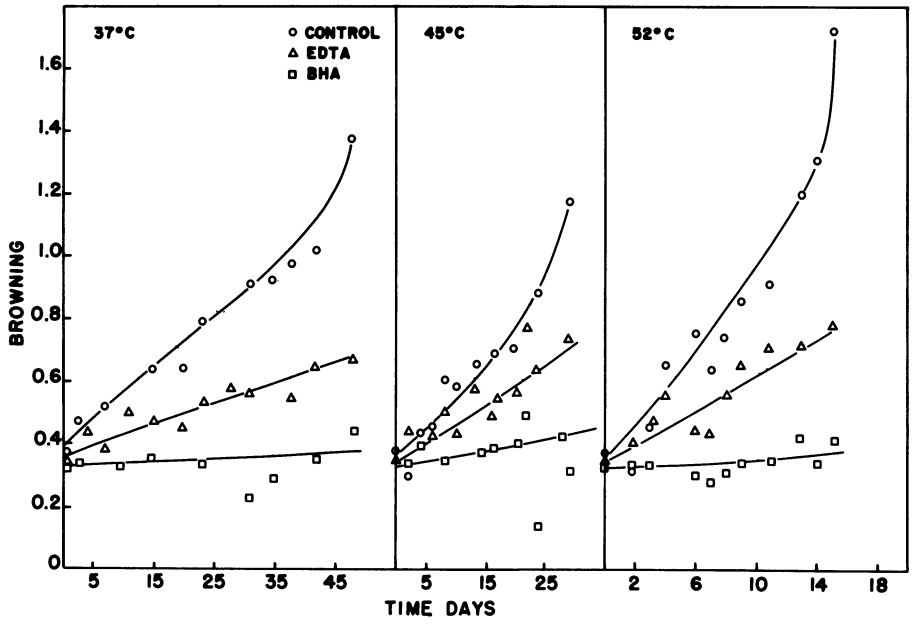


FIGURE 7

Effect of antioxidants and storage at 37°C, a_w 0.8 on browning in a chicken/glycerol system.

the accumulation of brown pigments. Choi et al., (1949) and Patton (1955) were the first to recognize the significance of the brown discoloration in milk products that were subjected to excessive heat during processing or storage. This color change was particularly noticeable in condensed and dried milk products. Brown discoloration has, therefore, been used as an indication of milk products being excessively thermally processed. Aside from the milk industry, the dry cereal and animal feed industries well-recognize the undesirable discoloration that can result in their products.

The semi-moist pet food industry also incurs loss in their product due to Maillard browning. The dog and cat foods that are made to an a_w of about 0.85 by directly extruding at 80-140°C sugars, cereals, and fresh meat by-products (Burgess and Mellentin, 1965). Coloring agents are used to mask the excessive browning that results from both the initial thermal processing and storage of these products, but the loss in protein quality cannot be.

The reaction of Maillard browning can be followed by several methods. The first and most obvious method is simple visual observation of the brown color and ranking of the product (McWeeney and Burton, 1963). Lea and Hannan (1949), Tarr (1954) and Arnold (1973) used reflectance readings as an indicator of the degree of browning. Spectrophotometric analysis of enzyme digested extracted filtrates such as the method of Choi et al., (1949) allows for more exact monitoring of the color change as induced specifically by non-enzymatic browning.

The accumulation of hydroxymethyl furfural or furfural has also been used by some investigators as an indicator of the extent of non-enzymatic browning (Reynolds, 1963). However, McWeeney and Burton (1963) concluded that, other than in strongly acidic conditions, HMF is a reaction by-product which accumulates in detectable amounts only because of its relatively low reactivity in browning reactions. Cole (1967) monitored carbon dioxide evolution as an indicator of the extent of Maillard browning. He concluded that although the Strecker degradation may be the main source of carbon dioxide produced during the Maillard reaction, it is not the only pathway by which carbon dioxide may be evolved. Eichner (1975) as well as many others have used carbonyl accumulation as an index. The problem here is that a maximum is reached since the reducing compounds participated in further reactions.

A wide variety of flavor compounds have been used as an index of the Maillard browning reaction. The Strecker degradation reaction is thought to be a source for producing characteristic browning flavors (Reynolds, 1965). Schonberg and Moubacher (1952) have determined that the type of organic group reacting with the amino acids during the Strecker reaction determines what type of end-product will be produced. Reynolds (1965) investigated some of the flavor compounds associated with cooked potatoes, soy sauce, bread and cooked milk and meat and Markova et al., (1972) have studied some of the flavor changes associated with cereal products that were subjected to non-enzymatic browning.

Another very important means of monitoring the non-enzymatic browning reaction is by observing the protein nutritional changes that occur in the food product after processing and storage. This will be discussed in the next section.

V. BIOLOGICAL EFFECTS OF NON-ENZYMATIC BROWNING

Lea and Hannan (1950), Lea (1958), Lea et al., (1958, 1960), Carpenter et al., (1962), Heller et al., (1961), Ben-Gara and Zimmermann (1971), Chichester and Smith (1972), Rao and Rao (1972), and Yanagita et al., (1973) have examined the influence of Maillard

browning on protein nutritional loss. As the free amino-N groups react, they can become bound to the brown pigments and cause the nutritional availability of the protein to decrease. In particular, the N-terminal group of peptides and the ε-amino groups of lysine react and become nutritionally unavailable. Folk (1956) and Tu and Eskin (1973) observed a decreased ability of digestive enzymes, e.g. trypsin, to hydrolyze a lysine containing system as Maillard browning ensued. As lysine is an essential amino acid, the availability of lysine is of great nutritional importance.

Lysine availability is determined by the positioning or bonding of the ε-amino groups in reference to recognition of this amino acid by lysine-specific peptidases. Lysine that is not subject to an otherwise active peptidase is defined as being unavailable. Lysine may be unavailable naturally within a food or it may become unavailable during processing and storage of the food.

Carpenter (with Booth, 1973) reviewed most of the methods available to determine the loss of lysine availability in foods subjected to thermal processing and storage. The most common method employs Sanger's reagent (1-fluoro-2, 4-dinitro-benzene, FDNB). The methods of Carpenter (1960), Rao et al., (1963a), Roach et al., (1967), and Booth (1971) all employ the use of FDNB as the tagging agent for free or available lysine.

In a comparative study between the Carpenter (1960) and the Roach et al., (1967) methods, Milner and Westgarth (1973) concluded that, though the two methods give good agreement for available lysine results, Carpenter's method might be best used for the analysis of protein foodstuffs whereas cereals and mixed feeds containing cereals should be analyzed with the method of Roach et al., (1967). Milner and Westgarth (1973) further concluded that the method of Roach et al., (1967) is more precise but is more costly than is Carpenter's (1960) method.

Rao et al., (1963b) claim their method to be superior to Carpenter's in that better separation of the ε-DNP-lysine from dinitrophenol and yellow derivatives of the reaction is achieved through the use of an ion exchange clean-up procedure. However, use of their ion exchange chromotographic technique does not really lend itself as a rapid routine procedure for determining the availability of lysine in foodstuffs.

Kakade and Liener (1969) used the dye 2,3,6-trinitro benzene sulfonate (TNBS) to tag available lysine. Ousterhout and Wood (1970) later modified the Kakade and Liener (TNBS) method. Holsinger et al., (1970) and Hall et al., (1973), however, have

shown the TNBS reaction to give erroneous available lysine results when glycosylamines and galactosylamines are present. Hurrell and Carpenter (1974) have also found the TNBS technique to not fully indicate the extent of the Maillard reaction for measuring available lysine content in foods subjected to mild (37°C) heat treatments. This and lesser temperatures would be typical of warehouse storage conditions for dry foods.

Booth (1971) modified Carpenter's (1960) original FDNB-lysine method to eliminate arginine interference. Hurrell and Carpenter (1974) found the FDNB technique to fully reflect the nutritional damage of food systems in which the Maillard reaction occurred at mild (37°C) conditions typical of that found in non-air-conditioned food storage warehouses. Thus, either Booth's (1971) or Carpenter's (1960) procedure utilizing FDNB as the tagging dye is the current method most commonly used for the routine analysis of available lysine in foodstuffs.

Warmbier et al., (1976) as seen in Figure 8 followed FDNB-available lysine as a function of pigment formation in a casein/glucose model system at 0.5 a_w. As seen, the loss of lysine occurs much sooner than does pigment formation as would be expected from the overall reaction, whether this represents true nutritional loss has not been well established. However, good correlation has been found between *in vitro* chemical (e.g. FDNB) and *in vivo* nutritional studies for evaluating nutritional loss in foods subjected to Maillard browning. Mauron and Mottu (1958) found an excellent correlation ($r = 0.99$) between *in vivo* decrease of protein efficiency and *in vitro* lysine deterioration in milk powders.

Milk products are especially susceptible to browning during drying or heat processing because of the high lactose content and lysine content in the protein. Henry et al., (1948) determined that cystine is the first limiting amino acid in milk products for rat growth. Milk products which had been deteriorated by browning at 37°C for 60 days gave lower PER's and Biological Values (BV) than control samples. When lysine was provided in the rat diets as 1.25% and 2.5% of the protein, growth equivalent to that on the control diet was observed. Cystine, however, did not improve the growth response. This emphasized the fact that the lysine was involved rather than the limiting amino acid, cystine.

Similar results for lysine supplemented to deteriorated milk products have been observed by Cook et al., (1951) and Rao et al., (1963b). Cook et al., (1951) examined PER's of rats fed casein and lactose which had been treated under mild conditions (52°C for 24 hr) and under strong conditions (140°C for 3 hr).

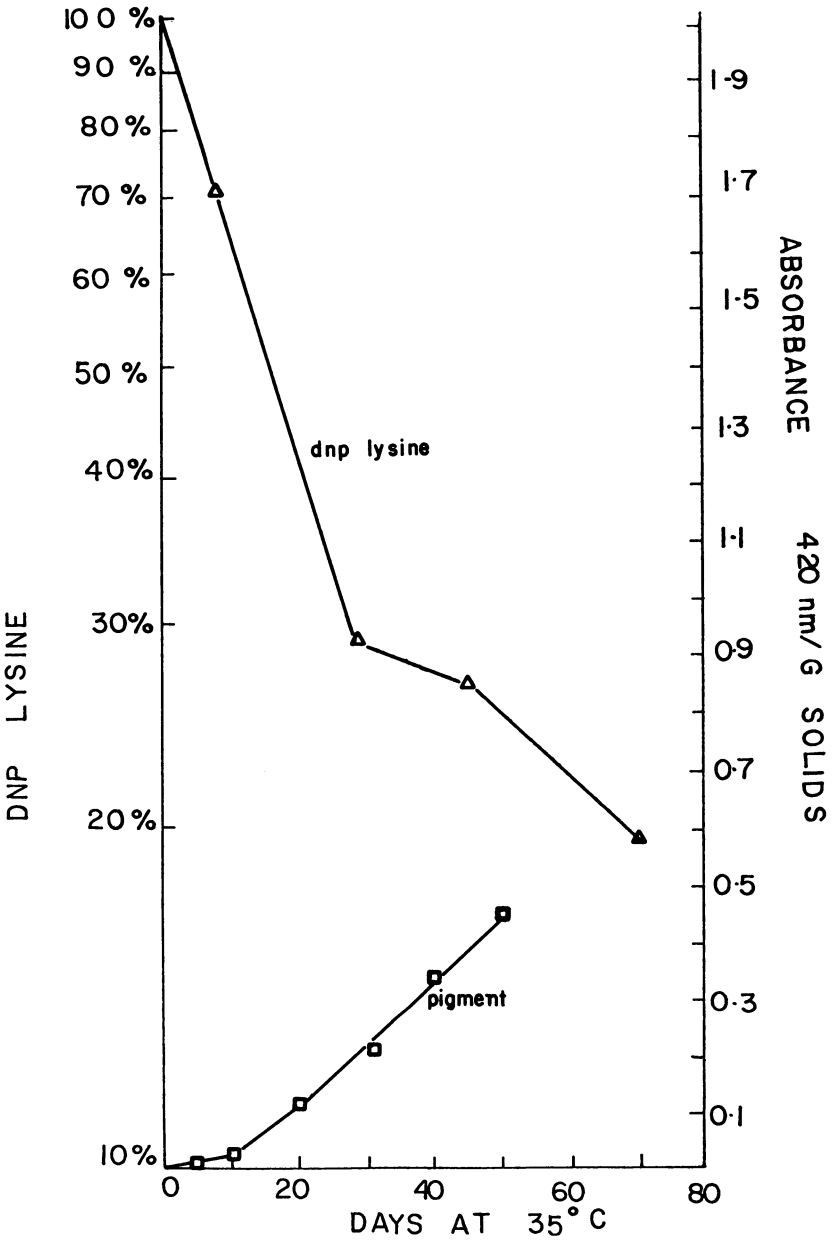


FIGURE 8

Browning pigment production (□) and lysine loss (Δ) at 35°C in a casein/glucose glycerol model system (a_w 0.5).

Warmbier et al., (1976)

Control samples gave PER's of 2.53, mildly treated samples gave PER's of 2.29 and the high heat treatment resulted in PER's of 2.18. Lysine supplementation at 0.2% in the similar groups increased the PER for the mildly treated sample to 2.51 and the high temperature sample to 2.00. This suggests that amino acids other than lysine may be involved in severe conditions, whereas lysine is the main reactant in early non-enzymatic browning.

These studies strongly support the belief that the nutritional loss of protein quality in non-enzymatic browning is due to a decrease in free lysine groups. Specifically, this has been shown by *in vitro* studies to be due to the inability of the pancreatic enzyme, trypsin, to split the reacted lysine from the peptide chain (Carpenter and Booth, 1973; Mauron and Mottu, 1958; Mauron et al., 1955; and Tu and Eskin, 1973). Trypsin activity in the small intestine of mammals is highly specific for the substrate binding site. The polar groups of the specific amino acids, lysine and arginine, must be present in the polar forms to be cleared from the polypeptide linkage. The reacted group (aldehyde or ketone) attached to the epsilon-amino group prevents enzymatic cleavage of the lysine-peptide bond linkage. Thus the effect of decreased enzymatic cleavage lowers protein utilization by limiting the lysine available for absorption (Finot, 1973; and Goodhart and Shils, 1974).

Hurrell and Carpenter (1974) in fact found very good agreement between *in vivo* growth studies and *in vitro* lysine values for an albumin-glucose system that was stored at 37°C for 10 days as seen in Table 2. It is obvious that the FDNB technique gave better agreement with the *in vivo* growth studies than did the TNBS technique.

It has also been suggested that the Amadori reaction compounds formed in the first phase of reaction are also biologically unavailable although they are chemically reversible (Adrian, 1974; and Lea and Hannan, 1950a, 1950b). As was seen in Figure 8, about a 70% loss in available lysine (by the FDNB modified method) occurred in a model food system (glucose/casein 3:3) held at a_w 0.5 for 30 days at 35°C. At this point the product was not yet visually brown. Warren and Labuza (1976) have confirmed that part of this loss leads to decreased nutritional value using a protozoan *Tetrahymena pyriformis* W (TPW) bioassay technique.

The use of TPW in evaluating protein quality has been shown to correlate well with both rat PER's (Evancho et al., 1976; and Landers, 1975) and FDNB (Shorrock and Ford, 1973). Warren and Labuza (1976) used the same model system of Warmbier (1975) and humidified the product to several a_w's. As can be seen in Figure 9, the loss in protein nutritional value (RNV) by TPW is less than

Table 2

Comparison of Biological and Chemical Methods for Protein Loss

	Albumin Mix & Glucose		Potency Ratio of Stored Sample of
	Unheated	Stored 30 d at 37°C	Unheated Control
Rat Assay			
g wt gain	64.5	18.3	0.28
g wt gain/g food eaten	75.2	25.9	0.34
Chick Assay			
g wt gain	107.4	23.6	0.22
g wt gain/g food eaten	103.3	25.4	0.25
total lysine	86.3	81.6	0.94
FDNB-lysine	50.9	19.6	0.38
TNBS-lysine	53.6	35.9	0.67

From Hurrell and Carpenter, 1974

predicted by the FDNB procedure. However, after 20 days storage in the 0.3 a_w samples where the samples have not changed color significantly (absorbance $\approx$0.2) the nutritional value has decreased by 50%. At higher a_w (0.7) as seen in Figure 10, the FDNB procedure significantly over-estimates losses in the early stages of browning. This suggests that some of the Amadori rearrangement products are not available nutritionally and also that reaction pathways are changing with a change in the water content.

VI. KINETICS OF MAILLARD BROWNING DURING PROCESSING AND STORAGE

An introduction to the mathematical kinetics of Maillard non-enzymatic browning was shown in section II. several other studies with respect to water and temperature will be reviewed here.

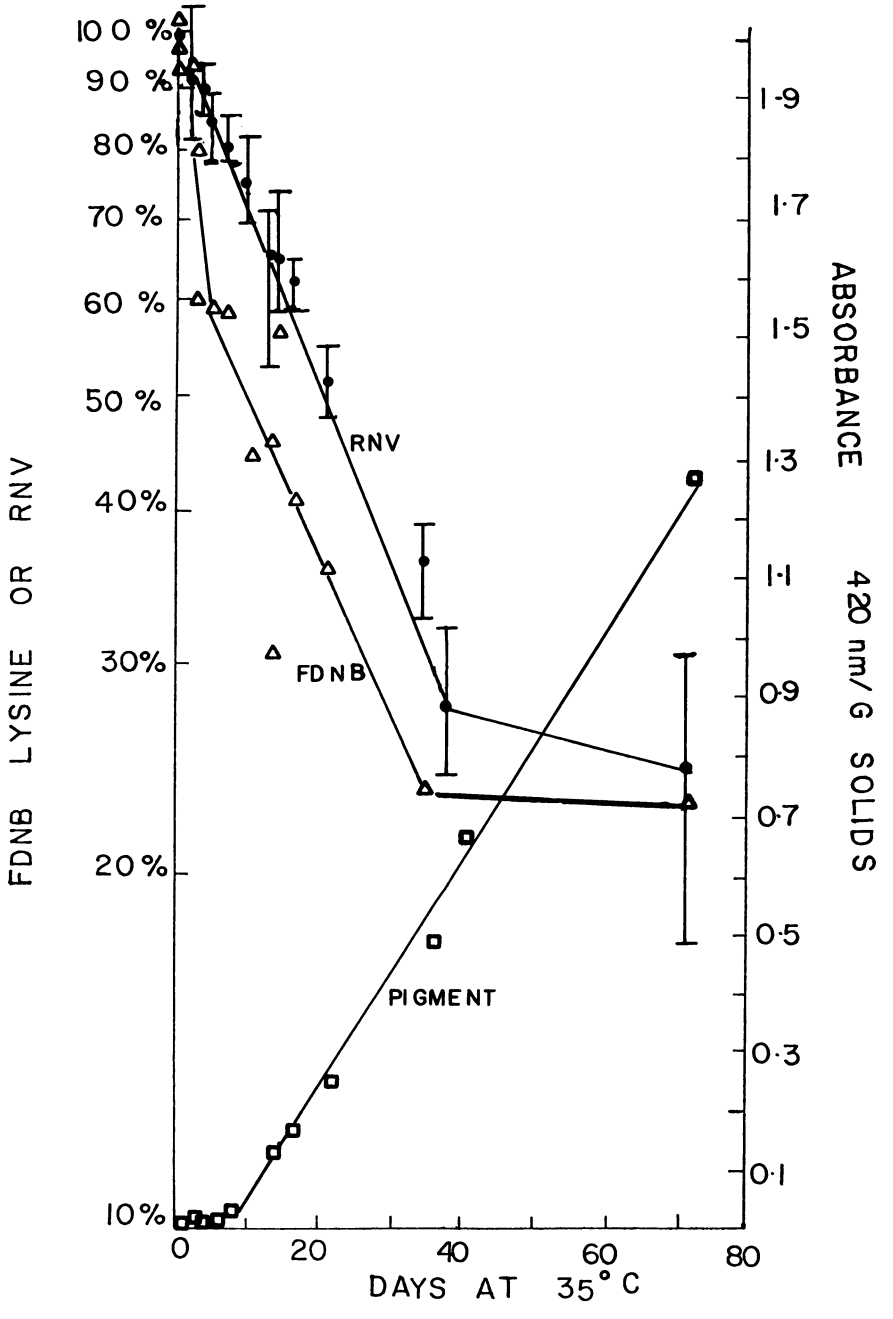


FIGURE 9

Lysine loss (Δ) by FDNB, protein nutritional loss (●) by TPW bioassay and browning production in a casein/glucose/glycol model system (a_w 0.3, 35°C). Warren and Labuza (1976)

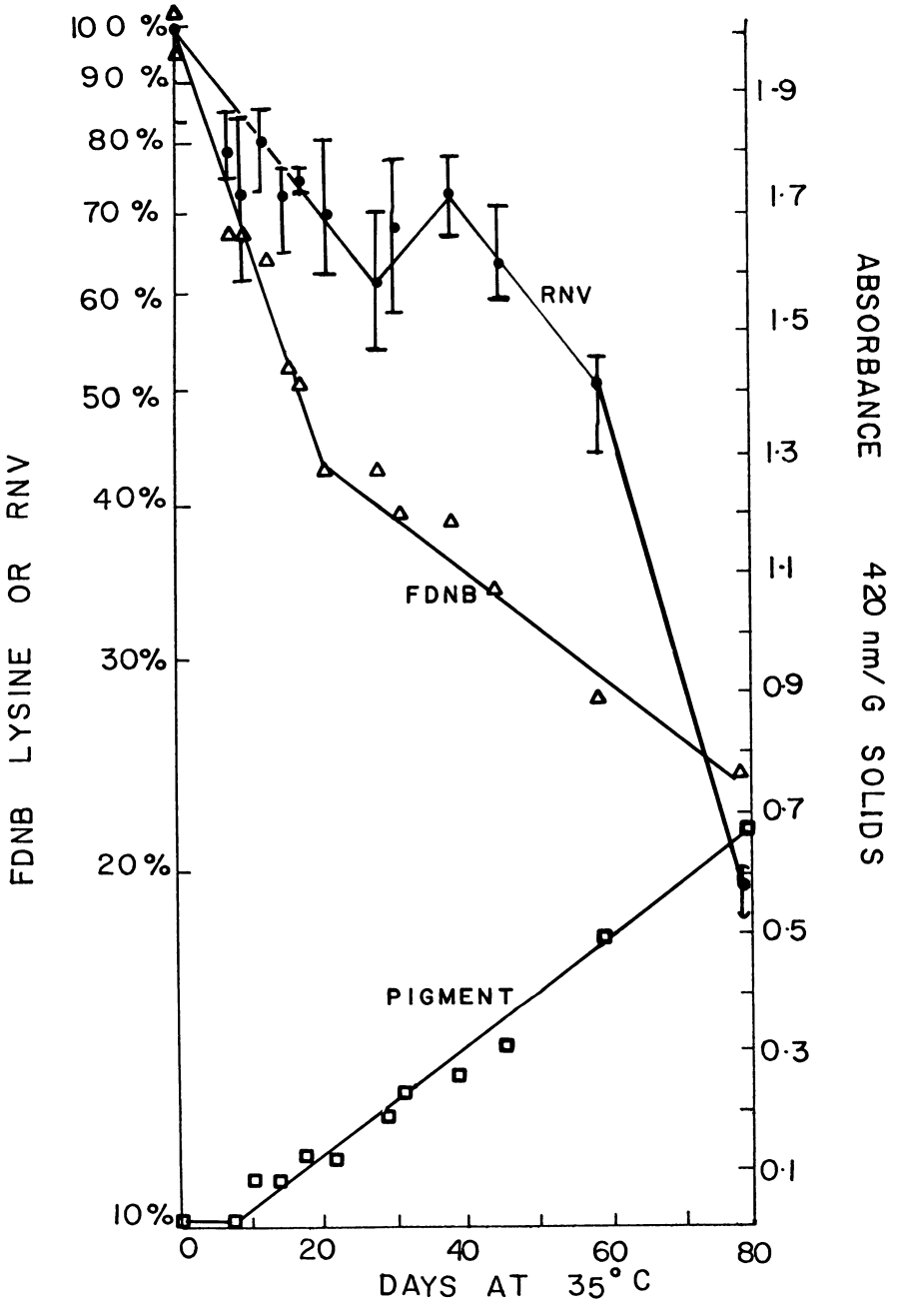


FIGURE 10

Lysine loss (Δ) by FDNB, protein nutritional loss (●) by TPW bioassay and browning production in a casein/glucose/glycol model system (a_w 0.7, 35°C). Warren and Labuza (1976)

Haugaard et al., (1951) studied the browning of an aqueous mixture of D-glucose and glycine at reflux temperature by measuring the absorbance of the reaction mixture at 490 nm. They concluded that the concentration of the brown pigment produced was proportional to the square of the amino acid concentration (A), the concentration of the reducing sugar (R), and the square of the reaction time as shown in equation (3):

$$B = K(A)^2 (R)\Theta^2 \qquad (3)$$

where K is the rate constant.

Differentiating this gives:

$$\frac{dB}{d\Theta} = K'A^2R\Theta \qquad (4)$$

which shows that browning rate increases with time. Eichner (1975) and others have found similar increased browning rate with time in the study of the browning of simple sugar/amino acid systems which undergo rapid browning. It should be noted that Haugaard's mechanism implies that two moles of amine react per mole of reducing sugar which is not as per the mechanism presented earlier.

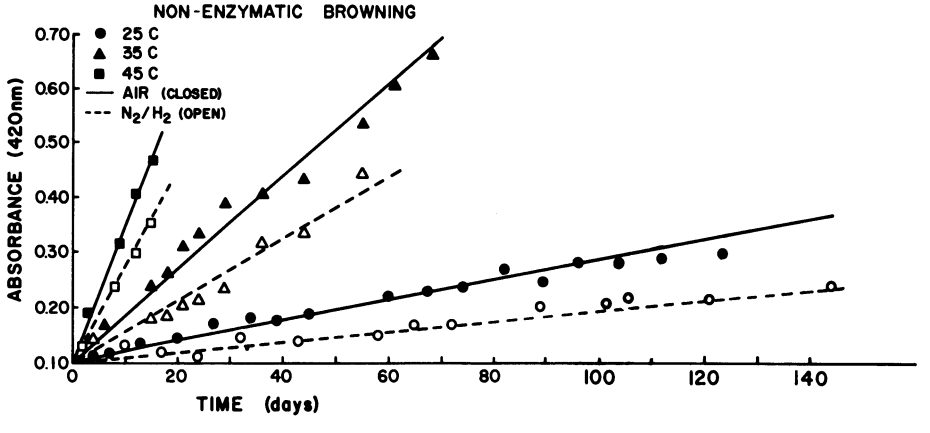


FIGURE 11

Increase in brown pigment as a function of temperature and gas composition for an intermediate moisture food (a_w 0.84)
Waletzko and Labuza (1976)

Accumulation of brown pigments is usually found to be relatively constant with time during the storage of most dry food products. For example, Figure 11 shows the increase in brown pigment in an IMF system ($a_w \simeq 0.84$) at three temperatures stored in air and an oxygen free atmosphere (Waletzko and Labuza, 1976). Similar effects of constant browning occur in complex model systems where the amine is supplied by protein. The data of Warmbier et al., (1976) shown in Figure 12 exemplifies the constant rate of pigment production after a short induction period and the effect of a_w and temperature. This constant rate occurs despite the fact that glucose and lysine of the protein are lost very rapidly by first order reactions as seen in Figures 13 and 14.

Based on these observations at low temperature (<50°C) storage of dehydrated and semi-moisture, the rate of production of reactive intermediates must reach a constant value in which the rate of breakdown is equivalent. In addition, some level of R* must be reached before visual browning occurs. Thus,

$$\frac{dB}{d\theta} = k_B = k_2 \, (R)^*_m \qquad (5)$$

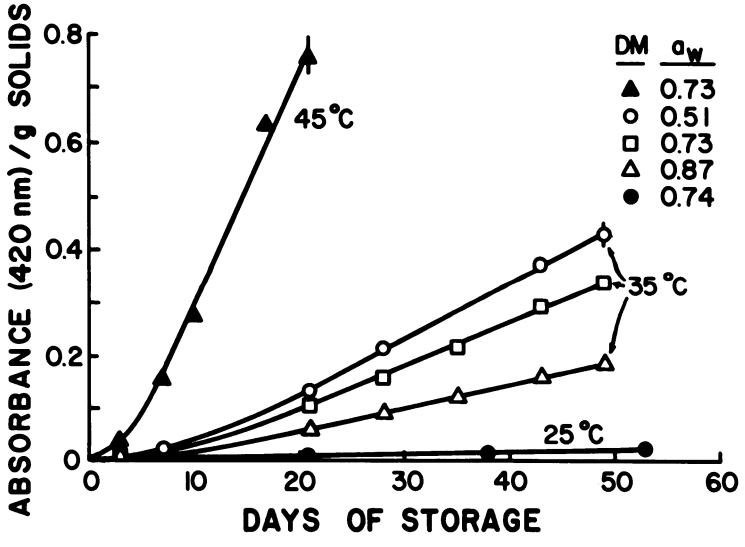


FIGURE 12

Increase in brown pigment for a casein/glucose/glycerol model system as a function of a_w and temperature.
(Warmbier et al., 1976)

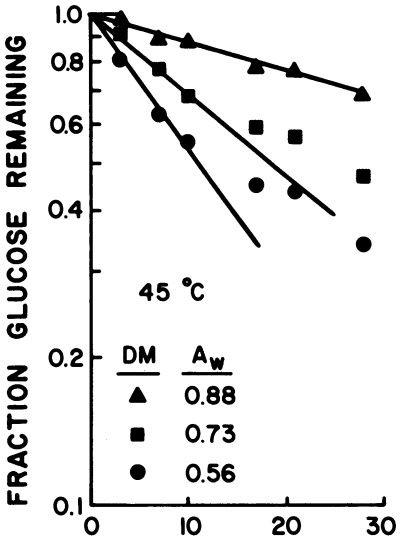


FIGURE 13

First order plot for glucose loss in a casein/glucose/glycerol model system as a function of a_w.

(Warmbier et al., 1976)

where

k_B = zero order browning rate
k_2 = rate constant for (R*) production
$(R^*)_m$ = constant level of R* needed for constant brown pigment production

Obviously at higher temperatures such as in the study of Haugaard et al., (1951) or in less complex systems, a change in kinetics occurs to make the reaction rate change with time.

Song et al., (1966) and Song and Chichester (1966, 1967a, 1967b) developed kinetics for the Maillard reaction of glucose-glycine. Their overall browning rate expression is:

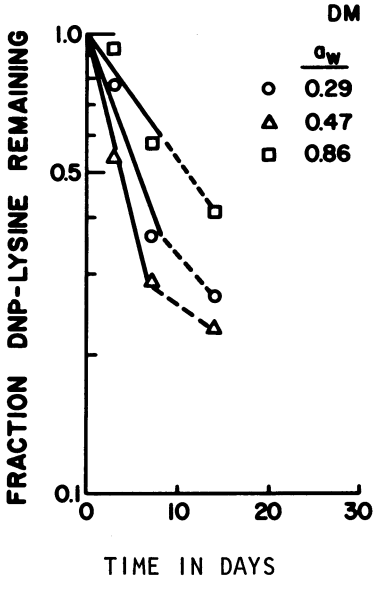


FIGURE 14

First order plot for lysine loss (FDNB) in a casein/glucose/glycerol model system as a function of a_w.

(Warmbier et al., 1976)

$$d(B)/dt = k_2(k_i(G)k_2'(I))^{\frac{1}{2}} + k_3(g_0 - B)\ (I - B) \qquad (6)$$

where I represents intermediates, g represents glycine, G represents glucose, B represents the brown melanoidin pigments, and the k's represent constants. The equation is too complex to be readily adaptable to practical use where more complex substrates are present. However, it should be noted that they also found even with a very simple system in the 50-100°C range, the amount of brown pigment followed a linear plot with time after a certain induction period. Their complex equation above accounts for this induction period.

Mizrahi et al., (1970a, 1970b) developed equations to predict browning rates of unsulfited dehydrated cabbage under accelerated storage conditions. Two equations that related browning rate to moisture content were derived by curve fitting:

$$\frac{dB}{d\Theta} = K_1(1 + \sin(-\pi/2 + m\pi/m_x))^n \quad (7)$$

$$\frac{dB}{d\Theta} = K_2(r_1 + m)/(r_2 + m))^s \quad (8)$$

where browning is in Klett units/day, m is the moisture content (g H_2O/100g solids), m_x is the moisture content at which browning rate is at a maximum (which was 18.0 in their study with humidified freeze-dried cabbage), and r_1, r_2, K_1, K_2, n and s are constants fit by computer analysis. Equation (7) gave minimum variance for relating browning rate to moisture content for samples stored at 30° and 37°. Equation (8) was best adapted to samples stored at 45° and 52°C. The equations quite accurately predicted the NEB rate of unsulfited dried cabbage from NEB rate data collected under accelerated shelf-life conditions.

Mizrahi et al., (1970a) also found that the apparent activation energy was related to moisture content as given by the following equation (9):

$$E_a = c_1 e^{-c_2 m} \quad (9)$$

where E_a is Arrhenius activation energy, m is moisture content (g H_2O/100g solids) and c_1 and c_2 are constants. The results are shown in Figure 15.

Eichner (1975) found a similar effect for model systems at low moisture contents. The Q_{10} dropped from 5.6 to 3.9 in going from 1.25 to 3.1% moisture. Figure 16 shows similar data of Hendel et al., (1955) used by Aguilera et al., (1975) in predicting browning during drying of potatoes. As seen, the major change in activation energy occurs below 15% water content ($a_w \approx 0.7$). This verifies that the changes in the pathways of browning mentioned earlier are indeed occurring.

With respect to water activity, as was shown before, the browning rate increases to a maximum and decreases again. The same factors of water feedback inhibition, reactant concentration and aqueous phase viscosity also affect the rates of loss of the reducing sugars and lysine. The data of Warmbier et al., (1976) for a casein/glucose/glycerol model system illustrates this in Figure 17. The E_a for browning was 33 KCal/mole whereas for the initial steps of glucose and lysine loss was 25 KCal/mole. This shows that the latter phases of the mechanism are rate controlling which allows build-up of reaction intermediates. As also noted in this study the rate maximum occurred at a lower a_w (0.5) than found in most dry foods which have been humidified. This is similar to the results of Eichner and Karel (1972) for

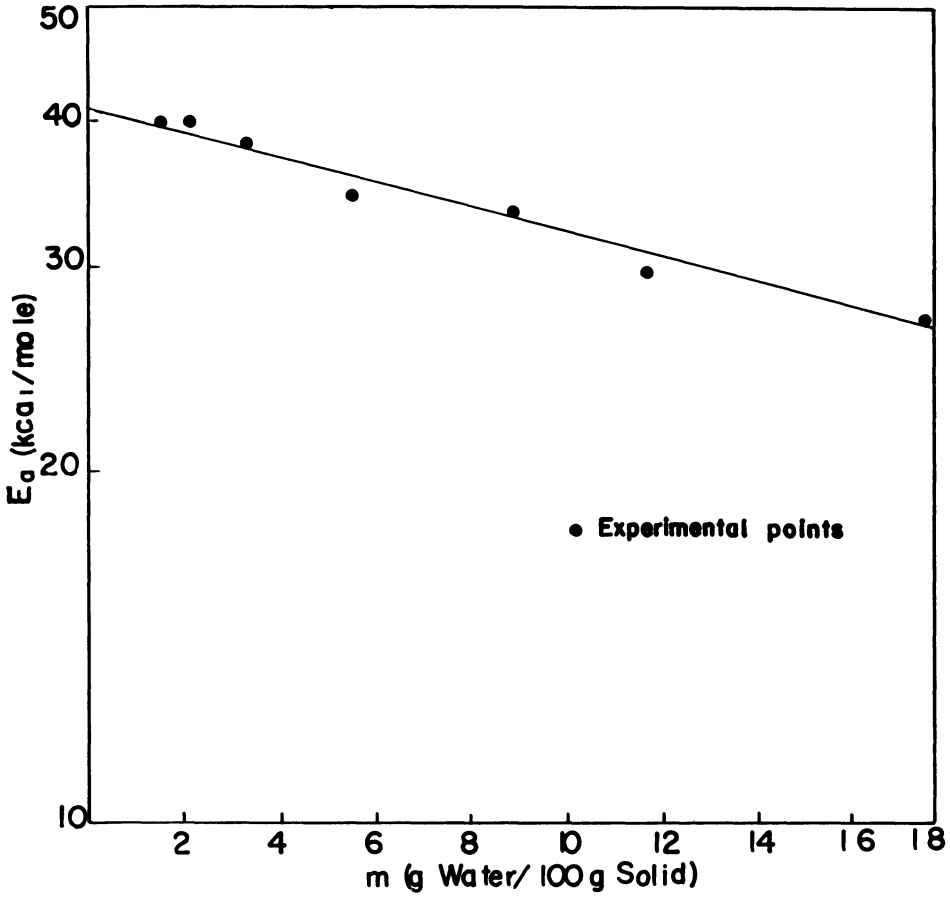


FIGURE 15

Decrease in activation energy E_a for non-enzymatic browning in dehydrated cabbage as a function of water content.
(Mizrahi et al., 1970b)

liquid systems shown in Table 3. They found that with glycerol present the maximum amount of browning occurred at 0.3 to 0.4 a_w as compared to 0.7 to 0.8 for humidified, dry, or semi-moist foods. Addition of gum arabic which increased phase viscosity decreased the amount of browning and increased the a_w at which this occurred.

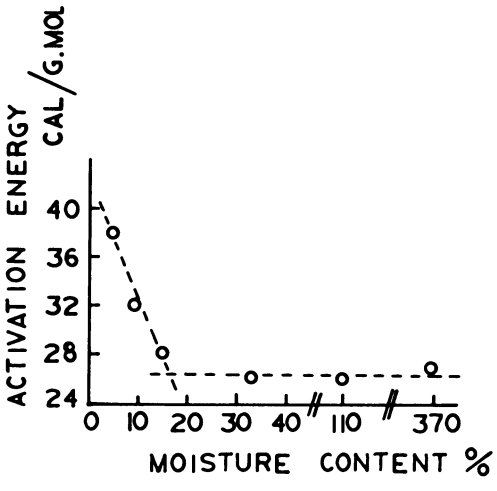


FIGURE 16

Decrease in activation energy for non-enzymatic browning of potatoes undergoing drying as a function of water content. (Hendel et al., 1955)

Table 3

Browning Value (420 nm) After Storage 3 Days at 37°C*

a_w	Control*	Control With Cellulose	Control with Gum Arabic
0.3	0.08	0.07	-
0.4	0.11	0.11	0.04
0.5	0.09	0.09	0.06
0.6	0.05	0.05	0.05
0.75	0.03	0.03	0.04

* Eichner and Karel (1972)
** glucose/glycine in H_2O-glycerol solutions

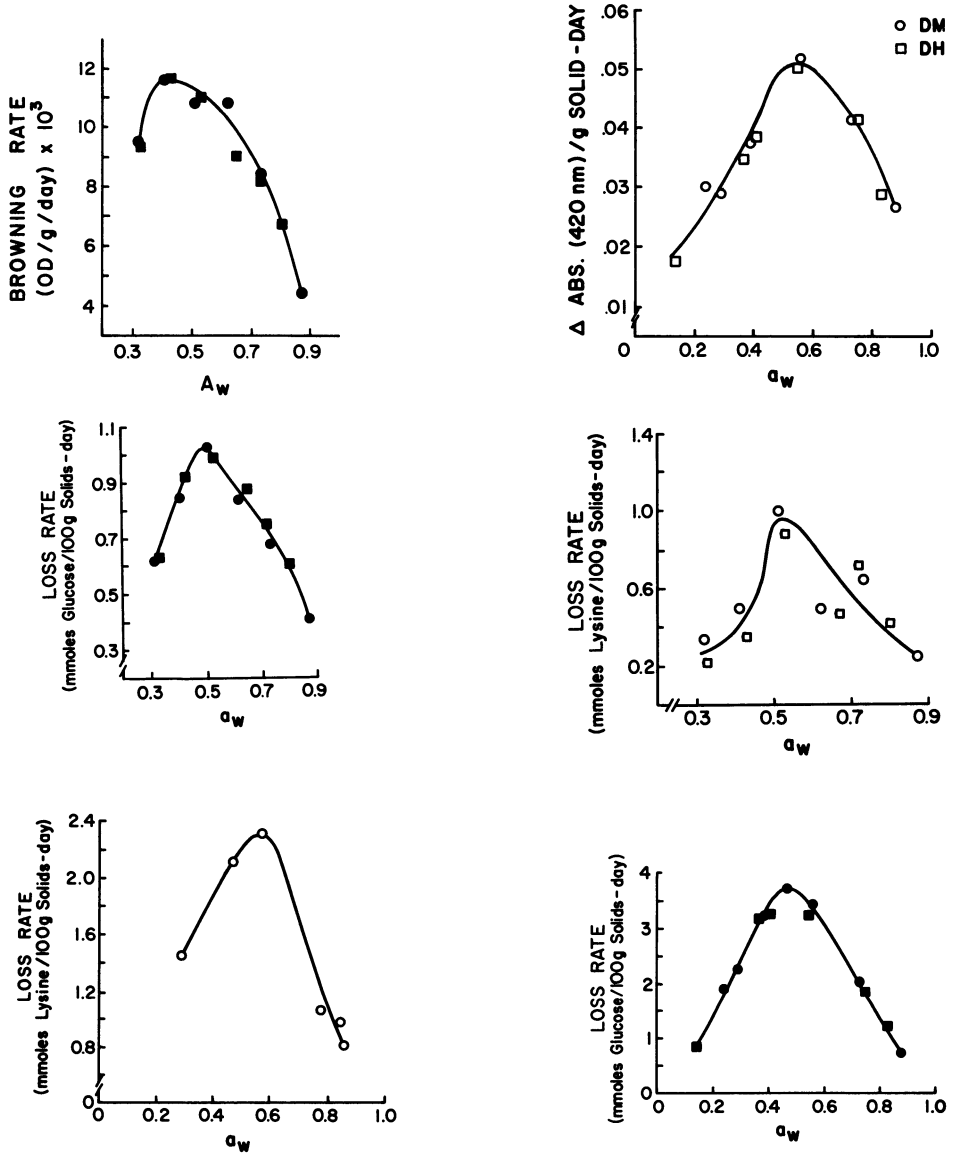


FIGURE 17

Effect on water activity on the rates of browning pigment production, glucose loss and available lysine loss at 45°C in a casein/glucose/glycerol model system. (Warmbier et al., 1976)

To further verify this, Labuza (1975) worked with solid model foods to which various humectants were added. As seen in Figure 18 the control showed a browning maximum at a_w = 0.82, typical of semi-moist or humidified dry foods. The systems with the liquid humectants (glycerol, butane diol and propylene glycol) all had a greater rate of browning at lower a_w. This was attributed to the greater phase volume at the lower water activity, since the liquid glycols are soluble in water and could dissolve the glucose. Phase volume was not the only factor however. The differences could also relate to solubilities, oxidation reduction potentials and pK's, and aqueous phase viscosity. This latter effect is shown for the sorbitol data. It reduces browning rate drastically by increasing the solution viscosity. Sorbitol is a solid and cannot act as a liquid humectant. The use of these humectants as browning inhibitors has been applied for as a patent.

Many other studies have been done to develop browning rate equations as a function of system composition and conditions. Most are specific systems and equipment. For example, Jokinen et al., (1976) determined available lysine loss due to

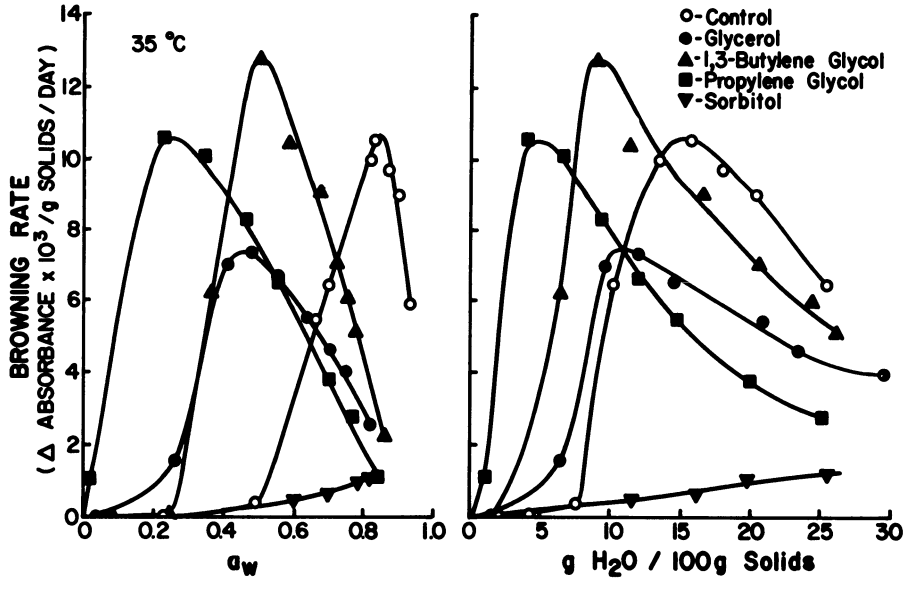


FIGURE 18

Effect of various humectants on browning rate in a casein/glucose model system as a function of water activity. (Labuza, 1975)

browning in samples composed of soybean protein, glucose, sucrose, potato starch, microcrystalline cellulose and water. The equation relating available lysine content to sample composition and thermal processing conditions is:

$$L/L_0 = 0.581 + 0.047\ (pH) - 0.093\ (G) - .059\ (T) - .0068\Theta$$
$$+ .0305\ (a_w)^2 + .025\ (a_w)(S) + .0331\ (S)\ (s) \qquad (10)$$

where L/L_0 is the fraction of available (FDNB) lysine remaining, G is glucose content, T is temperature, Θ is time, S is sucrose content, and s is starch content.

This is the first kinetic approach to the Maillard browning reaction where the nutrient stability (available lysine content) of a system can be predicted given the system's composition and thermal parameter. It should be noted that as with food storage the maximum in browning occurs in the a_w range of 0.65 to 0.7 and that sucrose hydrolysis contributed to browning at the low pH's. The limitation of the study is that it applies to extrusion processing where high temperatures (80 - 130°C) are used.

Another aspect of browning is the relative reactivity of proteins with the reducing sugars. Schnickles et al., (1976) showed that for IMF systems at 0.68 - 0.78 a_w the browning rate did not correlate with either total lysine available or time for 50% lysine loss for all the proteins. This is seen in Table 4.

If zero order kinetics apply then the methods of Labuza et al., (1972) and Labuza (1976) can be used to predict food product shelf-life undergoing browning for time-temperature-humidity variable systems. The details for the moisture dependency will not be gone into here, except to show that the moisture gain with time for a particular food in a certain package can be predicted by equation 11:

$$\ln \frac{m_e - m_i}{m_e - m} \quad \frac{k}{x} \frac{A}{W_s} \frac{P_o}{\beta} \Theta \qquad (11)$$

m_e = moisture content of food in equilibrium with outside atmosphere (g H_2O/g solids)
m_i = initial moisture content
m = moisture content at time Θ
k/x = permeance of package g H_2O/day m^2 mm Hg
A = area of package
W_s = weight of dry solids
P_o = vapor pressure of pure water mmHg
β = slope of linear portion of isotherm
Θ = time

Table 4

Browning and Lysine Loss In Model Systems

Protein Used	Total Avail Lysine mg/100g solids	Browning Rate (OD/day) x 10^3	Time for 50% Lysine Loss (days)
wheat gluten 10% lysine	3,300	35	< 1
casein	1,416	10.2	22
whey	857	8.5	25
soy (TVP)	762	7.5	7
fish protein conc.	1,283	5.9	19
egg albumin	360	4.9	> 60
wheat gluten	303	0.9	40

Given this and a graph of shelf-life as a function of water content for different temperatures one can use iterative processes to determine shelf-life consumed since:

$$f\text{con} = \frac{(\Theta)}{(\Theta_s)_{Tm}} \qquad (12)$$

$f$con = fraction shelf-life consumed
Θ = time held at some constant moisture (m) and temperature (T)
Θ_s = total shelf-life at that constant moisture (m) and temperature (T)

The fractions for each interval are summed up to get total consumption. This has been successfully applied to frozen foods where temperature is the only variable (Gutschmidt, 1974).

VII. ACKNOWLEDGEMENTS

The authors wish to acknowledge the support for much of this work through Project 18-72, University of Minnesota Agricultural Experiment Station and Contract NAS 9-12560, Lyndon Johnson Manned Space Craft Center, Houston, Texas.

REFERENCES

Adrian, J. 1974. Nutritional and physiological consequences of the Maillard reaction. World Review of Nutrition and Dietetics. 19:71.

Aguilera, J., J. Chirife, J. Flink and M. Karel. 1975. Computer simulation of non-enzymatic browning during potato dehydration. Lebensm. - Wiss. u.-Technol. 8:128.

Arnold, R.G. 1973. Inhibition of head induced browning of milk by L-cystein. J. Dairy Sci. 52:1857.

Bender, A.E. 1972. Processing damage to protein foods. J. Food Tech. 7:239.

Ben-Gara, I. and G. Zimmerman. 1972. Changes in the nitrogenous constituents of staple foods and feeds during storage. 1. Decrease of lysine. J. Food Sci. and Tech. 9:113.

Booth, V.H. 1971. Problems in the determination of FDNB-available lysine. J. Sci. Food Agr. 22:658.

Burgess, H. and R. Mellentin. 1965. Animal food and method of making same. U.S. Patent #3202514.

Carpenter, K.J. 1960. The estimation of the available lysine in animal protein foods. Biochem. J. 77:604.

Carpenter, K.J., C. Morgan, C. Lea and L. Parr. 1962. Chemical and nutritional changes in stored herring meal. Br. J. Nutr. 16:451.

Carpenter, K.J. and V.H. Booth. 1973. Damage to lysine in food processing: Its measurement and its significance. Nutr. Abstr. and Rev. 43(6):426.

Chichester, C.O. and R. Smith. 1972. Nutrition In Food Processing. IFT Short Course. May 25th.

Choi, R.P., A. Koncus, C. O'Mally and B. Fairbanks. 1949. A proposed method for the determination of color of dry products of milk. J. Dairy Sci. 32:580.

Cole, S.J. 1967. The Maillard reaction in food products - carbon dioxide production. J. Food Sci. 32:245.

Cook, B.B., J. Fraenkel-Conrat, B. Singer, and A.F. Morgan. 1951. The effect of heat treatment on the nutritive value of milk protein. III. The effect of heat on casein, lactalbumin, their lactose-induced derivatives, with special reference to digestibility and rate of release of lysine, methionine, and tryptophan. J. Nutr. 44:217.

Eichner, K. and M. Karel. 1972. The influence of water content and water activity on the sugar-amino browning reaction in model systems under various conditions. J. Agr. Food Chem. 20:218.

Eichner, K. 1975. The influence of water content on non-enzymatic browning reactions in dehydrated foods and model systems. In Water Relations in Foods. R. Duckworth, editor. Academic Press.

Ellis, G.P. 1959. The Maillard Reaction. In Adv. in Carbohydrate Chem. 14:63.

Eskin, N., H. Henderson and R. Townsend. 1971. Browning Reactions in Foods. In Biochemistry of Foods. Academic Press, NY.

Evancho, G.M., H.D. Hurt, P.A. Devline, and D.H. Ashton. 1976. Comparison of tetrahymena pyriformis W and rat bioassays for the determination of protein quality. Paper presented at Institute of Food Technologist, Anaheim, CA. June 6-9th.

Finot, P.A. 1973. Non-enzymic Browning. In Proteins in Human Nutrition. J.W.G. Porter and B.A. Rolls, editors. Academic Press, NY. 34:501.

Folk, J.E. 1956. The influence of the lysine-glucose reaction on enzymatic digestion. Arch. Biochem. and Biophy. 64:6.

Gal, S. 1975. Recent Advances in Techniques for the Determination of Sorption Isotherms. In Water Relations of Foods. R. Duckworth, editor. Academic Press.

Goodhart, R.S. and M.E. Shils. 1974. Modern Nutrition in Health and Disease. C. Lea and H. Febiger, editors. Philadelphia, PA.

Greenshields, R. and A. MacGillivray. 1972. Caramel. Part 1: The Browning Reactions. Process Biochem. Dec. pg. 11.

Gutschmidt, J. 1974. The storage life of frozen chicken with regard to the temperature in the cold chain. Lebensmitt Wiss u-Tech. 7:137.

Hall, R.J., N. Trinder and D.I. Givens. 1973. Observations on the use of 2,4,6-trinitrobenzene-sulfonic acid for the determination of available lysine. In Animal Protein Concentrations Analyst. 98:673.

Hannan, R.S. and C.H. Lea. 1952. Studies of the reaction between proteins and reducing sugars in the dry state. Biochem. et. Biophys. Acta. 9:293.

Haugaard, G., L. Tumerman and H. Silvestri. 1951. A study on the reaction of aldoses and amino acids. J. Amer. Chem. Sco. 73:4594.

Heller, B., M. Chutkow, C. Lushbough, A. Stedler, and B. Schweigert. 1961. Effect on heat treatment on lysine in meat. J. Nutr. 73:113.

Hendel, C.E., Silveira, V. and W.O. Harrington. 1955. Rates of non-enzymatic browning of white potato during dehydration. Food Tech. 2:433.

Henry, K., S.K. Kon, C.H. Lea and J.C.D. White. 1948. Deterioration in storage of dried skim milk. J. Dairy Res. 15(3):292.

Herrman, J. and W. Andrae. 1963. Oxydative abbauprodukte der L-ascorbinsaure. I. Papierchromatographischer Nashweis. Nahrung. 7:243.

Hodge, J.E. 1953. Dehydrated foods. Chemistry of browning reactions in model systems. J. Agric. and Food Chem. 1(15):928.

Hodge, J.E. 1967. Nonenzymatic Browning Reactions. In Chemistry and Physiology of Flavors. AVI Westport, Conn .

Holsinger, V.H., L.P. Posati and M.J. Pallansch. 1970. Anamolous results obtained in the determination of available lysine in casein using TNBS. J. Dairy Sci. 53:1638.

Hurrell, R.F. and K.J. Carpenter. 1974. The reactive lysine content of heat-damaged material as measured in different ways. Brit. J. Nutr. 32:589.

Jokinen, J.E., G. Reineccius and D.R. Thompson. 1976. Losses in available lysine during thermal processing of soy protein model systems. J. Food Sci. 41:816.

Kakade, M.L. and I.E. Liener. 1969. Determination of available lysine in proteins. Anal. Biochem. 27:273.

Karel, M. and T.P. Labuza. 1968. Non-enzymatic browning in model systems containing sucrose. J. Agr. and Fd. Chem. 16:717.

Labuza, T.P. 1968. Sorption phenomena in foods. Food Tech. 22:263.

Labuza, T.P. 1971. Properties of water as related to the keeping quality of foods. Paper presented at Third International Congress of Food Science and Technology. Washington, D.C. Aug. 9-14th. pp. 618-635.

Labuza, T.P., S. Mizrahi and M. Karel. 1972. Mathematical models of flexible film packaging of foods for storage. Trans. Am. Soc. Ag. Eng. 15:150.

Labuza, T.P. 1973. Optimization and storage stability of intermediate moisture foods. NASA Contract Report. Lyndon Johnson Space Center, Houston, Texas.

Labuza, T.P. 1974. Sorption Phenomena in Foods: Theoretical and Practical Aspects. In Theory, Determination and Control of Physical Properties of Food Materials. C. Rha, editor. D. Reidel Press, Holland.

Labuza, T.P. 1975. Interpretation of sorption data in relation to the state of constituent water. In Water Relations of Foods. R. Duckworth, editor. Academic Press.

Labuza, T.P. 1975. Final Report. Contract NAS-12560. National Aeronautics and Space Administration. Food and Nutrition Office. Houston, Texas. Aug. 1974 to Aug. 1975. pp. 126-215.

Labuza, T.P. 1976. Determination of Models for Shelf-life Testing. 1st International Congress on Food Engineering. Boston, MA.

Landers, R.E. 1975. Relationship between protein efficiency ratio of foods and relative nutritional value measured by Tetrahymena pyriformis W bioassay techniques. In Protein Nutritional Quality of Foods and Feeds. Part I. Assay methods - biological, biochemical and chemical. M. Friedman, editor. Marcel Dekker, Inc., NY.

Lea, C.H. and R.S. Hannan. 1949. Studies of the reaction between proteins and reducing sugars in the "dry" state. 1. The effect of activity of water, of pH and of temperature on the primary reaction between casein and glucose. Biochim. Biophys. Acta. 3:313.

Lea, C.H. and R.S. Hannan. 1950a. Studies of the reaction between proteins and reducing sugars in the "dry" state. 2. Further observations on the formation of the casein-glucose complex. Biochim. et Biophys. Acta. 4:518.

Lea, C.H. and R.S. Hannan. 1950b. 3. Nature of the protein groups reacting. Biochim. et Biophys. Acta. 5:433.

Lea, C.H., R.S. Hannan and D.N. Rhodes. 1951. Studies of the reaction between proteins and reducing sugars in the "dry" state. Biochim. et Biophys. Acta. 5:433.

Lea, C.H. and D.N. Rhodes. 1952. Studies of the reaction between proteins and reducing sugars in the dry state. Biochim. et. Biophys. Acta. 9:56.

Lea, C.H. 1958. Chemical changes in the preparation and storage of dehydrated foods. In Fundamental Aspects of the Dehydration of Foodstuffs. Soc. Chem. Ind. London. p. 178.

Lea, C.H., L. Parr and K.J. Carpenter. 1958. Chemical and nutritional changes in stored herring meal. Br. J. Nutr. 12:297.

Lea, C.H., L.J. Parr and K.J. Carpenter. 1960. Chemical and nutritional changes in stored herring meal. Br. J. Nutr. 14:91.

Lee, S. and T.P. Labuza. 1975. Destruction of ascorbic acid as a function of water activity. J. Food Sci. 40:370.

Loncin, M., J.J. Bimbenet, and J. Lenges. 1968. Influence of the activity of water in the spoilage of foodstuffs. J. Fd. Tech. 3:131.

Maillard, L.C. 1912. Action of amino acids on sugars. Compt. Rend. 154:66.

Markova, J., E. Honischova and J. Hampl. Non-enzymatic reactions in cereal production. Scientific Papers of Institute of Chem. Tech. Food. Prague E-33, Czechoslovakia.

Mauron, J., F. Mottu, E. Bujard, and E.H. Egli. 1955. The availability of lysine, methionine, and tryptophan in condensed milk and milk powder. In vitro digestion studies. Arch. Biochem. and Biophys. 59:433.

Mauron, J. and F. Mottu. 1958. Relationship between in vitro lysine availability and in vivo protein evaluation in milk powders. Arch. of Biochem. and Biophys. 77:312.

McWeeney, D.J. and H. Burton. 1963. Some possible glucose/glycine browning intermediates and their reactions with sulfites. J. Sci. Food Agr. 14:291.

Milner, C.K. and D. Westgarth. 1973. A collaborative study on the determination of available lysine in feeding stuffs. J. Sci. Food Agr. 24:873.

Mizrahi, S., T.P. Labuza and M. Karel. 1970a. Computer aided predictions on extent of browning in dehydrated cabbage. J. Food Sci. 35:799.

Mizrahi, S., T.P. Labuza and M. Karel. 1970b. Feasibility of accelerated tests for browning in dehydrated cabbage. J. Food Sci. 35:804.

Ousterhout, L.E. and E.M. Wood. 1970. Available lysine in fishmeals. Poultry Sci. 49:1423.

Patton, S. 1955. Browning and associated changes in milk and its products. J. Dairy Sci. 38:457.

Reynolds, T.M. 1963. Chemistry of non-enzymatic browning. Adv. Food Res. 12:1.

Reynolds, T.M. 1965. Chemistry of non-enzymatic browning. Adv. Food Res. 14:168.

Rao, S.P., F.L. Carter and F.L. Frampton. 1963a. Determination of available lysine in oilseed meal proteins. Anal. Chem. 35:1927.

Rao, M.N., H. Sreenivas, M. Swaninathon, K.J. Carpenter and C.B. Morgan. 1963b. The nutritionally available lysine and methionine of heated casein-glucose mixtures. J. Sci. Fd. Agr. 14:544.

Rao, N.M. and M.M. Rao. 1972. Effect of non-enzymatic browning on the nutritive value of casein-sugar complexes. J. Fd. Sci. and Tech. (Mysore). 9:66.

Roach, A.G., P. Sanderson, D. Williams. 1967. Comparison of methods for the determination of available lysine in animal and vegetable sources. J. Sci. Food Agr. 18:274.

Rockland, L.B. 1969. Water activity and storage stability. Food Tech. 23:1241.

Salwin, H. 1959. Defining minimum moisture contents for dehydrated foods. Food Tech. 13:594.

Schnickels, R.A., H.C. Warmbier and T.P. Labuza. 1976. Effect of protein substitution on non-enzymatic browning in an intermediate moisture food. J. Agr. Food Chem. (IN PRESS).

Schonberg, A. and R. Moubacher. 1952. The Strecker degradation of α-amino acids. Chem. Rev. 50:261.

Sharp, J.G. 1957. Deterioration of dehydrated meat during storage. J. Sci. Food Agr. 8(1):21.

Sharp, J.G. and E.J. Rolfe. 1958. Deterioration of dehydrated meat during storage. In Fundamental Aspects of Dehydration of Foodstuffs. Soc. Chem. Ind. London. pg. 197.

Shorrock, C. and J.E. Ford. 1973. An improved procedure for the determination of available lysine and methionine with tetrahymena. In Proteins in Human Nutrition. J.W.G. Porter and B.A. Rolls, editors. Academic Press, NY. 207-223.

Sloan, A.E. and T.P. Labuza. 1975. Investigating alternative humectants for foods. Food Prod. Devel. 9(9):75.

Song, P.S. and C.O. Chichester. 1966. Kinetic behavior and mechanism of inhibition in the Maillard reaction. J. Fd. Sci. 31:906.

Song, P.S., C.O. Chichester and F.H. Stadtman. 1966. Kinetic behavior and mechanism of inhibition in the Maillard reaction. J. Fd. Sci. 31:914.

Song, P. and C.O. Chichester. 1967a,b. Kinetic behavior and mechanism of inhibition in the Maillard reaction. J. Fd. Sci. 32(98):107.

Spark, A.A. 1969. Role of amino acids in non-enzymatic browning. J. Sci. Fd. Agr. 20:308.

Sullivan, J.F., R. Konstance, M. Calhoun, F. Talley, J. Cording and O. Panasiuk. 1974. Flavor and storage stability of explosion puffed potatoes - NEB. J. Fd. Sci. 39:58.

Taher, A.M. and D. Cates. 1974. A spectrophotometric investigation of the yellow color that accompanies the formation of furan derivatives in degraded sugar solutions. Carohy. Res. 34:249.

Tarr, H.L. 1954. The Maillard reaction in flesh foods. Food Tech. 1:15.

Tu, A. and N.A.M. Eskin. 1973. The inhibitory effect of reducing sugar on the hydrolysis of casein by trypsin. Can. Inst. Food Sci. Tech. J. 6(1):50.

Waletzko, P.T. and T.P. Labuza. 1976. Accelerated shelf-life testing of an intermediate moisture food in air and in an oxygen free atmosphere. J. Fd. Sci. 41.

Warmbier, H.C. 1975. Non-enzymatic browning kinetics of an intermediate moisture model food system. Ph.D. Thesis. University of Minnesota. St. Paul, MN.

Warmbier, H.C., R.A. Schnickles, and T.P. Labuza. 1976. Effect of glycerol on non-enzymatic browning in a solid intermediate moisture model food system. J. Fd. Sci. 41:528.

Warren, R. 1976. Evaluation of a biological technique using tetrahymena for determination of lysine loss during non-enzymatic browning. M.S. Thesis. University of Minnesota. St. Paul, MN.

Warren, R. and T.P. Labuza. 1976. Comparison of chemically measured available lysine with relative nutritive value measured by a Tetrahymena bioassay during early stages of non-enzymatic browning. J. Fd. Sci. (IN PRESS).

Yanagita, T. M. Sugano, S. Cho and M. Wada. 1973. Changes in available lysine and in vitro digestability of casein accompanied with oxidation of ethyl linoleate. Agr. Chem. (Japan) 47:73.

26

OXIDATIVE BROWNING OF AMADORI COMPOUNDS FROM AMINO ACIDS AND PEPTIDES

Hironaga Hashiba, Ikunori Koshiyama and Danji Fukushima

Noda Institute for Scientific Research

Noda-shi, Chiba-ken, Japan

ABSTRACT

The oxygen-dependent browning (oxidative browning) of Amadori compounds from amino acids or peptides was studied since it was an important reaction contributing to the discoloration and deterioration of some foodstuffs containing sugars and amino compounds during storage in contact with atmospheric oxygen.

The browning of fourteen Amadori compounds derived from amino acids and peptides was determined under the influence of metals or oxygen. All Amadori compounds exhibited remarkable browning during storage at 37°C for 5 days when both 40 ppm of Fe^{2+} and oxygen were present, but exhibited little browning without Fe^{2+} or oxygen. Every mixture of a parent sugar and amino compound showed no browning even though Fe^{2+} and oxygen were present. In particular, Amadori compounds composed of aromatic or heterocyclic amino acids were very reactive in oxidative browning, and this type of browning was synergistically accelerated by the presence of both Fe^{2+} and Mn^{2+}. The Amadori compound derived from pentose such as xylulose-glycine browned more rapidly than that from hexose such as fructose-glycine. Oxygen was thought to accelerate the breakdown of Amadori compounds to liberate parent amino acids and glucosone in the oxidative browning reaction.

In the browning reaction between glucose and seven peptides, glycylglycine, glycylleucine, leucylglycine, glycyltyrosine, glycylphenylalanine, glycylproline and glycylglycylglycine, the liberation of C-terminal amino acids by the cleavage of peptide bonds was observed. The amino acids were suggested to be liberated from the peptide in Amadori compounds, because the peptide bond in Amadori compounds was found to be more labile than that of free peptide.

INTRODUCTION

Oxygen accelerates the darkening of many foodstuffs; therefore, oxygen-dependent browning has been studied by many workers using materials such as ascorbic acid (Joslyn, 1957; Clegg, 1964), polyphenols (Burton et al., 1963), and furfural (Dunlop et al., 1946). Soy sauce which contains much sugars, amino acids and peptides also darkens in contact with atmospheric oxygen even though the color of soy sauce increases very scarecely under anaerobic conditions (Figure 1). The oxidative browning of soy sauce is considered to have a different mechanism from those of ascorbic acid, polyphenols and furfural, because the amount of these compounds in soy sauce is very small (Omata et al., 1955). The authors have investigated factors contributing to the oxidative browning of soy sauce, and suggested that Amadori compounds and heavy metals such as Fe^{2+} and Mn^{2+} plays important roles in the oxidative browning of soy sauce (Hashiba, 1976). Amadori compounds and the metals were considered to participate in oxidative browning of many other foods. From this point of view, this paper mainly deals with the examination of the browning of some Amadori compounds derived from amino acids and peptides.

In addition, this paper also deals with the utilization of Amadori compounds by yeasts in soy sauce mash to investigate the change of nutritive value of food by sugar-amine browning reaction.

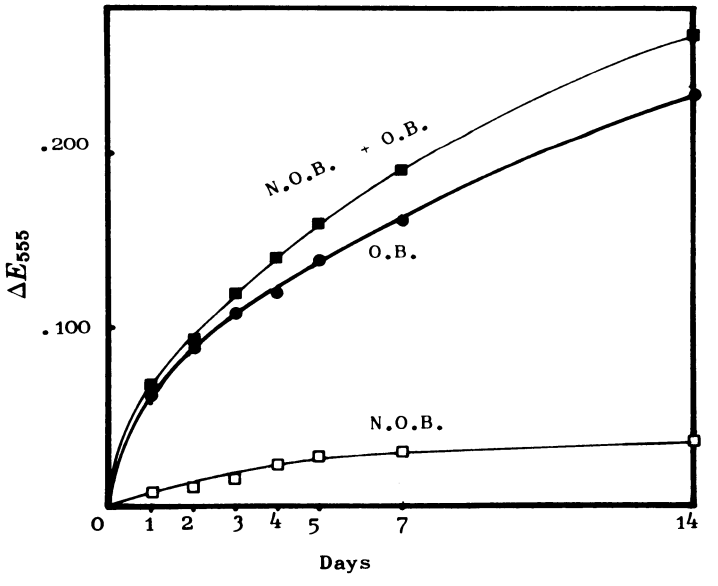


Figure 1. Oxidative and nonoxidative browning of soy sauce: O.B., oxidative browning; N.O.B., nonoxidative browning. The method of measurement of oxidative and nonoxidative browning is described in a reference (Hashiba, 1976).

RESEARCHES ON UNKNOWN SUBSTANCES CONTRIBUTING TO OXIDATIVE BROWNING OF SOY SAUCE

(I). Effect of Metals on the Oxidative Browning of Soy Sauce

Effect of iron on the amino-carbonyl browning reaction. It is well known that iron accelerates amino-carbonyl browning reaction (Ellis, 1959; Markuze, 1963; Umemoto et al., 1970). The effect of Fe^{2+} and oxygen on the browning reaction is shown in Figure 2. Fe^{2+} increases browning in the presence of oxygen. However, Fe^{2+} has no effect on the browning without oxygen. Generally, cereals, animals and fish have a tendency to accumulate heavy metals such as iron. Therefore, they would contain appreciable amount of iron (Clifcorn, 1948; Somers and Beeson, 1948; Davidson et al., 1959; Fox and Cameron, 1961). Iron plays an important role in the oxidative discoloration of many foods made from plants and aminals (Coulter et al., 1951; Kato and Sakurai, 1964; Sato and Tadenuma, 1967; Mathew and Pappia, 1971).

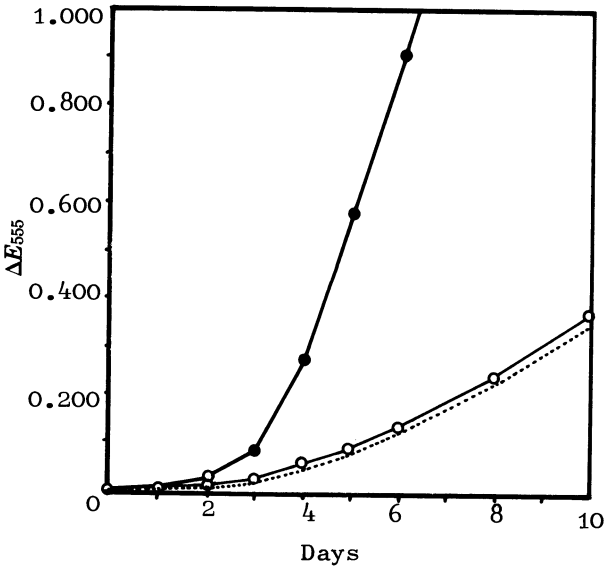


Figure 2. Effect of iron and oxygen on the browning of glucose-glycine model systems. Mixture solutions of 1 M glucose and glycine were held at 50°C. ----, Control contact with oxygen; ●, Control plus 40 ppm of Fe^{2+} contact with oxygen; o, Control plus 40 ppm of Fe^{2+} not contact with oxygen.

Effect of trace metals on the oxidative browning of soy sauce. It is unconvenient to examine the effect of Fe^{2+} on the oxidative browning of soy sauce by adding Fe^{2+} to soy sauce, because soy sauce contains 30 ~ 40 ppm of Fe^{2+} on the average. Therefore, Fe^{2+} free soy sauce is necessary to investigate an exact effect of Fe^{2+}, if Fe^{2+} increases browning in low concentration such as below 30 ppm. Fe^{2+} free soy sauce (concentration of $Fe^{2+} < 1$ ppm) is prepared by the treatment with a chelating resin, Dowex A-1 (Hashiba et al., 1970). Table I shows that the change of main constituents of soy sauce is very little, but only Fe^{2+} is removed almost completely by the treatment with the resin. It is an evidence of unchangeability of main constituents in soy sauce that the browning of treated soy sauce on heating is almost the same as that of untreated soy sauce.

Table I. Changes of Main Constituents in Soy Sauce by the Treatment with Dowex A-1 Resin

	T.N.	F.N.	R.S.	T.A.	Fe^{2+}	Heat browning (ΔE_{470})[a]
Untreated soy sauce	1.52	0.88	4.2	14.7	34 ppm	0.365 (100 %)
Treated soy sauce	1.48	0.87	4.1	14.0	<1 ppm	0.345 (95 %)

Abbreviations used were: T.N.= Total nitrogen (%); F.N.= Formol nitrogen (%); R.S.= Reducing sugar (%); T.A.= Total acid, ml of 1/20 N NaOH titrated for neutralization. [a]Net increase of color on heating at 80°C for 5 hr. The methods of analyses were based on a reference (Hashiba et al., 1970).

Oxidative browning of the metal free soy sauce is measured by the same method as Figure 1 (Figure 3). In contrast to the heat browning in Table I, oxidative browning of metal free soy sauce is about 30 % of the original untreated soy sauce. When some main metals were added to the treated soy sauce up to the same concentration of each metal as originally contained before the treatment, the oxidative browning of the metal free soy sauce is accelerated only by Fe^{2+} and Mn^{2+}, and other trace metals in soy sauce give very little effect on the oxidative browning. These experimental facts suggest that unknown substances contributing to oxidative browning remain in metal free soy sauce, even though soy sauce is treated with Dowex A-1 resin, and that these substances develop oxidative browning remarkably in the presence of Fe^{2+} and Mn^{2+}, but little without those metals.

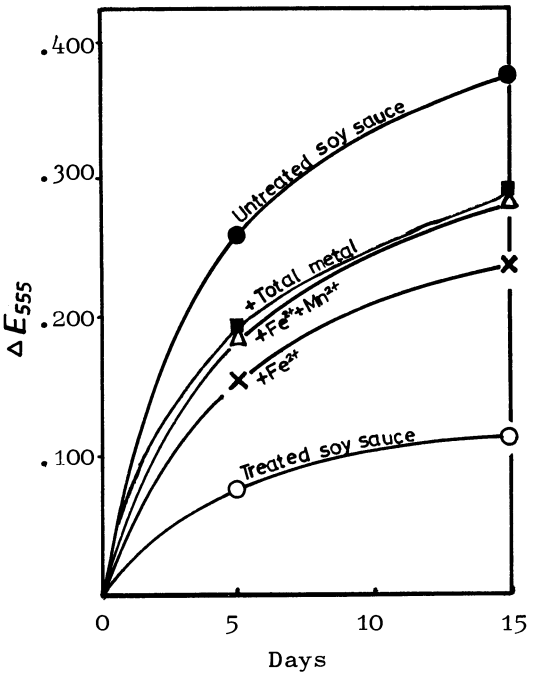


Figure 3. Effect of metals on the oxidative browning of metal free soy sauce prepared with Dowex A-1 resin. ■, △, ×; Total metal of soy sauce, 40 ppm of Fe^{2+} and 30 ppm of Mn^{2+}, and 40 ppm of Fe^{2+}were added to metal free soy sauce, respectively. Total metal was obtained as follows; after the removal of metals from soy sauce with the resin, the adsorbed metals on the resin was eluted by 1 N HCl, and the eluent was dried and ashed.

(II). Use of Ion-Exchange Resins to Identify the Unknown Substances Involved in Oxidative Browning

Fractionation of soy sauce by ion-exchange resins. Pasteurized soy sauce was fractionated with the method of Hashiba (1973) as shown in Figure 4. Total metal in soy sauce is obtained using Dowex A-1 resin. The cation fraction (C-fraction), neutral fraction (N-fraction) and anion fraction (A-fraction) contained the most part of the recovered nitrogen, sugar and organic acid, respectively. The oxidative browning of every fraction and combined fractions was shown in Table II. C-fraction darkened by itself, although it contained little sugar. (A + N)-fraction, A-fraction and N-fraction did not undergo much browning. Table II indicates that the unknown substances which play an important role in oxidative browning together with metals exist in C-fraction. The roles of organic acids and neutral sugars are minor in oxidative browning, because (A + N)-fraction is not related so much to oxidative browning.

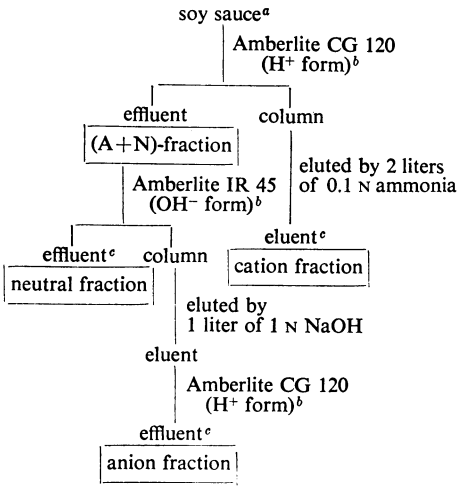


Figure 4. Fractionation of soy sauce by ion-exchange resins. [a]Fifty ml of soy sauce, diluted to 500 ml with distilled water was passed over the resin. [b]Column size was 2.5 X 50 cm. [c]Each fraction was lyophilized and adjusted to the original volume of 50 ml with distilled water.

Table II. Oxidative Browning of the Fractions and a Model System

	ΔE_{555}	
	5 days	14 days
C-fraction	0.050	0.115
A-fraction	0.000	0.001
N-fraction	0.000	0.000
C-fraction + total metal	0.295	0.460
(A+N)-fraction + total metal	0.002	0.004
C- + (A+N)-fraction	0.055	0.125
C- + (A+N)-fraction + total metal	0.285	0.470
MS I[a] + total metal	0.000	0.000
MS I + (A+N)-fraction + total metal	0.008	0.025
Original soy sauce	0.400	0.580

[a]MS I is a model system containing the same amount of amino acids as C-fraction.

Investigation of the constituents in C-fraction. C-fraction contained almost all of the amino acids in soy sauce. Therefore, it is considered that the contribution of C-fraction to oxidative browning is due to the amino acids. To make sure of the relation of amino acids to oxidative browning, oxidative browning of a model system (designated as MS I; Hashiba, 1973) composed of the same amount of amino acids in C-fraction is examined. Oxidative browning of MS I with and without any fractions is shown in Table II. MS I did not brown by itself. Although (A+N)-fraction and total metal are added to MS I, browning did not occur to such a extent as C-fraction. Therefore, it is suggested that the important substance in oxidative browning is not an amino acid. From Table II, it is concluded that organic acids, neutral sugars and amino acids, which have been regarded as important substances in browning reaction, are not directly related to oxidative browning of soy sauce. The amounts of other factors, such as carbonyl compounds, reducing substances and reductones, reported by many workers to be involved in browning are shown in Table III. Ferricyanide-reducing substances which have been reported by Hodge (1962) and Adachi (1958) as major precursors in browning reaction were recovered in C-fraction. It is considered that the reason why C-fraction darkened more remarkably than the model system might be due to the reducing substances.

Although the major part of the carbonyl compounds in soy sauce was recovered in (A+N)-fraction, the contribution of (A+N)-fraction to oxidative browning is very small. Therefore, the participation of carbonyl compounds in soy sauce should be minor in oxidative browning. Hashiba (1976) has suggested that the browning of α-hydroxycarbonyls, which are well known as highly reactive compounds in browning reaction (Hodge, 1953), does not increase by oxygen or metals appreciably. The carbonyl compounds have exhibited great browning in the presence of amino compounds even under anaerobic conditions.

Table III. Analyses of the Constituents in the Fractions

	3-DG[a]	T.C.[b]	R.S.[c]	O_2-uptake[d]	Reductone[e]
C-fraction	7	0.051	0.320	65	31
A-fraction	4	0.030	0.035	0	0
N-fraction	40	0.040	0.015	0	0
(A+N)-fraction	107	0.110	0.052	2	1
Model system (MS I)	0	0.000	0.000	0	0
Original soy sauce	120	0.159	0.510	77	180

[a] 3-Deoxyglucosone (ppm), determined by Kato's method (Kato et al., 1964). [b] Total carbonyls (E_{530}), determined by Peleg's method (Peleg and Mannheim, 1970). [c] Total reducing substances (E_{700}), determined by Adachi's method. [d] Microliters/2 ml of solution per 2 hr, determined by a Warburg manometer. [e] Absorbance at 530 nm, determined by Mitsuda's method (Mitsuda and Shikanai, 1957).

(III). Formation of Ferricyanide-Reducing Substances During the Aging of Model Systems

The effect of aging on the oxidative browning of model systems (Hashiba, 1974). To research the origin of the unknown substances contributing to oxidative browning, the authors examined the aging process of soy sauce mash with the production of the unknown substances. In this section, the effect of ageing on the oxidative browning of model systems was investigated to interpret the discrepancies between little browning of MS I and great browning of soy sauce. A model system containing similar amounts of amino acids and sugars to soy sauce was prepared (MS II). In addition, a simple glucose-glycine model system (0.5 M of glucose and glycine) and a more complicated model system, peptide solution (enzymic hydrolyzate of purified soy bean protein, added sugars; Okuhara et al., 1971) were prepared. The three model systems were aged (held) for 1 ~ 6 months at 30°C under anaerobic conditions. After the aging, oxidative browning of the model systems was measured by the ordinary method as described in Figure 1. As shown in Table IV, the oxidative browning of the model systems increased with increase of the aging.

Table IV. The Effect of Aging on the Oxidative Browning of Model Systems

Aging (months)	Oxidative browning[a] of model systems[b]		
	MS II	Glucose-glycine	Peptide solution
0	0.020	0.002	0.030
2	0.050	0.005	0.075
4	0.100	0.013	0.175
6	0.205	0.027	0.210

[a] Net oxidative browning (ΔE_{555}) during storage at 37°C for 5 days. [b] All model systems contain 40 ppm of Fe^{++}.

Substances produced in the model systems during aging. Table IV shows that some compounds related to oxidative browning are produced during aging. Hashiba (1974) has pointed out some relationship between aging period in soy sauce brewing and oxidative browning, and indicated that ferricyanide-reducing substances increased on increasing the aging period (Figure 5). In agreement with the results of Umemoto et al., (1970), Fe^{2+} did not affect the production of reducing substances.

To confirm the important role of ferricyanide-reducing substances in oxidative browning, the glucose-glycine model system was chromatographed on Sephadex G-10 before and after aging, and then

oxidative browning of each fraction was determined (Figure 6). A marked oxidative browning was observed in some fractions (around No. 31) in the case of the aged model system. Furthermore, the peak of ΔE_{555} (oxidative browning) corresponded closely to that of E_{700} (indicating reducing substances) and did not correspond to that of glycine. In the case of the unaged model system the oxidative browning of all fractions is very low (not shown in Figure 6).

The experimental data in Table IV, Figures 5 and 6 suggest that ferricyanide-reducing substances produced during aging of the model systems have significant effects on the oxidative browning reaction.

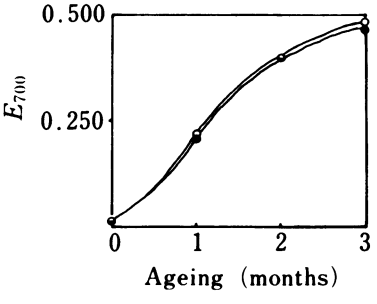


Figure 5. Time course of the formation of reducing substances during aging of MS II. o, MS II; ●, MS II added 40 ppm of Fe^{2+}.

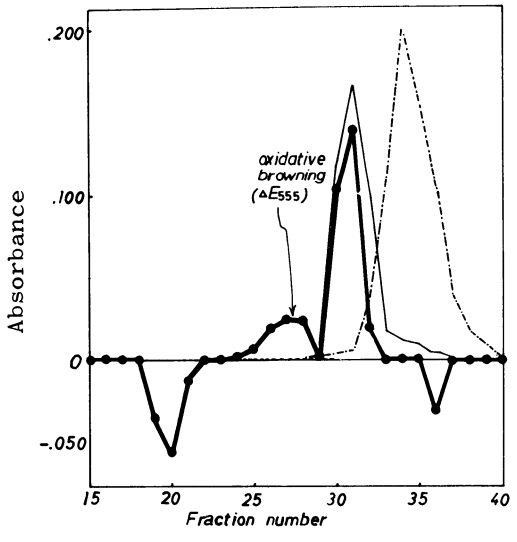


Figure 6. Gel filtration of aged glucose-glycine model system on Sephadex G-10. Column size, 2.5 X 100 cm; fraction volume, 10 ml; flow rate, 20 ml/hr. —, Reducing substances (E_{700}); -·-, glycine(E_{570}).

Identification of ferricyanide-reducing substances in the aged model systems. Cation fractions were obtained from the aged or unaged MS II and glucose-glycine model system by the similar method to Figure 4. The C-fractions from the aged model systems exhibited strong reducing power and great browning, whereas those from the unaged model systems did not brown (Hashiba, 1974). This fact suggests that ferricyanide-reducing substances produced during aging of the model systems and contributing to oxidative browning were recovered in the C-fraction. Therefore, the C-fractions from the aged model systems were investigated by paper chromatography (Figure 7) and an amino acid analyzer (Figure 8). Paper chromatography shows that only one unknown spot, which was positive to KIO_4-tetrabase reagent (Yoda, 1952), was produced during the aging of glucose-glycine model system and the spot increased in size and intensity on further aging. Many spots were observed in the C-fraction from soy sauce and the aged MS II, but no spot was detected in the C-fraction from the unaged model systems.

The automatic amino acid analyzer demonstrates that one ninhydrin positive unknown peak increased in height during the aging of the glucose-glycine model system (Figure 8). The unknown spot in Figure 7 and the unknown peak in Figure 8 were isolated and identified as an Amadori compound, 1-deoxy-1-glycino-D-fructose (fructose-glycine; F-Gly).

The oxidative browning of F-Gly is very remarkable (Figure 9). This result suggests that in oxidative browning of the aged glucose-glycine model system F-Gly formed during the aging plays an important role. Consequently, Amadori rearrangement products are thought to be prominent precursors in oxidative browning.

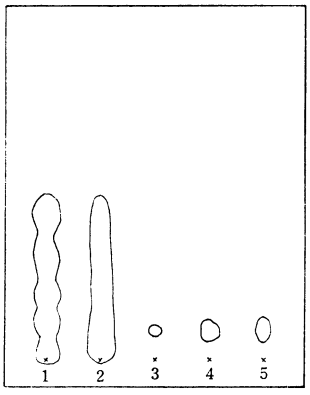


Figure 7. Paper chromatography of sugars in the C-fractions. 1, C-fraction from MS II (after ageing for 3 months); 2, from soy sauce; 3, from glucose-glycine model system (after aging for 1 month); 4, from glucose-glycine model system (after aging for 3 months); 5, 1-deoxy-1-glycino-D-fructose. The mobile phase, butanol-acetic acid-water (4 : 1 : 1 v/v); the detection reagent, KIO_4-tetrabase.

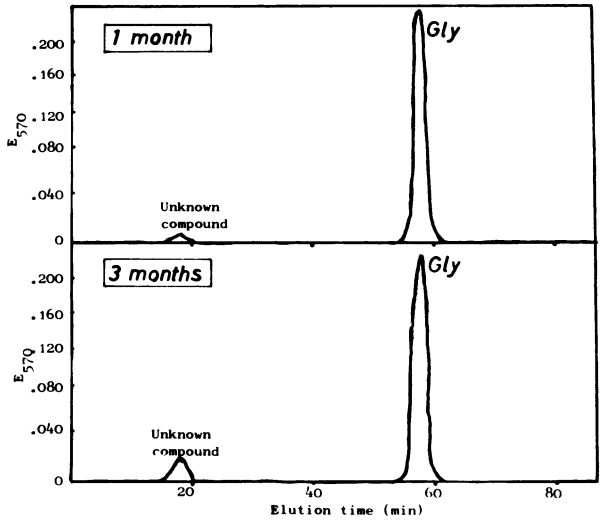


Figure 8. Effluent peaks obtained with the aged glucose-glycine model system: the glucose-glycine model system was held at 30°C under anaerobic conditions; Hitachi KLA-5 analyzer, 570 nm trace only.

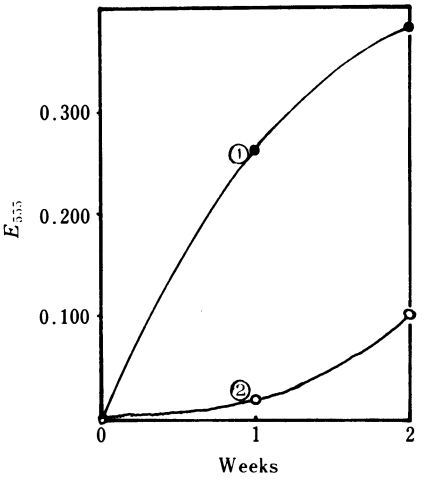


Figure 9. Oxidative browning of fructose-glycine. ①, The solution contained F-Gly (0.5 M), glucose (0.5 M) and glycine (0.5 M); ②, the solution contained 1 M of glucose and glycine. Both solutions contained 40 ppm of Fe^{2+}.

(IV). Isolation and Identification of Some Amadori Compounds from Soy Sauce

Preparation of the C-fraction from soy sauce. Ingles and Reynolds (1958) have determined Amadori compounds in freeze-dried apricots after storage for 12 months by ion-exchange chromatography. To make sure of the major participation of Amadori compounds in oxidative browning of soy sauce, isolation and identification of Amadori compounds in soy sauce were done. As shown in Table III, nearly all of Amadori compounds were recovered in the C-fraction. Therefore, the C-fraction was prepared and then applied to preparative ion-exchange chromatography (Hashiba, 1975). The fraction No. 10 ~ 20 containing notable amounts of Amadori compounds (Figure 10) were collected. Because main amino acids in soy sauce, for example, glutamic acid, glycine and alanine, were eluted around fractions No. 50 ~ 80, only small amounts of amino acids were contaminated in the fraction No. 10 ~ 20. Contaminated amino acids were removed by the gel filtration with Sephadex G-10.

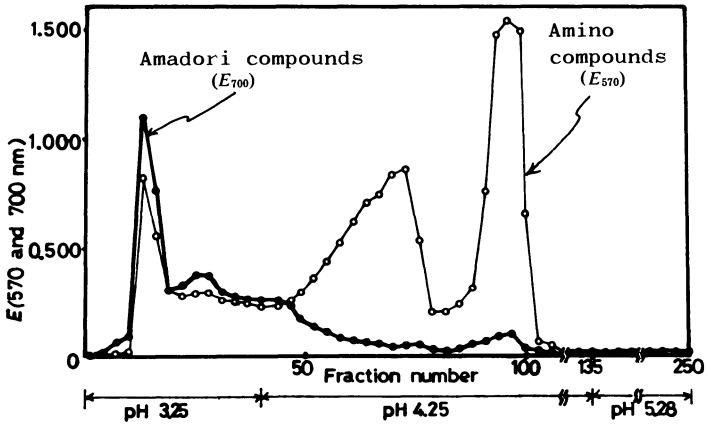


Figure 10. Purification of Amadori compounds in soy sauce by preparative ion-exchange chromatography.

Purification of Amadori compounds by paper chromatography. After the purification by Sephadex G-10, the sample was lyophilized and examined on paper chromatography (Figure 11). The sample was found to consist of four Amadori compounds (designated as SI, SII, SII and SIV). The four Amadori compounds were separated each other by paper chromatography and further treated with Sephadex G-10 to remove impurities. By the Rf on paper chromatography, the elution time in the amino acid analyzer, the liberation of a parent amino acid by acid hydrolysis (Figure 12), neutral equivalent and elementary analysis (except S IV), the compounds were identified as

fructose-glycine, fructose-alanine, fructose-valine, fructose-isoleucine and fructose-leucine. Although soy sauce had to contain many kinds of Amadori compounds, only five compounds derived from glucose and neutral amino acids were isolated. This is probably due to the facts that these Amadori compounds are comparatively stable and their elution time in the preparative ion-exchange chromatography is appreciably different from that of main amino acids in soy sauce.

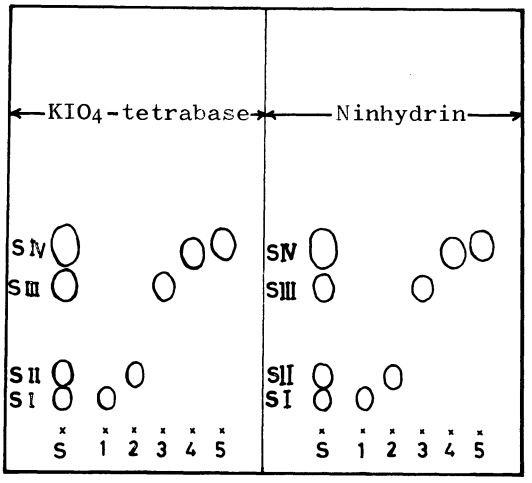


Figure 11. Paper chromatography of Amadori compounds obtained from soy sauce and standard synthesized Amadori compounds: S, Amadori compounds from soy sauce; 1, F-Gly; 2, F-Ala; 3, F-Val; 4, F-Ile 5, F-Leu. The mobile phase is the same as Figure 7.

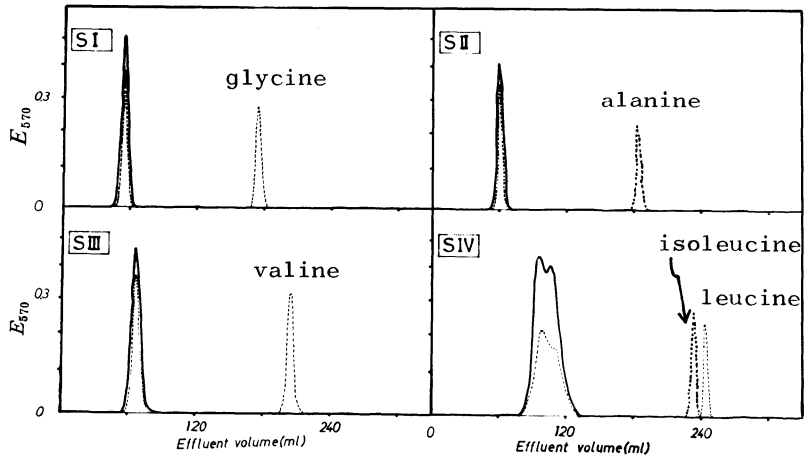


Figure 12. Effluent peaks obtained with the Amadori compounds in soy sauce (S I ~ S IV) before and after hydrolysis by 1 N H_2SO_4 at 100°C for 1 hr: —, before hydrolysis; ----, after hydrolysis.

STUDIES ON OXIDATIVE BROWNING OF AMADORI COMPOUNDS

(I). Oxidative Browning of Amadori Compounds from Amino Acids

Amadori compounds have been suggested to be important substances in oxidative browning of soy sauce. Generally, foods contain many kinds of amino acids and sugars (Wasserman and Spinelli, 1970; Hashiba, 1973). Therefore, many kinds of Amadori compounds must exist in such foods and participate in oxidative browning. From this point of view, this chapter deals with the examination of the browning of some Amadori compounds and discusses the oxidative degradation of Amadori compounds.

Synthesis and isolation procedure of Amadori compounds. The procedure was based on the method of Hodge (Hodge and Fisher, 1963) and Hashiba (Hashiba, 1976). Preparation and some characteristic properties of Amadori compounds are shown in Table V. Attempts to crystallize Amadori compounds were unsuccessful except for F-Gly, F-β-Ala and X-Gly. However, analytical data in Table V indicate that all prepared Amadori compounds were highly purified.

Oxidative browning of Amadori compounds. The browning reaction of Amadori compounds has been reported by many investigators (Hodge and Rist, 1953; Umemoto et al., 1970). However, the effect of oxygen on browning is obscure. Oxidative and nonoxidative browning of Amadori compounds are shown in Table VI. The Amadori compounds browned only slightly without oxygen, and in this case Fe^{2+} and Mn^{2+} did not affect the browning. On the other hand, the browning of all the Amadori compounds except F-Arg was increased very significantly when oxygen and Fe^{2+} were present. F-Arg browned notably without oxygen and decolorization was observed in the presence of oxygen. Each mixture of a parent sugar (glucose or xylose) and an amino acid did not brown under these aerobic or anaerobic conditions.

Previously, Hashiba et al., (1970) have reported that the oxidative browning of soy sauce is accelerated remarkably by the presence of Fe^{2+} and Mn^{2+} whose concentration in soy sauce are about 40 and 30 ppm, respectively. The accelerating effect of Mn^{2+} on sugar-amino browning reaction has scarecely been reported. On the contrary, Bohart and Carson (1955) have found inhibition of browning with Mn^{2+} in a glucose-glycine model system. Results in Table VI indicate that Mn^{2+} had little effect on the oxidative browning of the Amadori compounds by itself, but together with Fe^{2+}, Mn^{2+} accelerated synergistically the oxidative browning of F-Tyr, F-Phe, F-Trp and F-His. In the case of F-Arg, Mn^{2+} also appreciably accelerated the oxidative browning synergistically with Fe^{2+}. On the other hand, the oxidative browning of F-Gly, F-Lys, F-β-Ala and X-Gly was not affected and that of F-Ser, F-Glu and F-Leu was inhibited by the presence of Mn^{2+}. The effect of Mn^{2+} on the oxidative browning of sugar-amino acid system is not considered to be uniform with the amino acids employed.

Table V. Preparation and Some Characteristic Properties of Amadori Compounds

Amadori compd[a]	Reaction time[b]	Position of emergence[c]	Neut. equiv		Phenol-H_2SO_4 test[d]	Reduction of ferricyanide at 100°C[e]	Reduction of ferricyanide at 50°C[f]	Elementary anal. of Amadori compds, %					
								Carbon		Hydrogen		Nitrogen	
			Calcd	Found				Calcd	Found	Calcd	Found	Calcd	Found
F-Ser	1	Cys acid-Asp	267.1	267.3	6	115	113	40.5	40.3	6.42	6.43	5.24	5.23
F-Glu	1	Cys acid	309.3	309.6	7	112	111	42.7	42.6	6.20	6.14	4.53	4.40
F-Gly	1	Cys acid-Asp	237.2	237.2	12	110	100	40.5	40.5	6.37	6.37	5.91	5.90
F-Leu	2	Asp-Thr	293.2	293.3	13	83	107	49.2	49.1	7.91	7.92	4.78	4.77
F-Tyr	5	Pro-Gly	343.3	344.5	12	96	112	52.5	52.2	6.17	6.30	4.08	4.29
F-Phe	1	Pro-Gly	327.3	327.4	12	85	114	55.0	55.0	6.47	6.50	4.28	4.27
F-Trp	1	Leu-Tyr	366.3	369.5	17	110	109	55.7	55.5	6.05	6.32	7.65	7.91
F-Lys	0.5	Ala-Cys	308.3	309.3	17	98	97	46.8	46.9	7.85	7.92	9.09	9.21
F-His	0.5	Phe	317.3	317.6	13	123	125	45.4	45.4	6.04	6.15	13.2	13.3
F-Arg	1	Lys-NH_3	366.2	366.5	23	75	90	42.9	42.7	7.20	7.43	16.7	16.9
F-β-Ala	0.5	Val-Met	251.2	251.2	7	95	96	43.0	43.0	6.77	6.78	5.58	5.60
X-Gly	0.5	Cys acid-Asp	207.2	207.2	12	108	100	40.6	40.5	6.32	6.33	6.76	6.75
Glucose					100	100	0						

[a] Abbreviations used are: F-Ser, fructose-serine; F-Glu, fructose-glutamic acid; F-Gly, fructose-glycine; F-Leu, fructose-leucine; F-Tyr, fructose-tyrosine; F-Phe, fructose-phenylalanine; F-Trp, fructose-tryptophan; F-Lys, fructose-lysine; F-His, fructose-histidine; F-Arg, fructose-arginine; F-β-Ala, fructose-β-alanine; X-Gly, xylulose-glycine. [b] Hours in boiling water. [c] Position of emergence in Hitachi KLA-5 amino acid analyzer. Abbreviations used are: Cys acid, cysteic acid; Asp, aspartic acid; Thr, threonine; Pro, proline; Gly, glycine; Ala, alanine; Cys, cysteine; Val, valine; Met, methionine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; Lys, lysine. [d] Percent of glucose. [e] Percent of glucose by Somogyi-Nelson's method. [f] Percent of F-Gly by Adachi's method.

Table VI. Effect of Oxygen, Fe^{2+}, and Mn^{2+} on the Browning of Amadori Compounds

	Browning (ΔE_{555})[a]				
	Oxidative				
Compds[b]	None	+ Fe^{2+} [c]	+ Mn^{2+}[c]	+ Mn^{2+},[c] Fe^{2+}[c]	Non-oxidative[d]
F-Ser	0.000	0.036	0.002	0.023	0.003
F-Glu	0.000	0.030	0.000	0.015	0.002
F-Gly	0.000	0.100	0.000	0.100	0.003
F-Leu	0.000	0.025	0.000	0.010	0.000
F-Tyr	0.003	0.385	0.020	0.620	0.005
F-Phe	0.003	0.150	0.004	0.260	0.004
F-Trp	0.025	0.230	0.020	0.350	0.010
F-His	0.006	0.210	0.007	0.320	0.008
F-Lys	0.005	0.045	0.003	0.046	0.010
F-Arg	−0.110	0.000	−0.100	0.050	0.115
F-β-Ala	0.006	0.095	0.005	0.096	0.003
X-Gly	0.006	0.120	0.005	0.120	0.020
Mixture of parent materials[e]	0.000	0.000	0.000	0.000	0.000

[a] Samples were stored at 37°C for 5 days, and browning was measured after diluting ninefold water. [b] Concentration of compounds is 0.2 M in water. Abbreviations used are the same as Table V. [c] Concentrations of Fe^{2+} and Mn^{2+} are 40 and 30 ppm, respectively. [d] In this case Fe^{2+} and Mn^{2+} had no effect on browning. [e] All the combinations of a parent sugar (0.2 M) and an amino acid (0.2 M) for Amadori compounds in this table showed no browning under the conditions.

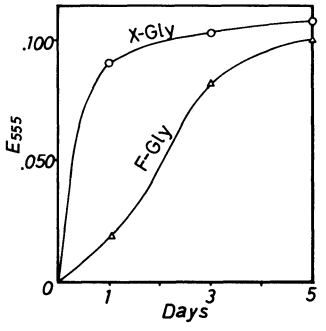


Figure 13. Comparison of the oxidative browning of F-Gly and X-Gly. The method of measurement of oxidative browning is the same as that in Figure 1.

Contrary to many reports (Lewis and Lea, 1950; Spark, 1969) that xylose is more reactive than glucose in the browning reaction, the oxidative browning of X-Gly is close to that of F-Gly as shown in Table V. However, a more detailed investigation exhibited that the rate of the browning reaction of X-Gly is much higher than F-Gly in an early stage. Figure 13 shows that the amount of the browning occurring in one day is six times as much for X-Gly as for F-Gly. Amadori compounds from pentose are presumed to be more reactive in an initial velocity than those from hexose in the oxidative browning reaction.

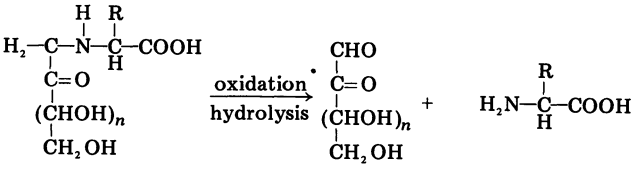
Dgradation of F-Gly and F-Phe by oxygen. In order to relate the high oxidative browning of Amadori compounds and their susceptibility to oxygen, the changes of two Amadori compounds during storage under anaerobic or aerobic conditions were compared. F-Gly and F-Phe were chosen as representative Amadori compounds of the simplest and aromatic amino acid, respectively. Degradation products of the Amadori compounds are shown in Table VII. F-Gly and F-Phe were determined by the amino acid analyzer, and glucosone and 3-deoxyglucosone (3-DG) were determined by Kato's method (Kato, 1962). F-Gly and F-Phe were decomposed by oxygen and a free amino acid (glycine or phenylalanine) and glucosone were found to be produced. The amount of 3-DG produced under aerobic conditions was larger than that under anaerobic conditions. However, even under the latter conditions the amount of 3-DG was appreciable. The results of Table VII also support the idea that Amadori compounds were closely related to the browning reaction in the presence of oxygen. One of the interactions of oxygen to the degradation of Amadori compounds is considered as scheme I.

Table VII. Effect of Oxygen on the Degradation of F-Gly and F-Phe

	Conditions of storage	Remaining Amadori compounds[b]	Liberated amino acid[b]	Glucosone[c]	3-DG[c]
F-Gly[a]	Anaerobic	120	4	0.060	0.360
	Aerobic	80	7	0.200	0.540
F-Phe[a]	Anaerobic	75	20	0.090	1.900
	Aerobic	36	31	0.410	2.600

[a] Water solutions of Amadori compounds (0.2 M) were stored at 37°C for 2 months. [b] Micromoles per milliliter.
[c] E_{435}, 0.05 ml of samples was used.

Scheme I



(II). Oxidative Browning of Amadori Compounds from Peptides

In last section, the authors have showed the great browning reaction of Amadori compounds from amino acids. However, the oxygen-dependent amino-carbonyl browning reaction is not considered to be completely due to amino acids. Some foodstuffs which contain peptides and sugars darken during storage in contact with atmospheric oxygen. This darkening had to be partially due to a sugar-peptide reaction. Hitherto studies on amino-carbonyl browning reaction in foods or model systems have been concentrated on amino acids, amines and proteins (Hodge, 1953; Reynolds, 1965; Talley and Porter, 1968; Hannan and Lea, 1952), but not on peptides.

Some kinds of sugar-amino acid condensation products such as Amadori compounds or N-glycosides were isolated (Gottschalk, 1952; Abrams et al., 1955) as intermediates of the browning reaction in model systems, but little was reported in sugar-peptide systems. Recently, Prey and Petershofer (1968) and Chuyen et al. (1973) identified some pyrazinones from glyoxal-peptides reactions, but the relations of these pyrazinones to the browning reaction have not been examined.

In this section, the reactivity of peptides for browning was compared with that of amino acids to study the sugar-peptide oxidative browning reaction. Furthermore, an intermediate in the browning reaction between glucose and diglycine (glycylglycine) was isolated and identified to investigate the effect of oxygen on the browning reaction.

Comparison of the reactivity on glucose and the amount of browning between peptides and amino acids. Okuhara et al. (1971) suggested that the rapid browning of soy sauce is caused by peptide-sugar reaction. Chuyen et al. (1973) compared the reactivity of peptides with that of amino acids (Table VIII), and concluded that the peptides are more reactive than the amino acids. In addition, they suggested that pK value of amino groups play a main role in the reactivity of amino compounds.

When glycine, diglycine, triglycine (glycylglycylglycine), leucine and glycyl-leucine were heated with glucose, consumption of peptides is greater than that of amino acids (Table IX), The reaction speed of peptides is undoubtedly more rapid than that of amino acids in the heat browning.

Table VIII Browning Reaction of Amino Acids or Peptides[a]

	Browning(E_{470}) on heating	
	15 min	30 min
Ala + glyoxal	0.012	0.057
Ala[b] + glyoxal	0.033	0.155
Ala-Ala + glyoxal	0.100	0.480
Gly + glyoxal	0.032	0.160
Gly[b] + glyoxal	0.092	0.340
Gly[c] + glyoxal	0.189	0.505
Gly-Gly + glyoxal	0.750	1.400
Gly-Gly-Gly + glyoxal	0.650	0.950
Gly-Gly-Gly-Gly + glyoxal	0.780	1.110
Carmosine + glyoxal	0.120	0.285
Balenine + glyoxal	0.005	0.025
Ala + acetaldehyde	0.000	0.000
Ala-Ala + acetaldehyde	0.000	trace
Gly + acetaldehyde	0.000	0.000
Gly-Gly + acetaldehyde	0.065	0.155
Gly-Gly-Gly + acetaldehyde	0.050	0.095
	4 hr	9 hr
Gly + glucose	0.000	0.003
Gly-Gly + glucose	0.000	0.055
Gly-Gly-Gly + glucose	0.000	0.045
Gly-Gly-Gly-Gly + glucose	0.000	0.090

[a] Chuyen et al. (1973). Equimolar solution (0.1M) of amino acids or peptides were heated with glyoxal, acetaldehyde and glucose at 100°C for 15, 30 min or 4, 9 hr at pH 5.0. [b] 0.2 M. [c] 0.4 M.
Abbreviations used are: Ala-Ala, alanylalanine; Gly-Gly, glycylglycine. Oxidative browning did not occur in all the mixtures in this Table during storage at 37°C for 5 days.

Table IX. Comparison of Reactivity between Amino Acids and Peptides

	Gly	diGly	triGly	Gly-Leu	Leu
Initial	200 mM	200 mM	200 mM	200 mM	200 mM
Remaining (after 15 min)	160 mM	100 mM	96 mM	80 mM	180 mM

Every amino acid and peptide was heated with glucose (200 mM) in 0.1 M of acetate buffer (pH 5.0) at 120°C for 15 min.
Abbreviation is: Gly-Leu, glycyl-leucine.

Examination of the glucose-diglycine browned mixture.
A mixture solution containing glucose and diglycine was held at 50°C for 1 month, then the browned mixture was examined with the amino acid analyzer (Figure 14; Hashiba, 1975). Figure 14 illustrates that appreciable amounts of an unknown compound (UC), F-Gly and glycine were produced as reaction products. Glycine was suggested to be liberated from diglycine by cleavage of the peptide bond in the browning reaction. When a solution containing only diglycine was stored under the same conditions, no compounds except diglycine were detected. Degradation of diglycine occurred after the 3-months' storage. Chuyen et al.,(1973) also reported the cleavage of peptide chains in the reaction between peptides and glyoxal. It is interesting that the degradation of diglycine occurred in such a mild condition by the browning reaction with glucose.

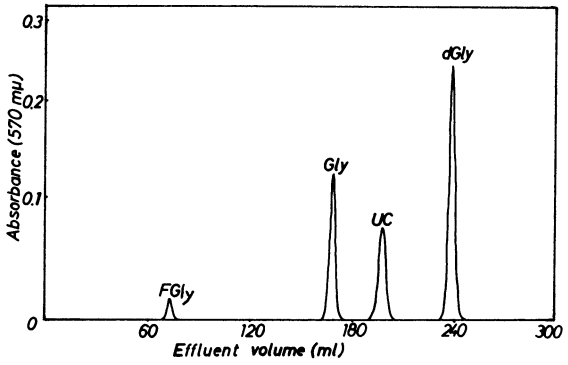


Figure 14. Effluent peaks obtained with a glucose-diglycine browned mixture; neutral and acidic analyzer column of Hitachi KLA-5, 570 nm trace only.

The browned mixture was examined by paper electrophoresis, and amino compounds and sugars were detected by the ninhydrin and KIO_4-tetrabase reagents, respectively (Figure 15). The successful separation of the compounds shows that UC is positive for both reagents like F-Gly.

In addition, UC was positive to triphenyltetrazolium chloride (TTC) test and reduced ferricyanide at pH 6.6 (Adachi, 1953), but did not reduce dichlorophenolindophenol at pH 3.5. Such properties of UC were very similar to those of Amadori compounds.

The hydrolysis products of UC were examined with the amino acid analyzer (Figure 16). Diglycine was liberated from UC as a major product. When the total reducing sugars, aldoses and ketoses in UC were determined by the methods of Ting (1956) and Horn et al. (1968), the value for sugar was found to be the same at 55°C as at 100°C. Since UC reduced all the ferricyanide at 55°C, this suggests all the sugar to be in the form of a ketose and none in the form of an aldose. Furthermore, the content of the sugar and the amino

compound in UC, caluculated as fructose (by Somogyi-Nelson's method; Nelson, 1944) and as diglycine (by ninhydrin method), respectively suggested that fructose combined with diglycine in a 1 : 1 ratio. Conceivably, the structure of UC might be proposed as an Amadori rearrangement product, 1-deoxy-1-diglycino-D-fructose (F-diGly) from the experimental results. F-diGly obtained in this preparation is colorless and tasteless white powder, and its taste resembled diglycine.

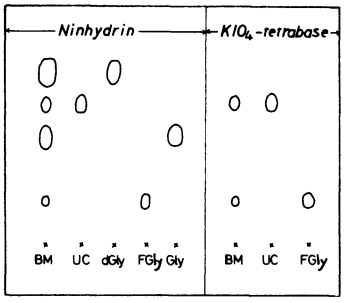


Figure 15. Paper electrophoresis of a glucose-diglycine browned mixture (BM): the conditions of electrophoresis; 40 V/cm at 30°C for 50 min; volatile buffer, pyridine-acetic acid-water(1:10:289).

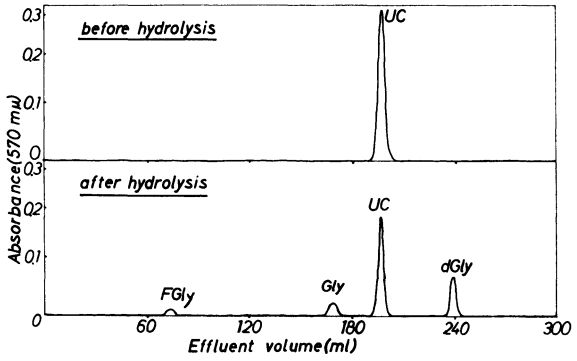
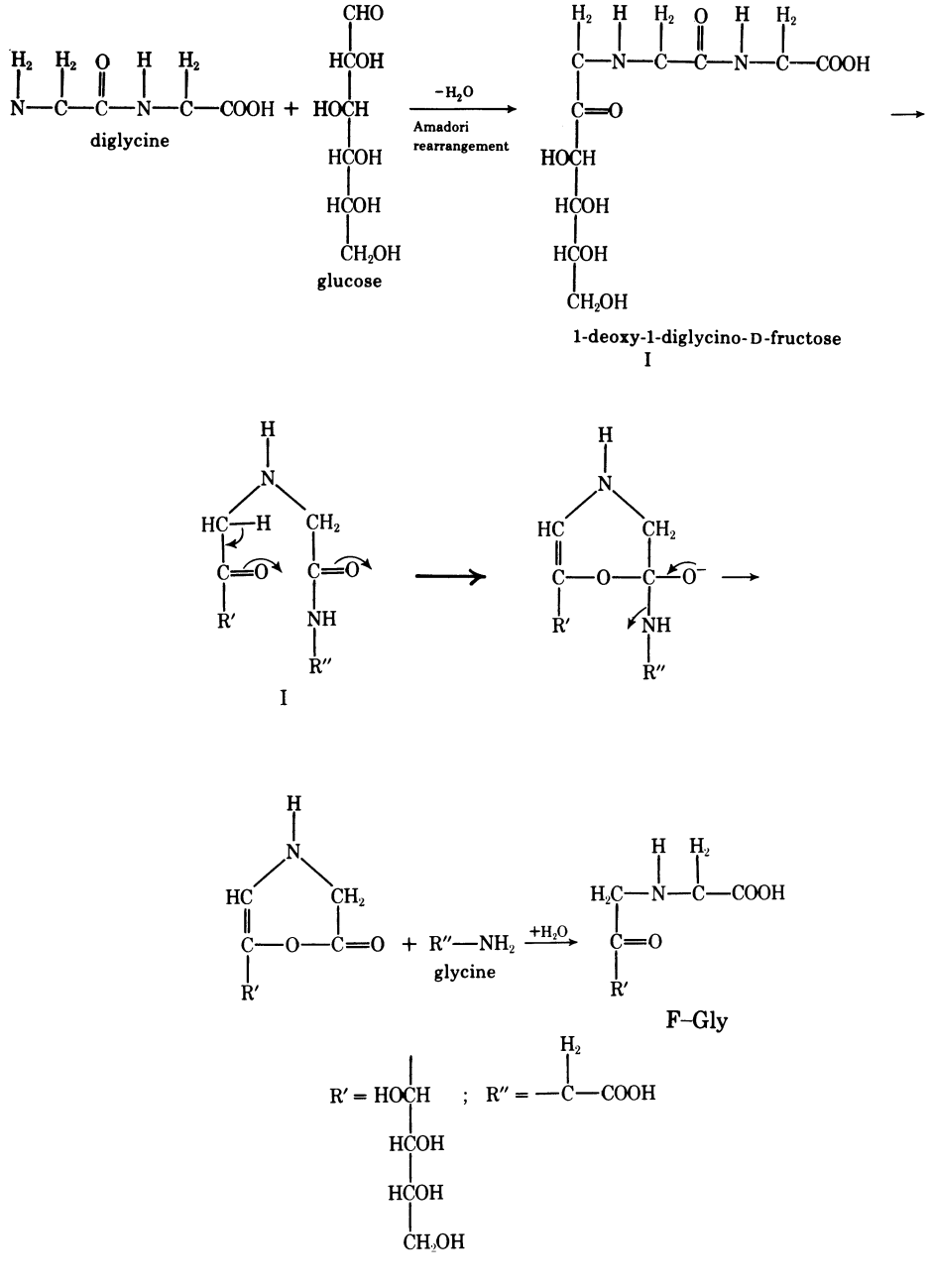


Figure 16. Effluent peaks obtained with UC hydrolyzed by 1 N sulfuric acid at 100°C for 3 hr; neutral and acidic analyzer column of Hitachi KLA-5, 570 nm trace only.

Stability of F-diGly. In order to investigate the process of the liberation of glycine in the glucose-diglycine browning reaction, the aqueous solution of 0.1 M F-digly was stored for 1 month at 50°C. F-diGly was less stable than diglycine and degraded to F-Gly and glycine, whereas diglycine was not decomposed under the same conditions. Thus, the cleavage process of the peptide bond in diglycine was suggested as shown in Scheme II.

Scheme II


diglycine
glucose
$-H_2O$
Amadori rearrangement
1-deoxy-1-diglycino-D-fructose
I
I
glycine
$+H_2O$
F-Gly
R′ = HOCH ; R″ = —C—COOH
HCOH
HCOH
CH₂OH

Other Amadori compounds from peptides. To make sure of the mechanism of the cleavage of peptide chain during browning reaction, some other peptides were examined. When glycylleucine (Gly-Leu) was heated with glucose, leucine was produced at first and then glycine was produced. On the other hand, when leucylglycine was heated with glucose, glycine was produced at first and then leucine was produced. These facts indicate that peptides combine with glucose in the first stage of browning reaction and in the next stage they liberate C-terminal amino acids.

In addition, Amadori compounds from peptides, fructose-glycylleucine (F-GlyLeu), fructose-glycyltyrosine (F-GlyTyr), fructose-glycylphenylalanine (F-GlyPhe) and fructose-glycylproline (F-GlyPro), which emerging positions in the amino acid analyzer were shown in Figure 17, were prepared by the similar method used in preparing F-diGly. These Amadori compounds gave a single spot and single peak in paper chromatography and amino acid analyzer, respectively. Furthermore, they liberated a parent peptide by the hydrolysis with 1 N H_2SO_4 at 100°C for 1 hr. Therefore, the purity of the four Amadori compounds from peptides were considered to be very high. Subsequently, 0.1 M solutions of each Amadori compound were stored under the same conditions as F-diGly (50°C, 1 month). In this case, the degradation of Amadori compounds and cleavage of peptide chains had occurred. A considerable amount of C-terminal amino acids was liberated. However, the amount of liberated N-terminal amino acids was very small. These results obtained in four Amadori compounds support the speculation of Scheme II.

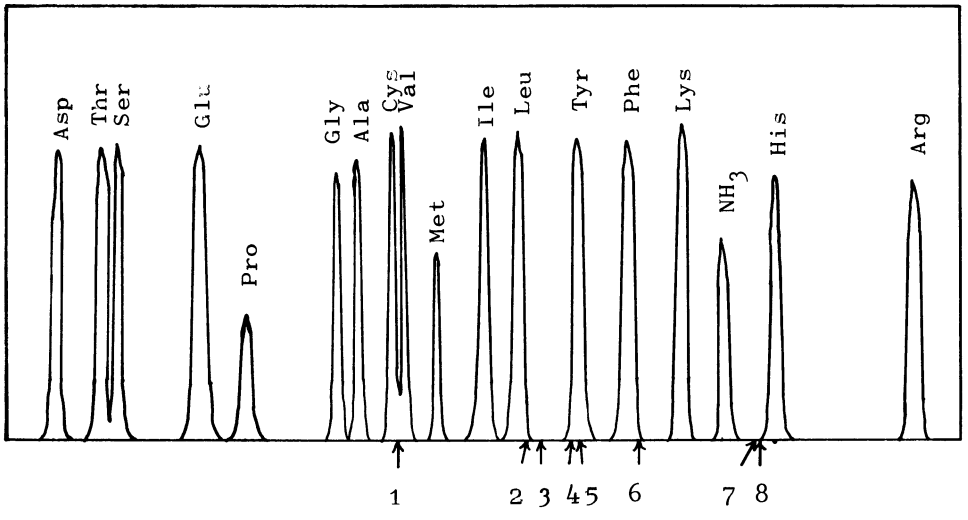


Figure 17. The position of emergence of peptides or Amadori compounds from peptides in the amino acid analyzer: 1, F-Gly·Pro; 2, F-Gly·Leu; 3, Gly-Pro; 4, F-Gly·Tyr; 5, F-Gly·Phe; 6, Gly-Leu; 7, Gly-Tyr; 8, Gly-Phe.

Examination of a glucose-triglycine browned mixture. To investigate a sugar-tripeptide browning reaction, the glucose-triglycine system was studied by means of the same procedures as those used in the glucose-diglycine system. The browned mixture of glucose and triglycine was examined by the amino acid analyzer (Figure 18). Similar results as shown in Figure 14 were obtained; that is, the degradation of triglycine was observed and F-Gly, glycine, F-diGly and diglycine were produced. A substance eluted at about 200 ml could not be identified. However, it is supposed to be a glucose-triglycine condensation product, based on the elution order, positive reaction to ninhydrin, phenol-sulfuric acid, and reduction of ferricyanide at pH 6.6.

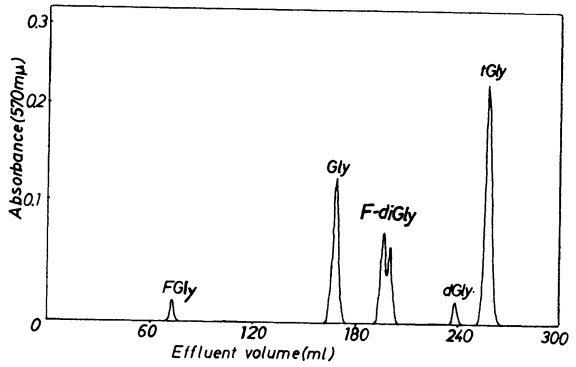


Figure 18. Effluent peaks obtained with a glucose-triglycine browned mixture; neutral and acidic analyzer column of Hitachi KLA-5, 570 nm trace only.

Effect of oxygen on the browning of F-diGly. The browning of an F-diGly solution during storage in or out of contact with air was investigated and compared with that of glucose + glycine, F-Gly and glucose + diglycine solutions. Table X shows that the oxidative browning of F-diGly was remarkable.

When 40 ppm of Fe^{2+} was added to the solutions, the oxidative browning of F-diGly was accelerated very significantly and F-Gly also showed a strong ability to brown. On the other hand, little browning was observed in the glucose + diglycine and glucose + glycine solutions. At first, F-diGly developed light red color and changed to a red brown color on prolonged storage. The browning of F-diGly in the presence of oxygen was 50 ~ 100 times as great as that of glucose + diglycine or glucose + glycine. It is suggested that F-diGly is an important precursor in the browning in the presence of oxygen.

In Table VIII, peptides are more reactive than amino acids in general nonoxidative browning reaction. However, the results in

Table X indicate that the oxidative browning is almost the same among the Amadori compounds from amino acid and peptides.

The oxidative browning of an Amadori compound from another peptide, fructose-glycylleucine was shown in Table X. Like F-diGly, the browning of fructose-glycylleucine was very remarkable in the presence of oxygen, whereas that of glucose + glycylleucine is not.

Table X. Effect of Oxygen on the Browning of Amadori Compounds from Peptides

	Browning (ΔE_{555})	
	N.O.B.	O.B.
F-diGly	0.016	0.200
Glc + diGly	0.003	0.001
F-Gly	0.007	0.205
Glc + Gly	0.001	0.000
F-Gly Leu	0.017	0.214
Glc + Gly-Leu	0.004	0.002

Abbreviations are: N.O.B., nonoxidative browning; O.B., oxidative browning; F-Gly.Leu, fructose-glycylleucine; Glc, glucose; Gly-Leu, glycylleucine. Each solution contains 0.1 M of compounds and 40 ppm of Fe^{2+}.
The method of measurement of oxidative browning is the same as Figure 1.

Oxygen uptake of F-diGly. The oxygen uptake in browning reaction was studied with only furfural (Dunlop et al., 1946), but the autoxidation of Amadori compounds has not been reported. In order to correlate oxygen uptake with the oxidative browning, an F-diGly solution was shaken in an oxygen atmosphere at 37°C in a Warburg apparatus to measure the amount of oxygen absorbed. Table XI shows that the F-diGly solution absorbed a greater amount of oxygen than a glucose + diglycine solution.

When 40 ppm of Fe^{2+} was added, although the oxygen uptake of the glucose + diglycine solution did not increase appreciably, that of the F-diGly solution increased 5 ~ 6 times. Similar results were obtained in the case of F-Gly, that is, remarkable oxygen uptake of F-Gly was observed whereas glucose + glycine absorbed no oxygen. The experimental results in Table XI agreed fairly well with those in Table X. Thus, the effectiveness of oxygen on the browning of F-diGly was again proved. The process of oxygen uptake by Amadori compounds is considered to be as shown in Scheme I.

Table XI. Comparison of the Oxygen Uptake of the Solutions of Fructose-Diglycine and Related Compounds

Solutions[b]	Oxygen uptake[a]	
	Without Fe^{2+}	With Fe^{2+}[b]
F-diGly	8.3	45.1
Glc + diGly	0.2	0.3
F-Gly	2.2	36.1
Glc + Gly	0.2	0.2

[a] Microliters/2 ml of solution per 2 hr, determined by a Warburg manometer.
[b] Abbreviations are the same as Table X. Concentration of compounds and Fe^{2+} are 0.2 M and 40 ppm, respectively.

BIOCHEMICAL AND NUTRITIONAL ASPECTS OF AMADORI COMPOUNDS

The biochemical and nutritional properties of Amadori compounds have not so far been examined in any detail. Abrams et al. (1955) and Borsook (1958) have reported that Amadori compounds do not serve as intermediates in protein synthesis. Horn et al. (1968) had prepared fructose-methionine and determined its availability to Leuconostoc mesenteroides and rats. For L. mesenteroides the compound had 80 % of the growth-stimulating ability of methionine; for rats none of the methionine was available. Hagan et al. (1970) had examined the availability of F-Gly to L. mesenteroides and determined that F-Gly yielded 68 % of the growth given by an equimolar amount of free glycine. On the other hand, Miura et al. (1973) have reported that F-Asp stimulated the initial growth of a yeast, Sporobolomyces odorus.

Since the presence of some Amadori compounds in soy sauce has been demonstrated, the authors studied the availability of several Amadori compounds for salt-tolerant yeasts which can grow in a medium of high salt such as 18 % of sodium chloride and play important roles in the fermentation of soy sauce in this chapter.

(I). Availability of Fructose-Glycine and Some Amadori Compounds

Fructose-glycine was examined as (a) nitrogen source (N-source) and carbon source (C-source), (b) C-source, and (c) N-source. No

yeasts can grow in (a) and (b) medium (Table XII). This fact indicates that these yeasts cannot utilize F-Gly as sole C-source. However, as sole N-source, a few yeasts can assimilate F-Gly. When glycine was used as sole N-source, all yeasts have grown very well. It is suggested that F-Gly were not so available as glycine for the yeasts.

In addition, the availability of some other Amadori compounds, F-β-Ala, F-Leu and F-Lys were assayed using the same yeasts (Table XII). No tested yeasts can utilize the Amadori compounds, whereas all yeasts can grow in the medium of the yeast carbon base (DIFCO) added parent amino acids of Amadori compounds. This result suggests that amino acids are made less nutritious by condensation with sugar and formation of Amadori compounds. Amadori compounds in soy sauce mash were almost unavailable for yeasts. Studies on the process of metabolism of Amadori compounds are now in progress.

Table XII. Utilization of Fructose-Glycine and Four Amadori Compounds by the Salt-Tolerant Yeasts in Soy Sauce Mashes

			Yeast carbon base			
	(a)	(b)	+ F-Gly	+ F-βAla	+ F-Leu	+ F-Lys
Saccharomyces rouxii	-	-	-	-	-	-
Saccharomyces bailii	-	-	-	-	-	-
Hansenula subpelliculosa	-	-	++	-	-	-
Hansenula fabianii	-	-	++	-	-	-
Pichia miso	-	-	-	-	-	-
Pichia farinosa	-	-	-	-	-	-
Candida diddensii	-	-	-	-	-	-
Torulopsis candida	-	-	-	-	-	-
Endomycosis burtonii	-	-	-	-	-	-
Debaryomyces hansenii	-	-	++	-	-	-

(a); F-Gly was used as N- and C-source. (b); F-Gly was added to the yeast nitrogen base. The yeast nitrogen base and yeast carbon base were obtained from DIFCO LABORATORIES. The concentration of each Amadori compound is 0.05 M.

(II). Availability of Fructose-Diglycine

Takeuchi (1976) has reported the growth-stimulating effect of peptides on Pediococcus halophilus in Miso. However, the reports on the nuritional studies of peptides have seldom been presented. In addition, in spite of many reports on the availability of sugar-amino acid or sugar-protein reaction products, that of sugar-peptide reaction products has not been dealt. The utilization of diglycine and F-diGly by the yeasts which can assimilate F-Gly as N-source was shown in Table XIII. H. subpelliculosa and H. fabianii can utilize diglycine as well as glycine. When the availability of F-diGly was assayed for diglycine using the two yeasts, it yielded 35 ~ 55 % of the growth given by an equimolar amount of diglycine. On the other hand, D. hansenii cannot utilize diglycine so well as glycine and the growth in the F-diGly medium is 60 % of that in the F-Gly medium. All tested yeasts can utilize F-diGly but the growth in the F-diGly medium is smaller (about 40 ~ 60 %) than that in the F-Gly medium.

Table XIII. Utilization of Fructose-Diglycine by the Yeasts

	Growth (E_{660})				
	N-base	C-base			
	+ F-diGly	+ diGly	+ F-diGly	+ Gly	+ F-Gly
H. subpelliculosa	0.000	0.300	0.185	0.270	0.310
H. fabianii	0.000	0.355	0.140	0.390	0.335
D. hansenii	0.000	0.100	0.110	0.160	0.160

The experimental materials and methods are the same as Table XII.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. M.Fujimaki and Prof. H.Kato for their kind guidance; Dr. M.Mogi, Dr. N.Iguchi, Dr. S.Nasuno and Dr. H.Mori for their encouragement; and Mrs. K.Sekiguchi for her excellent technical assistance.

REFERENCES

Abrams, A., Lowy, P.H., Borsook, H., J. Amer. Chem. Soc. 77, 4794 (1955).
Adachi, S., Nippon Nogei Kagaku Kaishi 32, 313 (1958).
Bayne, S., Methods Carbohydr. Chem. 2, 421 (1963).
Bohart, G.S., Carson, J.F., Nature (London) 175, 4454 (1955).
Borsook, H., Natl. Acad. Sci.-Natl. Res. Council, Publ. 557, 111 (1958).
Burton, H.S., McWeeny, D.J., Pandhi, P.N., Nature (London) 199, 659 (1963).
Chuyen, N.V., Kurata, T., Fujimaki, M., Agric. Biol. Chem. 36, 1257 (1972).
Chuyen, N.V., Kurata, T., Fujimaki, M., Agric. Biol. Chem. 37, 327 (1973).
Clegg, K.M., J. Sci. Food Agric. 15, 878 (1964).
Clifcorn, L.E., Advan. Food Res. 1, 48 (1948).
Coulter, S.T., Jenness, R., Geddes, W.F., Advan. Food Res. 3, 47 (1951).
Davidson, S., Meiklejohn, A.P., Passmore, R., "Human Nutrition and Dietetics," (1959), E. & S. Livingstone Ltd., Edinburgh & London, p. 179.
Dunlop, A.P., Stout, P.R., Swadesh, S., Ind. Eng. Chem. 38, 705 (1946).
Ellis, G.P., Advan. Carbohydr. Chem. 14, 63 (1959).
Fox, B.A., Cameron, A.G., "A Chemical Approach to Food and Nutrition", (1961), University of London Press Ltd., p. 226.
Gottschalk, A., Biochem. J. 52, 455 (1952).
Hagan, S.N., Horn, M.J., Lipton, S.H., Womack, M., J. Agric. Food Chem. 18, 273 (1970).
Hannan, R.S., Lea, C.H., Biochim. Biophys. Acta 9, 293 (1952).
Hashiba, H., J. Agric. Food Chem. 24, 70 (1976).
Hashiba, H., J. Agric. Food Chem. 23, 539 (1975).
Hashiba, H., Agric. Biol. Chem. 37, 871 (1973).
Hashiba, H., Koshiyama, I., Sakaguchi, K., Iguchi, N., Nippon Nogei Kagaku Kaishi 44, 312 (1970).
Hashiba, H., Agric. Biol. Chem. 38, 551 (1974).
Hodge, J.E., J. Agric. Food Chem. 1, 928 (1953).
Hodge, J.E., Fisher, B.E., Methods Carbohydr. Chem. 2, 99 (1963).
Hodge, J.E., Advan. Carbohydr. Chem. 10, 187 (1955).
Hodge, J.E., Rist, C.E., J. Amer. Chem. Soc. 75, 316 (1953).
Horn, M.J., Lichtenstein, H., Womack, M., J. Agric. Food Chem. 16, 741 (1968).
Ingles, D.L., Reynolds, T.M., Austral. J. Chem. 11, 575 (1958).
Joslyn, M.A., Food Res. 22, 1 (1957).
Kato, H., Sakurai, Y., Nippon Nogei Kagaku Kaishi 38, 536 (1964).
Kato, H., Agric. Biol. Chem. 26, 187 (1962).
Lewis, V.M., Lea, C.H., Biochim. Biophys. Acta 4, 532 (1950).
Markuze, Z., Ghem. Abst. 59, 4980 (1963).
Mathew, A.G., Parpia, H.A.B., Advan. Food Res. 19, (1971), p.75.

Mitsuda,H., Shikanai,T., Vitamins (Japan) 13, 394 (1957).
Miura,Y., Tahara,S., Mizutani,J., Agric. Biol. Chem. 37, 2669 (1973).
Nelson,N., J. Biol. Chem. 153, 375 (1944).
Okuhara,A., Saito,N., Yokotsuka,T., J. Ferment. Technol. 49, 272 (1971).
Okuhara,A., Kagaku to Seibutsu (Japan) 10, 383 (1972).
Omata,S., Ueno,T., Nakagawa,Y., Nippon Nogei Kagaku Kaishi 29, 259 (1955).
Peleg,Y., Mannheim,C.H., J. Agric. Food Chem. 18, 176 (1970).
Prey,V.V., Petershofer,G., Z. Zuckerind. 18, 63 (1968).
Reynolds,T.M., Advan. Food Res. 14, 168 (1965).
Sato,S., Tadenuma,M., Nippon Jozo Kyokai Zasshi 62, 1287 (1967).
Somers,G.F., Beeson,K.C., Advan. Food Res. 1, 314 (1948).
Spark,A.A., J. Sci. Food Agric. 20, 308 (1969).
Takeuchi, T., J. Ferment. Technol. 54, 143 (1976).
Talley,E.A., Porter,W.L., J. Agric. Food Chem. 16, 262 (1968).
Ting,S.V., J. Agric. Food Chem. 4, 263 (1956).
Umemoto,S., Ishiie,S., Irie,Y., Imai,T., Nippon Nogei Kagaku Kaishi 44, 64 (1970).
Wasserman,A.E., Spinelli,A.M., J. Food Sci. 35, 328 (1970).
Yoda,A., J. Chem. Soc. Jap. 73, 18 (1952).

27

THE POSSIBLE UTILIZATION OF THE 1-AMINO-1-DEOXY-2-KETOSE COMPOUNDS BETWEEN AMINO ACIDS AND CARBOHYDRATES AS FOODSTUFF INTEGRATORS

Giancarlo Barbiroli, Palmira Mazzaracchio,
and Walter Ciusa

Istituto di Merceologia
University, Bologna, 40126, Italy

ABSTRACT

The behavior of the primary products (1-amino-1-deoxy-2-ketose-derivatives) from amino acids and monosaccharides in the Maillard reaction to the action of pepsin, trypsin, chymotrypsin and of heating has been investigated, in order to test the possibility of their utilization as foodstuff integrators.

The behavior is very different from the corresponding behavior of the original amino acids.

INTRODUCTION

One of the main problems in the food industry is related to the synthesis of new substances with chemical characteristics which can be utilized as "additives" or as "integrators".

The second class of substances presents difficulties because they must be nutritionally availabile which is rarely obtained. For example free amino acids have been considered integrators to foodstuffs and employed in large amounts in diets (Block and Bolling, 1944). Later it was pointed out that their absorption in the stomach was too fast and that they were eliminated without being metabolized (Albanese, 1943; Wolley, 1946; Cannon, Steffee, Frazier, 1947; Geiger, 1947; Albanese, Holt et al., 1947; Rosenberg, Culik, 1957;Secchi, 1959).

Nevertheless several authors have pointed out the biological value of proteins increases on addition of that free essential amino acids (Bressani, Scrimshaw, 1961; Jansen, 1962; Dam; Lee, Fry, Fox,1965; Enebo, 1970; Gordon, 1970). We have therefore investigated the pos-

sible utilization of primary products of the Maillard reaction, the 1-amino-1-deoxy-2-ketose derivatives (Lea, Hannan, 1949;Gottschalk, Partridge, 1950; Friedmann, Kline, 1950; Chichester, Stadtman, Mackinnew, 1952; Hodge, Rist, 1953; Hodge, 1953; Ellis, 1959; Pill-Soon Song, Chichester, 1966, 1966, 1967; Kuhn, Dansi, 1936; Kuhn, Weygand, 1937; Kuhn, Birkofer, 1938, Weigand, 1940;Gottschalk,Lind, 1949; Gottschalk, 1951; Gottschalk 1952; Lea, Hannan, 1950; Lea, Hannan, 1950; Borsook, Abrams, Lowy, 1955; Abrams, Lowy, Borsook, 1955, Heyns, Breur, Paulsen, 1957; Heyns, Breur, 1958; Heyns; Noak, 1962; Heyns, Müller, 1967; Finot, Mauron, 1969; Finot, Mauron 1972). Samejima (1966) added the condensation products between amino acids and reducing sugars to milk and milk powder; Onishi, Nishi, Kakizawa (1966) used the same products as flavoring agents for sponge cakes, pudding moulds, chocolate products.

In previous papers we have studied and determined optimal reaction conditions for the formation of 1-amino-1-deoxy-2-ketose derivatives between amino acids and mono and disaccharides (ribose,arabinose, glucose, galactose, maltose and lactose) (Ciusa et al.,1974 1975, 1976). In order to investigate the nutritional availability of these compounds, we have tested their digestibility"in vitro"and their behavior on cooking. Adrian and Frangne (1973) also studied the action of the pre-melanoidins soluble substances on protein digestibility"in vivo" and on proteolyses"in vitro". These authors showed that addition of these pre-melanoidins at low concentrations doesn't affect alimentary metabolism:addition of high concentrations inhibits digestibility and decreases metabolic efficiency of protein nitrogen.It should be emphasized that these pre-melanoidinsare very different from amino-deoxy-ketose derivatives.

EXPERIMENTAL

Synthesis of Amino-ketose Derivatives. Absolute methanol (100 ml) is added to 0,055 moles amino acid (DL-or L-form); the anhydrous sugar (0,275 moles) and malic or malonic acid (0,024 moles) are then added to the solution and refluxed in a water-bath for 8 hours at 80°C. After cooling, the solvent is removed in vacuo at 35°C. The brown residue is dissolved into 50 ml of water, containing 5 g. of fresh baker's yeast, and the suspension stirred mechanically at 25°C for 7 hours, in which time excess of the free sugar is completely fermented. Yeast is centrifuged, the supernatant filtered and the filtrate is concentrated under reduced pressure dried in a desiccator over P_2O_5. The powder obtained is dissolved into 20 ml of methanol and slowly added with 160 ml of methylethylketone. The precipitate is then separated and dried in a desiccator. This precipitating procedure is repeated three times, to remove completely the remaining free amino acid.

Digestibility "in vitro". The 1-amino-1-deoxy-2-ketose-derivatives were subjected to the following experiments:

6N HCl (pH 0.1): 25 mg of the various products were dissolved in 5 ml of HCl 6N and held in closed vials at a temperature of 100°C for 20 hours. 20 µl of the solutions were subsequently subjected to circular paper chromatography.

N/10 HCl (pH 1.1): 25 mg of the various products were dissolved in 5 ml of HCl N/10. The solutions obtained were kept at a constant temperature of 37°C. 20 µl were drawn off after 1/2 -1-2-4-8-16-32 hours and chromatographed.

Trypsin (pH 7.8-10): 25 mg of the various products were dissolved in 5 ml of 2‰ trypsin solution at pH 7.8 and pH 10 respectively (pH 7.8 obtained thus: 0.6 ml of NaOH N/10 in 1000 ml of water; pH 10: 6 ml of NaOH N/10 in 1000 ml of water). The solutions were maintained at a constant temperature of 37°C. 20 µl were drawn off at 1/2 -1-2-4-8-16-32 hours and chromatographed.

Chymotrypsin (pH 7.8 and 10): 25 mg of the products were dissolved in 5 ml of 2‰ chymotrypsin solution at pH 7.8 and 10.The solutions were kept at a constant temperature of 37°C. 20 µl were drawn off at 1/2- 1-2-4-8-16-32 hours and chromatographed.

Sodium hydroxide (pH 12): 25 mg of the products were dissolved in 5 ml sodium hydroxide solution at pH 12 obtained by diluting 60 ml of NaOH N/10 with water to 100 ml. The solutions were kept at a constant temperature (37°C). 20 µl were drawn off at 1/2- 1-2-4-8-16-32 hours and chromatographed.

Trisodium phosphate (pH 12): 25 mg of the products were dissolved in a solution of trisodium phosphate at pH 12 (12.8 g $Na_3PO_4.12H_2O$ in 100 ml of water). The solutions were maintained at a constant temperature of 37°C. 20 µl were drawn off at 1/2- 1-2-4-8-16-32 hours and chromatographed.

Circular paper chromatography. Paper chromatography was performed by an improved method (Barbiroli, 1964, 1965). The solutions to be chromatographed were deposited over four points 2.5 cm from the center of a disk (30 or 45 cm diameter) of Whatman no. 2 paper and eluted with a n-butanol-methylethylketone-propionic acid-acetic acid phenol-water (20:50:10:10:20:20) solvent mixture at room temperature for 8 or 28 hours respectively. The chromatographic apparatus was composed of two 30 or 45 cm watch glasses. After the elution time the disks were dried, immersed in a ninhydrin-acetone-acetic acid solution (0.5 of ninhydrin into 95 ml of acetone and 5 ml of acetic acid) and put in a 105°C oven for 15 minutes. The pink and violet spots corresponding to the different amino acids or amino-derivatives were cut out and the color extracted with 5 ml of 75% ethanol and measured in a Bausch and Lomb 600 E Spectrophotometer at 570 nm. Quantitative results were obtained by comparing the optical density readings with standard curves previously prepared. The

standard error of the mean was within ± 3%.

Behavior on cooking. The experiments have been carried out on the following products: bread from wheat flour, biscuits from weath and corn flour, polenta from corn meal, cake from potato flour.
Preparation of breads. To 50 g of flour (type "00") were added 25 ml of water and 1.25 g of fresh yeast. The dough was left to rise for 1 hour at 35°C. Breads were baked in a muffle oven at 220°C for 40 minutes or in a microwave oven (Raytheon, mod. 500N), for 3 minutes.
Preparation of biscuits. To 50 g of wheat (of corn) flour were added 20 ml of water, 5 g of sucrose, 2 g of shortenings and 1 g of chemical yeast. Biscuits were baked in a muffle oven at 240°C for 20 minutes;or in an infrared oven (Heraeus, mod.H 420) at 240°C for 30 minutes; or in a microwave oven (Raytheon, mod. 500N) for 1 minute.
Preparation of polenta. To 25 g of corn flour were added 350 ml of water and 0.5 g of sodium chloride. Polenta was cooked on a direct fire for 45 minutes and continually stirred.
Preparation of potato flour cakes. To 70 g of potato flour were added 70 g of sucrose, 3.3 g of fresch yeast and 1 whole egg (50 g). Cakes were baked in a muffle oven at 160°C for 40 minutes.

To determine the behavior of the derivatives, the following tests were established: 1) product withour additive; 2) product with 2% derivative (dry matter); 3) product with the corresponding percentage of sugar; 4) product with the corresponding percentage of amino acid; 5) product with the corresponding percentages of the sugar and amino acid (to a total of 2%).

The following determinations were carried out on all samples.

Water content. 10 g of the products prepared by grinding up a whole and sieving with a 0.3 mm sieve were weighed in porcellain containers and put in a 105°C oven, until constant weight was reached.
Free amino acids and amino-keto compounds. 10 g of the sample were weighed, put in a conical flask wich 100 ml of water, left for 12 hours and subsequently filtered, 100 µl of the solution were separated by circular paper chromatography as previously described.
Sugars. 200 µl of the water solution obtained for amino acids and derivatives determination were separated by circular paper chromatography (Barbiroli, 1965).
Thermogravimetric analysis. 100 mg of the compound were placed in an Adamel, mod. TH 59 thermal scale, registering the weight variations from 20°C up to stabilization of the weight (630-700°C).

RESULT AND DISCUSSION

Digestibility "in vitro". The percentages of hydrolysis of the 1-amino-1-deoxy-2-ketose-derivatives found after hydrolysis with 6N HCl

are shown in Table 1. Data on the behavior of the derivatives in HCl N/10 and in pepsin is not given, since all the compounds so treated were stable at least up to 32 hours. The decrease in the aumonts of compounds in the presence of trypsin and chymotrypsin at pH 7.8 and pH 10 and the regeneration of the corresponding amino acid are shown in Tables 2-5. In Tables 6-9 are summarized up the results obtained using trypsin and chymotrypsin at pH 12. Tables 10-11 show the decrease in compounds tested at pH 12 without trypsin or chymotrypsin and the regeneration of free amino acids. The results of the tests carried out at pH 7.8 and 10 without trypsin or chymotrypsin are not given as the products remained highly stable (at least up to 32 hours). The data obtained using trypsin and chymotrypsin at pH 10 is not shown as it is pratically identical to that obtained using trypsin and chymotrypsin at pH 7.8. In Table 12 are given the R_f values of the free amino acids and of the amino-derivatives. An examination of Table 1 shows that the derivatives obtained from the reaction of some essential amino acids with two hexoses (glucose and galactose) and with two pentoses (arabinose and ribose) are hydrolysed from 65 to 100%.In particular, the derivatives formed with pentoses and many derivatives formed with glucose are totally hydrolysable. Only a few of the compounds are not totally hydrolysable: those from lysine and glucose or galactose are hydrolysable (64-68%). Those from valine and glucose or galactose(84-90%). Those from glycine and glucose or galactose(82-88%). Those from methionine and glucose or galactose (70-75%). Those from alanine, arginine, and leucine and galactose (85-92%). The derivatives from galactose are less hydrolysable with 6N HCl (Table 1). Under these conditions we did not observe the formation of the original sugar simultaneously with the formation of the original amino acid. Trypsin and chymotrypsin at pH 7.8 and 10 hydrolysed the products formed with hexoses but not with pentoses. The hydrolysis was partial. In fact, after 32 hours one can observe (Tables 2-5) a maximum (50%) diminution of compounds (for lysine-glucose) and the corresponding formation of free amino acid and free sugar. The action of trypsin is slightly superior to that of chymotrypsin. The derivatives from glucose are more susceptible to hydrolysis than the corresponding ones from galactose. No differences were observed with trypsin and chymotrypsin at pH 10. On subjecting these same derivatives (also those from pentoses) to hydrolysis with trypsin and chymotrypsin at pH 12, a much more rapid and total hydrolysis was observed.Indeed, for some derivatives hydrolysis was total after 16 hours of incubation at 37°C (Tables 6-9). To discover if this result was due to enhancement of the action of trypsin and chymotrypsin at a very basic pH, or vice versa, some derivatives were incubated with the basic solutions alone. It was ascertained that the strong hydrolysis was due to the strongly basic pH and not to enzyme action,since no differences resulted between the tests carried out with the basic solutions alone and those with trypsin and chymotrypsin.Finally,no differences were found when using a basic solution obtained with sodium hydroxide or phosphate.

TABLE 1
Hydrolysis of Amino-deoxy-ketose Compounds in 6 N HCl, at 110°C for 24 Hours.

Compound	% hydrolyzed
Alanine-arabinose	100
Alanine-ribose	100
Alanine-galactose	92
Alanine-glucose	100
Arginine-arabinose	100
Arginine-galactose	86
Arginine-glucose	100
Phenylalanine-arabinose	100
Phenylalanine-galactose	100
Phenylalanine-glucose	100
Glycine-arabinose	100
Glycine-ribose	100
Glycine-galactose	82
Glycine-glucose	88
Leucine-arabinose	100
Leucine-galactose	92
Leucine-glucose	100
Lysine-arabinose	100
Lysine-ribose	92
Lysine-galactose	64
Lysine-glucose	68
Methionine-arabinose	100
Methionine-galactose	70
Methionine-glucose	76
Proline-galactose	100
Proline-glucose	100
Valine-arabinose	100
Valine-ribose	100
Valine-galactose	84
Valine-glucose	90

The terminology of the amino-compounds is reported with the original sugar. According to the chemical structure it should be more correct to write ribulose instead of arabinose and ribose; tagatose instead of galactose; fructose instead of glucose.

TABLE 2
Decrease of Several Compounds with Trypsin at pH 7.8 (mg).

Compound	Hours				
	0	4	8	16	32
Alanine-arabinose	100	100	100	100	100
Alanine-ribose	100	100	100	100	100
Alanine-galactose	100	76	62	54	50
Alanine-glucose	100	70	65	60	46
Arginine-arabinose	100	100	100	100	100
Arginine-ribose	100	100	100	100	100
Arginine-galactose	100	100	86	78	68
Arginine-glucose	100	80	68	60	54
Glycine-arabinose	100	100	100	100	100
Glycine-ribose	100	100	100	100	100
Glycine-galactose	100	92	82	74	64
Glycine-glucose	100	90	82	74	64
Phenylalanine-arabinose	100	100	100	100	100
Phenylalanine-ribose	100	100	100	100	100
Phenylalanine-galactose	100	82	70	60	50
Phenylalanine-glucose	100	84	74	62	52
Leucine-arabinose	100	100	100	100	100
Leucine-ribose	100	100	100	100	100
Leucine-galactose	100	90	82	76	70
Leucine-glucose	100	100	92	84	78
Lysine-arabinose	100	100	100	100	100
Lysine-ribose	100	100	100	100	100
Lysine-galactose	100	100	92	84	74
Lysine-glucose	100	84	73	60	56
Methionine-arabinose	100	100	100	100	100
Methionine-ribose	100	100	100	100	100
Methionine-galactose	100	92	82	74	66
Methionine-glucose	100	86	80	72	70
Proline-arabinose	100	100	100	100	100
Proline-ribose	100	100	100	100	100
Proline galactose	100	100	92	84	76
Proline-glucose	100	94	80	70	58
Valine-arabinose	100	100	100	100	100
Valine-ribose	100	100	100	100	100
Valine-galactose	100	100	94	86	80
Valine-glucose	100	100	92	84	78

TABLE 3
Regeneration of Free Amino Acid with Trypsin, at pH 7.8 (mg).

Compound	Hours				
	0	4	8	16	32
Alanine-arabinose	0	0	0	0	0
Alanine-ribose	0	0	0	0	0
Alanine-galactose	0	8	12	15	16
Alanine-glucose	0	10	12	14	15
Arginine-arabinose	0	0	0	0	0
Arginine-ribose	0	0	0	0	0
Arginine-galactose	0	0	6	10	14
Arginine-glucose	0	8	14	18	20
Glycine-arabinose	0	0	0	0	0
Glycine-ribose	0	0	0	0	0
Glycine-galactose	0	2	4	7	9
Glycine-glucose	0	4	8	12	16
Phenylalanine-arabinose	0	0	0	0	0
Phenylalanine-ribose	0	0	0	0	0
Phenylalanine-galactose	0	8	13	18	23
Phenylalanine-glucose	0	6	11	16	21
Leucine-arabinose	0	0	0	0	0
Leucine-ribose	0	0	0	0	0
Leucine-galactose	0	3	6	10	13
Leucine-glucose	0	0	3	6	8
Lysine-arabinose	0	0	0	0	0
Lysine-ribose	0	0	0	0	0
Lysine-galactose	0	0	2	6	10
Lysine-glucose	0	6	12	18	20
Methionine-arabinose	0	0	0	0	0
Methionine-ribose	0	0	0	0	0
Methionine-galactose	0	3	6	11	15
Methionine-glucose	0	6	8	12	14
Proline-arabinose	0	0	0	0	0
Proline-ribose	0	0	0	0	0
Proline-galactose	0	0	3	6	8
Proline-glucose	0	2	7	11	15
Valine-arabinose	0	0	0	0	0
Valine-ribose	0	0	0	0	0
Valine-galactose	0	0	2	4	6
Valine-glucose	0	0	2	4	6

TABLE 4
Decrease of Several Compounds with Chymotrypsin at pH. 7.8 (mg)

Compound	Hours				
	0	4	8	16	32
Alanine-arabinose	100	100	100	100	100
Alanine-ribose	100	100	100	100	100
Alanine-galactose	100	100	92	80	74
Alanine-glucose	100	100	86	72	66
Arginine-arabinose	100	100	100	100	100
Arginine-ribose	100	100	100	100	100
Arginine-galactose	100	100	80	70	58
Arginine-glucose	100	100	84	74	62
Glycine-arabinose	100	100	100	100	100
Glycine-ribose	100	100	100	100	100
Glycine-galactose	100	88	78	68	60
Glycine-glucose	100	90	82	74	66
Phenylalanine-arabinose	100	100	100	100	100
Phenylalanine-ribose	100	100	100	100	100
Phenylalanine-galactose	100	84	76	80	52
Phenylalanine-glucose	100	88	86	66	56
Leucine-arabinose	100	100	100	100	100
Leucine-ribose	100	100	100	100	100
Leucine-galactose	100	92	86	80	74
Leucine-glucose	100	10o	94	86	80
Lysine-arabinose	100	100	100	100	100
Lysine-ribose	100	100	100	100	100
Lysine-galactose	100	100	90	80	70
Lysine-glucose	100	76	64	58	50
Methionine-arabinose	100	100	100	100	100
Methionine-ribose	100	100	100	100	100
Methionine-galactose	100	94	86	78	70
Methionine-glucose	100	78	68	60	56
Proline-arabinose	100	100	100	100	100
Proline-ribose	100	100	100	100	100
Proline-galactose	100	100	94	86	78
Proline-glucose	100	96	82	72	60
Valine-arabinose	100	100	100	100	100
Valine-ribose	100	100	100	100	100
Valine-galactose	100	100	100	94	86
Valine-glucose	100	100	94	88	80

TABLE 5
Regeneration of Free Amino Acid with Chymotrypsin at pH 7.8 (mg).

Compound	Hours				
	0	4	8	16	32
Alanine-arabinose	0	0	0	0	0
Alanine-ribose	0	0	0	0	0
Alanine-galactose	0	0	2	6	8
Alanine-glucose	0	0	4	8	10
Arginine-arabinose	0	0	0	0	0
Arginine-ribose	0	0	0	0	0
Arginine-galactose	0	0	8	12	18
Arginine-glucose	0	0	6	10	16
Glycine-arabinose	0	0	0	0	0
Glycine-ribose	0	0	0	0	0
Glycine-galactose	0	3	6	9	11
Glycine-glucose	0	4	7	12	15
Phenylalanine-arabinose	0	0	0	0	0
Phenylalanine-ribose	0	0	0	0	0
Phenylalanine-galactose	0	6	10	16	22
Phenylalanine-glucose	0	4	10	14	18
Leucine-arabinose	0	0	0	0	0
Leucine-ribose	0	0	0	0	0
Leucine-galactose	0	2	5	8	12
Leucine-glucose	0	0	2	5	8
Lysine-arabinose	0	0	0	0	0
Lysine-ribose	0	0	0	0	0
Lysine-galactose	0	0	4	7	10
Lysine-glucose	0	10	14	14	22
Methionine-arabinose	0	0	0	0	0
Methionine-ribose	0	0	0	0	0
Methionine-galactose	0	2	5	10	12
Methionine-glucose	0	10	14	18	20
Proline-arabinose	0	0	0	0	0
Proline-ribose	0	0	0	0	0
Proline-galactose	0	0	2	5	7
Proline-glucose	0	1	6	10	14
Valine-arabinose	0	0	0	0	0
Valine-ribose	0	0	0	0	0
Valine-galactose	0	0	0	2	5
Valine-glucose	0	0	2	4	6

TABLE 6
Decrease of Compounds with Trypsin at pH 12 (mg).

Compound	Hours						
	0	1	2	4	8	16	32
Methionine-glucose	100	82	64	38	18	0	0
Methionine-arabinose	100	92	76	62	50	36	20
Methionine-galactose	100	90	74	68	44	28	10
Valine-galactose	100	92	84	72	36	16	8
Valine-arabinose	100	94	88	78	68	56	48
Valine-glucose (with NaOH)	100	82	72	60	28	12	4
Valine-glucose (with Na_3PO_4)	100	84	74	62	30	14	6

TABLE 7
Regeneration of Free Amino Acid with Trypsin at pH 12 (mg).

Compound	Hours						
	0	1	2	4	8	16	32
Methionine-glucose	0	8	16	28	36	44	44
Methionine-arabinose	0	4	12	20	26	34	42
Methionine-galactose	0	4	11	19	26	34	42
Valine-galactose	0	2	5	10	24	32	35
Valine-arabinose	0	2	5	9	14	19	23
Valine-glucose (with NaOH)	0	7	10	15	26	34	36
Valine-glucose (with Na_3PO_4)	0	6	10	15	26	34	36

TABLE 8
Decrease of Compounds with Chymotrypsin at pH 12 (mg).

Compound	Hours						
	0	1	2	4	8	16	32
Methionine-glucose	100	84	66	40	20	0	0
Methionine-arabinose	100	92	78	64	52	38	22
Methionine-galactose	100	94	78	62	48	32	14
Valine-galactose	100	94	86	76	46	32	20
Valine-arabinose	100	94	88	80	70	60	52

TABLE 9
Regeneration of Free Amino Acid with Chymotrypsin at pH 12(mg)

Compound	Hours						
	0	1	2	4	8	16	32
Methionine-glucose	0	6	16	28	36	44	44
Methionine-arabinose	0	4	11	18	24	31	40
Methionine-galactose	0	2	9	17	24	32	41
Valine-galactose	0	2	4	8	20	25	30
Valine-arabinose	0	2	5	8	13	18	22

TABLE 10
Decrease of Compounds at pH 12 (mg).

Compound	Hours						
	0	1	2	4	8	16	32
Methionine-glucose	100	84	66	40	20	0	0
Valine-glucose (with NaOH)	100	82	72	60	28	12	4
Valine-glucose (with Na_3PO_4)	100	84	74	62	30	14	6

TABLE 11
Regeneration of Free Amino Acid at pH 12 (mg).

Compound	Hours						
	0	1	2	4	8	16	32
Methionine-glucose	0	7	15	26	34	42	42
Valine-glucose (with NaOH)	0	7	10	15	26	34	36
Valine-glucose (with Na_3PO_4)	0	6	10	15	26	34	36

TABLE 12
Rf Values of the Free Amino Acids and of the Corresponding 1-Amino-1-deoxy-2-ketose Compounds.

Aminoacid	Parent sugar						
	Free	Glucose	Galactose	Arabinose	Ribose	Maltose	Lactose
Alanine	0.47	0.24	0.28	0.36	0.36	0.16	0.20
Arginine	0.32	0.21	0.25	0.28	0.26	0.12	0.12
Aspartic ac.	0.22	=	0.18	0.19	0.19	=	=(≠)
Cysteine	0.13	0.19	0.19	0.20	0.20	=	0.64
Glutamic ac.	0.35	0.64	0.64	0.58	0.68	=	0.50
Glutamine	0.35	0.22	0.22	0.40	0.40	=	=
Glycine	0.30	0.24	0.24	0.30	0.20	0.13	0.13
Histidine	0.16	0.13	0.13	0.13	0.13	0.07	0.07
Isoleucine	0.80	0.62	0.62	0.76	0.75	0.40	0.43
Leucine	0.90	0.60	0.60	0.68	0.70	0.35	=
Lysine	0.19	0.14	0.14	0.16	0.16	0.10	0.06
Methionine	0.68	0.41	0.41	0.57	0.52	0.24	0.25
Phenylalanine	0.72	0.67	0.55	0.67	0.66	0.28	0.28
Proline	0.59	0.40	0.37	0.46	0.50	0.16	0.16
Serine	0.28	0.21	0.21	0.21	0.25	0.09	0.09
Threonine	0.39	0.24	0.28	0.34	0.35	0.18	0.12
Tryptophan	0.63	0.52	0.56	0.57	0.57	0.32	0.28
Tyrosine	0.51	0.34	0.36	0.42	0.43	=	=
Valine	0.62	0.40	0.52	0.58	0.58	0.27	0.28

(≠) After 64 hours the amino acid has not reacted.

Behavior on Cooking. It is possible to observe that the free amino acid is very stable with the different cooking methods employed:we have recovered amounts corresponding to about the 95-96% of the amounts addet (Tables 13-22). No amino-deoxy-ketose compound is formed. On the contrary, added sugars have been recovered in small amounts but it is well known that sugars carmelize during heating. The simultaneous addition of amino acid and sugar gave different results with the different products in the bread baked in the traditional oven and in the infrared oven it leads to the formation of small amounts of 1-amino-1-deoxy-2-ketose derivatives only in a few cases. In the bread baked in the microwave oven, however, large amounts of derivatives have been obtained wich a corresponding decrease of the original amino acid (70-75%). In the biscuits from wheat flour, in corn meal, and in the cake of potato flour small amounts of amino-ketose derivative are formed in the traditional oven. Large amounts are formed in biscuits baked in the microwave oven. In the polenta from corn meal no derivative is formed,and the original amino acid is stable.

These results show that the formation of the amino-ketose derivatives is possible only when the amino acid and the sugar are added at the some time. The direct addition of the amino-ketose compounds to the products has shown a high stability in breads baked in traditional and infrared ovens (over 80% is recovered). A high degradation in the microwave oven has been observed with meats which was accompanied by regeneration of the original amino acid. The original sugars were not regenerated since they are transformed into caramel and carbonyl compounds. In the biscuits and in cake of potato flour, large amounts of the amino-ketose compounds are degradated to the original amino acid. The amino-ketose derivatives are very stable in the polenta of corn meal.

TABLE 13
Behavior of Amino Acids, Sugars and Corresponding Amino-Ketose Compounds when Added to Breads Baked in a Traditional Oven at 220°C.

Added Substances	Amounts added (g% dry matter)	Determined substances (g% dry matter)	
		Amino Acid	Derivative
Leucine	1.03	1.00	-
Leucine + glucose	1.03 + 1.25	0.97	0.1
Leucine-fructose	2.28	tr.	1.97
Valine	0.96	0.90	-
Valine + glucose	0.96 + 1.32	0.90	-
Valine-fructose	2.28	0.19	1.79
Valine	0.95	0.95	-
Valine + arabinose	0.95 + 1.35	0.95	-
Valine-ribulose	2.28	-	2.29
Lysine	0.68	0.70	-
Lysine + glucose	0.68 + 1.60	0.68	-
Lysine-fructose	2.28	0.12	1.68

TABLE 14

Behavior of Amino Acids, Sugars and Corresponding Amino-Ketose Compounds when Added to Breads Baked in a Microwave Oven.

Added Substances	Amounts added (g% dry matter)	Determined substances (g% dry matter)	
		Amino Acid	Derivative
Valine	0.96	0.93	-
Valine + glucose	0.96 + 1.32	0.31	1.28
Valine-fructose	2.28	0.90	0.09
Leucine	1.03	0.89	-
Leucine + glucose	1.03 + 1.25	0.19	0.82
Leucine-fructose	2.28	0.68	0.06

TABLE 15

Behavior of Amino Acids, Sugars and Corresponding Amino-Ketose Compounds when added to Breads Baked in an Infrared Oven at 220°C.

Added Substances	Amounts added (g% dry matter)	Determined substances (g% dry matter)	
		Amino Acid	Derivative
Valine	0.96	0.92	-
Valine + glucose	0.96 + 1.32	0.90	-
Valine-fructose	2.28	0.21	1.71

TABLE 16

Behavior of Amino Acids, Sugars and Corresponding Amino-Ketose Compounds when Added to Wheatmeal Biscuits Baked in a Traditional Oven at 240°C.

Added Substances	Amounts added (g% dry matter)	Determined substances (g% dry matter)	
		Amino Acid	Derivative
Valine	0.96	0.88	-
Valine + glucose	0.96 + 1.32	0.82	0.082
Valine-fructose	2.28	0.77	0.45
Valine	0.96	0.90	-
Valine + galactose	0.96 + 1.32	0.84	0.10
Valine-tagatose	2.28	0.78	0.35
Lysine	0.68	0.60	-
Lysine + glucose	0.68 + 1.60	0.58	-
Lysine-fructose	2.28	0.36	0.72
Leucine	1.03	0.84	-
Leucine + glucose	1.03 + 1.25	0.77	0.04
Leucine-fructose	2.28	0.72	0.19
Leucine	1.01	0.77	-
Leucine + arabinose	1.01 + 1.27	0.67	tr.
Leucine-ribulose	2.28	0.62	0.34

TABLE 17

Behavior of Amino Acids, Sugars and Corresponding Amino-Ketose Compounds when Added to Wheatmeal Biscuits Baked in a Microwave Oven.

Added Substances	Amounts added (g% dry matter)	Determined substances (g% dry matter)	
		Amino Acid	Derivatives
Valine	0.96	0.93	-
Valine + glucose	0.96 + 1.32	0.39	1.08
Valine-fructose	2.28	0.85	0.27
Leucine	1.03	0.88	-
Leucine + glucose	1.03 + 1.25	0.49	0.89
Leucine-fructose	2.28	0.83	0.16

TABLE 18
Behavior of Amino Acids, Sugars and Corresponding Amino-Ketose Compounds when Added to Wheatmeal Biscuits Baked in a Infrared Oven at 240°C.

Added Substances	Amounts added (g% dry matter)	Determined substances (g% dry matter)	
		Amino Acid	Derivative
Valine	0.96	0.91	-
Valine + glucose	0.96 + 1.32	0.85	0.09
Valine-fructose	2.28	0.66	0.47

TABLE 19
Behavior of Amino Acids Sugars and Corresponding Amino-Ketose Compounds when Added to Cornmeal Biscuits Baked in a Traditional Oven at 240°C.

Added Substances	Amounts added (g% dry matter)	Determined substances (g% dry matter)	
		Amino Acid	Derivative
Valine	0.96	0.86	-
Valine + glucose	0.96 + 1.32	0.83	0.09
Valine-fructose	2.28	0.70	0.33
Valine	0.96	0.85	-
Valine + galactose	0.96 + 1.32	0.84	traces
Valine-tagatose	2.28	0.74	0.23
Leucine	1.03	0.83	-
Leucine + glucose	1.03 + 1.25	0.81	traces
Leucine-fructose	2.28	0.66	0.22
Leucine	1.03	0.79	-
Leucine + galactose	1.03 + 1.25	0.77	traces
Leucine-tagatose	2.28	0.73	0.18
Phenylalanine	1.14	0.888	-
Phenylalanine+glucose	1.14 + 1.14	0.86	-
Phenylalanine-fructose	2.28	0.76	0.23
Methionine	1.06	0.84	-
Methionine + glucose	1.06 + 1.22	0.74	0.12
Methionine-fructose	2.28	0.71	0.16

TABLE 20
Behavior of Amino Acids, Sugars and Corresponding Amino-Ketose Compounds when Added to Cornmeal Biscuits in a Microwave Oven.

Added Substances	Amounts added (g% dry matter)	Determined substances (g% dry matter)	
		Amino Acid	Derivative
Valine	0.96	0.95	-
Valine + glucose	0.96 + 1.32	0.38	1.18
Valine-fructose	2.28	0.88	0.16
Leucine	1.03	0.89	-
Leucine + glucose	1.03 + 1.25	0.52	0.94
Leucine-fructose	2.28	0.81	0.19

TABLE 21
Behavior of Amino Acids, Sugars and Corresponding Amino-Ketose Compounds when Added to Cornmeal Polenta Boiled in Water at 100°C.

Added Substances	Amounts added (g% dry matter)	Determined substances (g% dry matter)	
		Amino Acid	Derivative
Valine	0.96	0.89	-
Valine + glucose	0.96 + 1.32	0.90	traces
Valine-fructose	2.28	0.24	1.46
Valine	0.96	0.89	-
Valine + galactose	0.96 + 1.32	1.03	-
Valine-tagatose	2.28	0.36	1.29
Leucine	1.03	0.93	-
Leucine + glucose	1.03 + 1.25	0.84	-
Leucine-fructose	2.28	0.29	2.00
Leucine	1.03	0.98	-
Leucine + galactose	1.03 + 1.25	1.00	-
Leucine-tagatose	2.28	0.32	1.94
Phenylalanine	1.14	1.08	-
Phenylalanine+glucose	1.14 + 1.14	1.20	-
Phenylalanine-fructose	2.28	0.32	1.94
Methionine	1.09	0.97	-
Methionine + glucose	1.09 + 1.19	0.86	-
Methionine-fructose	2.28	0.40	1.60

TABLE 22
Behavior of Amino Acids, Sugars and Corresponding Amino-Ketose Compounds when Added to Potato Flour Cakes Baked in a Traditional Oven at 160°C.

Added Substances	Amounts added (g% dry matter)	Determined substances (g% dry matter)	
		Amino Acid	Derivative
Valine	1.01	1.00	-
Valine + glucose	1.01 + 1.45	0.90	0.09
Valine-fructose	2.46	0.67	0.58
Leucine	1.03	0.83	-
Leucine + glucose	1.03 + 1.25	0.78	0.05
Leucine-fructose	2.28	0.67	0.44

We conclude that the formation of the 1-amino-1-deoxy-ketose derivative when the amino acids and the sugar are added toghether in the baked products depends especially on the temperature and on the type of cooking. Thus, polenta is cooked at 100°C and no com - pound is formed; bread is baked at 220° C, and only traces are for med; biscuits are baked at 240°C and large amounts are formed.

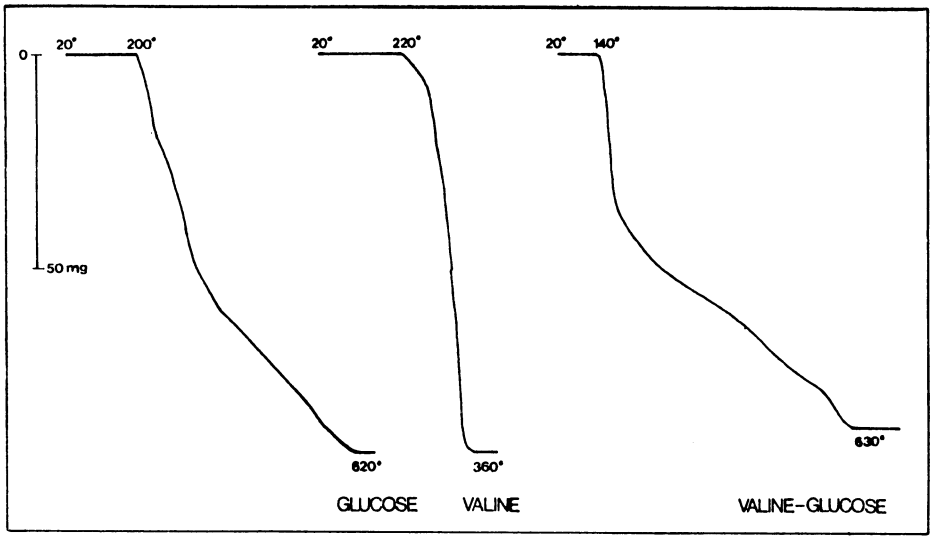


Figure 1-Thermogravimetric curves of glucose,valine and valine-glucose

The same considerations are valid when the amino-ketose compounds are added; they are not stable at 240°C or in the microwave oven, but are very stable in polenta boiled at 100°C.

The second main consideration is that the thermal stabilities of the amino-ketose derivatives is much higher when they are added to foodstuffs compared to those prepared with cereal flours when are directly heated in a thermobalance. Figure 1 shows that they are stable only until 120-130°C.

A quantitative aspect must be emphasized: the yields of transformation from amino acid plus sugar to amino-ketose derivative and from amino-ketose derivative to amino acid are very high(about 80-90%), much higher than the corresponding yields obtained when they are synthetized in methanolic solution (15-20%) (Ciusa et al., 1975).

REFERENCES

Abrams, A., Lowy, P.H. and Borsook, H. (1955). Preparation of 1--amino-1-deoxy-2-ketohexoses from aldohexoses and amino acids. J. Am. Chem. Soc., 77, 4794-6.

Adrian, J. and Frangne R. (1973). La réaction de Maillard. Ann. Nutr. et Alim., 27, 111-123.

Albanese, A.A. (1943). Preparation of methionine and tryptophan free casein hydrolyzates. Science, 98, 286.

Albanese, A.A., Holt et al. (1947). Protein metabolism of the infant-(1)-biological value of some milk proteins preparations-tryptophan requirement of the infant. Bull. Johns Hopkins Hosp., 80, 149-57.

Barbiroli, G. (1964). Tecnica per la separazione ed il dosaggio di complesse miscele di amminoacidi mediante cromatografia circolare su carta. Rass. Chim., 6, 251.

Barbiroli, G. (1965). Papierchromatographische Trennung und Bestimmung von 22 Aminosäuren. Mikrochimica Acta, 4, 653.

Barbiroli, G. (1965). Separazione e dosaggio di zuccheri mediante cromatografia circolare su carta. Rass. Chim., 2, 62.

Block, R.G. and Bolling, D. (1944). Food products, U.S., 344, 229.

Borsook, A., Abrams, A. and Lowy, P.G. (1955). Fructose-amino-acids in liver: stimuli of amino acid incorporation in vitro. J. Biol. Chem., 215, 111.

Bressani, R. and Scrimshaw, N.S. (1961). "Progress in Meeting Protein Needs of Preschool Children", National Academy of Science, National Research Council, Publication n. 843,Washington, p. 15.

Cannon, P.R., Steffee, C.H. and Frazier, J. (1947). Amino acid utilization, dietary utilization of mixtures of purified amino acids in protein depleted adult albino rats Feder.Proc.,6,390.

Chichester, C.O., Stadtman, F.H. and Mackinnew, G. (1952). On the products of the Maillard reaction. J. Am. Chem. Soc.,74, 3418.
Ciusa, W., Mazzaracchio, P. and Barbiroli, G. (1974). Considerazioni sulle reazioni fra amminoacidi e zuccheri.Rass.Chim.,3,136.
Ciusa, W., Barbiroli, G. and Mazzaracchio, P. (1975). Ricerche sistematiche sulla reattività di 20 amminoacidi con mono e disaccaridi e sui rendimenti in N-ammino-deossi-chetoderivati. Rass. Chim., 2, 94.
Ciusa, W., Barbiroli, G. and Mazzaracchio, P. (1976). Reazioni fra amminoacidi e zuccheri: ricerche sulla formazione dei prodotti primari. Rass. Chim., 4, 176.
Dam, R., Lee, S., Fry, P.C. and Fox, H. (1965). Utilization of algae as a protein sourcee for humans. J. Nutr., 86, 376.
Ellis, G.P. (1959). The Maillard reaction. Advances in Carbohydrate Chemistry, 14, 376.
Enebo, L. (1970). "Evaluation of Novel Proteins Products", Pergamon Press, Oxford, p. 93.
Finot, P.A. and Mauron, J. (1969). Le blocage de la lysine par la réaction de Maillard. I. Synthèse de N-(désoxy-1-D-fructosyl-1)- et N-(désoxy-1-D-lactulosyl-1)-L-lysines. Helv. Chim.Acta, 52 (6), 1488-95.
Finot, P.A. and Mauron, J. (1972). Le blocage de la lysine par la réaction de Maillard. II. Propriétés chimiques des dérivés N-(désoxy-1-D-fructosyl-1)-et N-(désoxy-1-D-lactulosyl-1) de la lysine. Helv. Chim. Acta, 55 (5), 1153-64.
Friedman, L. and Klinz, L.O. (1950). The amino acid-sugar reaction J. Biol. Chem., 184, 599.
Geiger, R. (1947). Experiments with delayed supplementation of incomplete amino acid mixtures. J. Nutrition, 34, 97.
Gordon, J.F. (1970). "Protein as Human Food", Butterworths, London, p. 328.
Gottschalk, A. and Lind, P.F. (1949). Product of interaction between influenza virus enzyme on ovomucin. Nature, 164, 232.
Gottschalk, A. and Partridge, S.M. (1950). Interaction between simple sugars and amino acids. Nature, 165, 684.
Gottschalk, A. (1951). N-Substituted isoglucosamine released from mucoproteins by the influenza virus enzyme. Nature, 167, 845.
Gottschalk, A. (1952). Some biochemically relevant properties of N-substituted fructosamines derived from amino acids and N-arylglucosylamines. Biochem., J., 52, 455-460.
Heyns, K., Breur, H. and Paulsen, H. (1957). Darstellung und Verhalten der "Glucos-amino-säuren" aus Glycin, Alanin, Leucin und Fructose, Ber., 90, 1374-96.
Heyns, K. and Breur, H. (1958). Darstellung und Verhalten weiterer N-substituierter 2-Amino-2-desoxy-aldosen aus D-Fructose und Aminosäuren. Ber., 91, 2750-62.
Heyns, K. and Noak, H. (1962). Die Umsetzung von D-Fructose mit L-Lysin und L-Arginin und deren Beziehung nichtenzymatischen Bräunungreaktionen. Ber., 95, 720-7.

Heyns, K., Müller, G. and Paulsen H. (1967). Reaktion von Hexosen mit Aminosäuren. Liebigs Ann. Chem., 703, 202-14.

Hodge, J.E. and Rist, C.E. (1953). Dehydrated food chemistry browning reactions in model systems. J. Agr. Food Chem., 1, 928.

Hodge, J.E. and Rist, C.E. (1953). The Amadori rearrangement under new conditions and its significance for non enzymatic browning reactions. J. Am. Chem. Soc., 75, 316.

Jansen, G.R. (1962). Lysine in human nutrition. J. Nutrition, 76, suppl. I-part II.

Kuhn, R. and Dansi, A. (1936). Über eine molekulare Umlagerung.Ber., 69, 1745-54.

Kuhn, R. and Weygand, F. (1937). Die Amadori-Umlagerung. Ber., 70, 769-72.

Kuhn, R. and Birkhofer, L. (1938). Untersuchungen über N-Glykoside und die Amadori-Umlagerung. Ber., 71, 621-33.

Lea, C.H. and Hannan, R.S. (1949). Reaction between proteins and reducing sugars in the dry state. Effect of activity of water, pH and temperature on the primary reaction between casein and glucose. Biochim. Biophys. Acta, 3, 313.

Lea, C.H. and Hannan, R.S. (1950). Studies of the reaction between proteins and reducing sugars in the "dry" state. Biochim.Biophys. Acta, 5, 433-454.

Lea, C.H. and Hannan, R.S. (1950). Biochemical and nutritional significance of the reaction between proteins and reducing sugars. Nature, 165, 438.

Onishi, I, Nishi, A. and Kakizawa, T. (1969). Process for reacting amino acid and an active carbonyl sugar in a polyhydric alcohol. U.S. Patent, 3, 478, 015, Nov. 11, 1969.

Rosenberg, H.R. and Culik, R. (1957). Improvement of the protein quality of white rice by lysine supplementation. J. Nutrition, 63, 477.

Samejima, H., Furukawa, H. and Deguchi, T. (1970). Milk and milk powder with high nutritive value. Japan, 7003, 189, Feb. 1970.

Secchi, G. (1959). Considerazioni sulla integrazione con amminoacidi delle proteine degli alimenti. Quaderni della Nutrizione , XIX, 5, 251.

Song, P.S. and Chichester, C.O. (1966). Kinetic behavior and mechanism of inhibition in the Maillard reaction-I. J. Food Science, 31, 906.

Song, P.S. and Chichester, C.O. (1966). Kinetic behavior and mechanism of inhibition in the Maillard reaction-II. J.Food Science, 31, 914.

Song, P.S. and Chichester, C.O. (1967). Kinetic behavior and mechanism on the Maillard reaction between D-glucose and glycine . J. Food Science, 32, 98.

Weygand, F. (1940). Über N-Glykoside, II mitteil: Amadori-umlagerungen. Ber., 73, 1259-79.

Wolley, D.W. (1946). Some correlations of growth-promoting powers of proteins with their strepogenin content. J. Biol. Chem. , 162, 383.

28

NOVEL FREE RADICALS FORMED BY THE AMINO-CARBONYL REACTIONS OF SUGARS WITH AMINO ACIDS, AMINES, AND PROTEINS

Mitsuo Namiki, Tateki Hayashi, and Yukio Ohta

Department of Food Science and Technology

Nagoya University, Nagoya, Japan

ABSTRACT

Formation of novel free radicals in an early stage of the amino-carbonyl reactions of sugars with either amino acids,amines and some peptides were demonstrated by use of ESR spectroscopy. These radicals showed characteristic hyperfine ESR spectra and apparently differed from those observed in melanoidin. Formation of radicals of this type was faster at alkaline pH, but their signals were most stably observed in weakly acidic pH region and disappeared rapidly in other regions. The radicals developed even the mixtures were heated in open test tube but abolished rapidly by aeration. The results of reactions of various sugars or related carbonyl compounds with various amino compounds indicated that presence of sugar-like enediol structure in the carbonyl compound and a primary amine group in the amino compound are necessary for the formation of the radical products, and that the spectral patterns were mainly influenced by the structure of amino compound, e.g. α-vs β-alanine, but not by the structure of sugar, except for three carbon aldehydes. Analyses of the hyperfine structures of ESR spectra lead to the assumption of the identity of the radical products as N,N'-disubstituted pyrazine radical derivatives. Formation of such free radicals was also investigated on some peptides and proteins.

INTRODUCTION

A great deal of work has been done on nonenzymatic browning reactions of food components. Amino-carbonyl reactions of sugars with amino acids and proteins have been paid special attention as a principal cause of important quality changes during processing and storage of food products, such as browning by melanoidin formation, flavor development and deterioration of nutritional value.

Mechanism of the nonenzymatic browning reaction has also been extensively studied and it is said that an early stage of the reaction is initiated by condensation of sugar with amino acid that gives an enaminol compound, followed by formation of aminoketose through Amadori rearrangement or formation of osone by oxidative deamination. Including these reactions, the process of melanoidin formation have been interpreted as proton transfer chain reactions (Isbell and Frush, 1958). So far, little is known on the formation of free radicals in the browning reaction or amino-carbonyl reaction except for the stable free radicals observed in melanoidin prepared from glycine-glucose reaction mixture (Mitsuda et al.1965) and the radicals in the reaction of ninhydrin-amine system (Orr, 1965).

Recently, we have demonstrated the development of novel free radicals in an early stage of the amino-carbonyl reactions of sugars with amino acids (Namiki et al., 1973. Namiki et al.,1975), and of dehydroascorbic acid(DHA) with amino acids (Namiki et al., 1974. Yano et al., 1974) or amines (Yano et al., 1976-I).

In the case of DHA-amino acid system, no ESR spectral change was observed by changing the reactant amino acid, indicating no amino acid residue in the structure of free radical product probably due to the elimination of it through Strecker degradation reaction. The reaction mixture of DHA with amino acid in ethanol showed the characteristic ESR spectrum composing two different type spectra, responsible to the radical products A and C. They could be separately detected on TLC in a blue spot and in a vicinity of the red pigment, respectively. Based on chemical and spectral investigations, the structures of these radical products have been proposed. (Yano et al., 1976-II).

On the other hand, the ESR spectrum of the sugar-amino acid system varied apparently by the structural difference of amino acid, and the analyses of the hyperfine structures of ESR spectra indicate that the structure and formation mechanism of the free radical products in this system differed apparently from those formed in the DHA-amino acid systems (Ohta et al., 1976).

This is a tentatively summarized report of our past studies on the development of novel free radicals in the reactions of sugars and related carbonyl compounds with amino acids, amines, peptides and some proteins.

GENERAL EXPERIMENTAL METHODS

Sugars, amino acids and other related compounds used were guaranteed grade reagent. Distilled water was prepared in Pyrex apparatus.

Mixtures of sugar(or carbonyl compound) with amino acid (or amine) were prepared with distilled water sufficient to make solutions 1 M or more concentrated for each when solubilized. For the sake of convenience molar concentrations will be used in later sections.

The mixtures were heated in Pyrex test tubes in boiling water bath or oil bath for a given time. Development of free radical was checked at regular intervals by recording ESR spectrum with a JES-ME-1X ESR spectrometer. The splitting constants and g values of the spectra were determined using potassium peroxylamine disulfate as a standard. The concentration of free radical product was recorded for convenience as the relative intensity to the signal of standard polycrystalline Mn^{2+}. Development of the browning was determined by measurement of the absorbancy at 420 nm.

RESULTS AND DISCUSSIONS

Development of ESR Spectra and Browning during the Heating of the Mixtures of Sugar and Amino acid

Reactions of α- and β-alanine with sugars. A mixture of D-arabinose (1.0 g) and β-alanine (0.6 g) in distilled water (2.0 ml) was heated in boiling water bath and ESR spectra and browning were measured at 5 min intervals.As shown in Figure 1, development of ESR signal was observed with the mixture immediately after the heating was started, and its relative intensity reached a maximum at about five minutes and then gradually decreased with further heating to a constant level. During this process the ESR signal first showed a characteristic hyperfine structure as shown in Figure 1 (a), g=2.0034, 23 lines of a splitting constant of 3 G), but later changed to a broad singlet spectrum (c) with disappearance of hyperfine structure, after about 90 minutes of heating.

On the other hand, browning increased also markedly with the heating, due to high reactivity of β-alanine (Lento 1958), but the first development of browning was slightly later than that of the ESR signal. The changes in the relative intensity of ESR signal appeared to agree with the changes in the sum of those of each spectra (a) and (c) as indicated by the broken lines A and C, respectively.

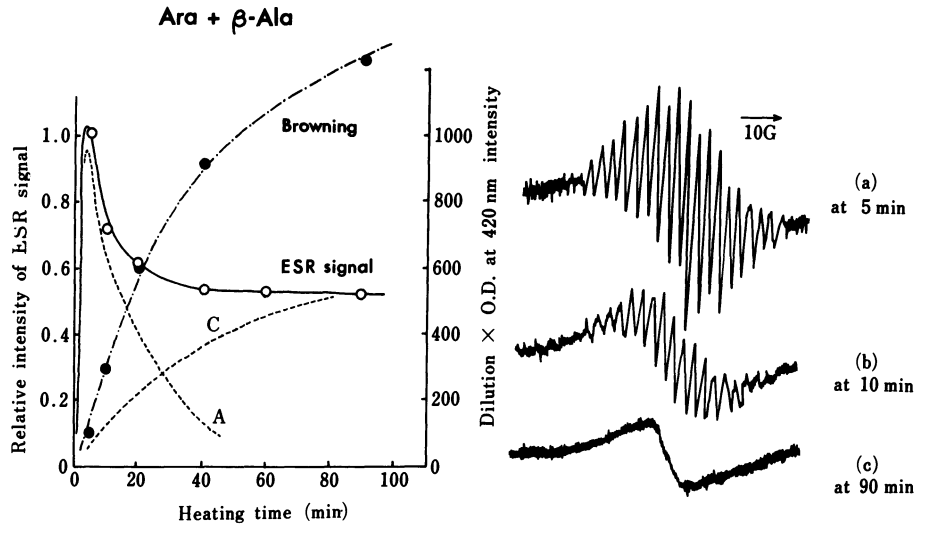


Figure 1. Development of ESR signals and browning in the reaction of D-arabinose with β-alanine, and ESR spectra of the reaction mixture heated at 98°C. —o— ESR signal; —●— browning; broken lines A and C , see text.

Figure 2 shows the progress of reaction of D-arabinose with α-alanine (each 1.5 M), and that of D-glucose with α-alanine (each 3.0M) treated under similar conditions. In the former case, the development of either free radical or browning proceeded in similarly to the previous case but more slowly. It is clearly seen again that the first development of free radical products preceded to that of browning

ESR spectrum at the initial stages also showed a characteristic hyperfine structure as shown in Figure 3, which was apparently different from that of the arabinose-β- alanine system (g=2.0036, 17 lines of the splitting constant of 3 G). The broad singlet spectrum of melanoidin radical increased along with the increase in the browning.

In contrast to this, browning in the D-glucose-α-alanine reaction occured only very weakly along with a slow increase in ESR signal of melanoidin type. Here the ESR signal with hyperfine structure was so weak that it could not be detected by measuring with highest available sensitivity.

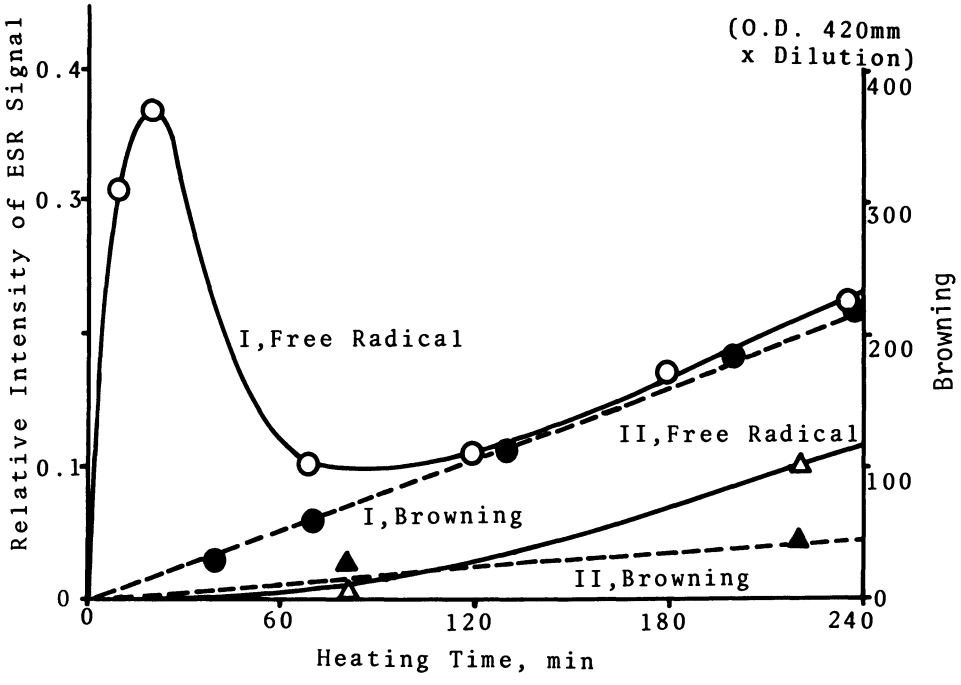


Figure 2. Development of ESR signal and browning in the reaction of (I) D-arabinose with α-alanine(each 1.5 M) and (II) D-glucose with β-alanine (each 3.0 M), heated at 98°C.
—○— ESR, —●— browning (I)
—△— ESR, browning (II)

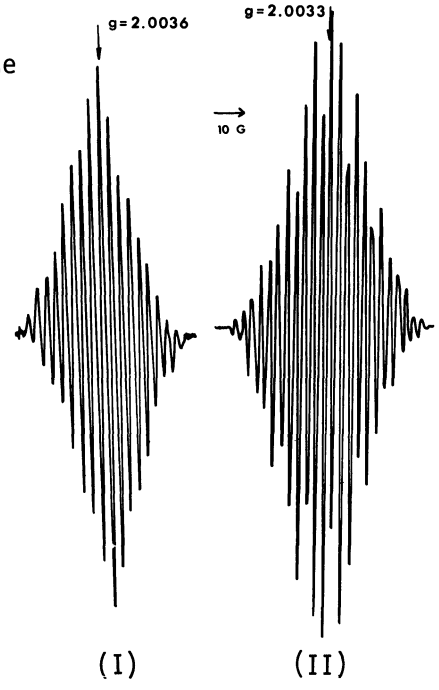


Figure 3. ESR spectra of the reaction mixtures of (I) D-arabinose with α-alanine, and (II) D-glucose with β-alanine

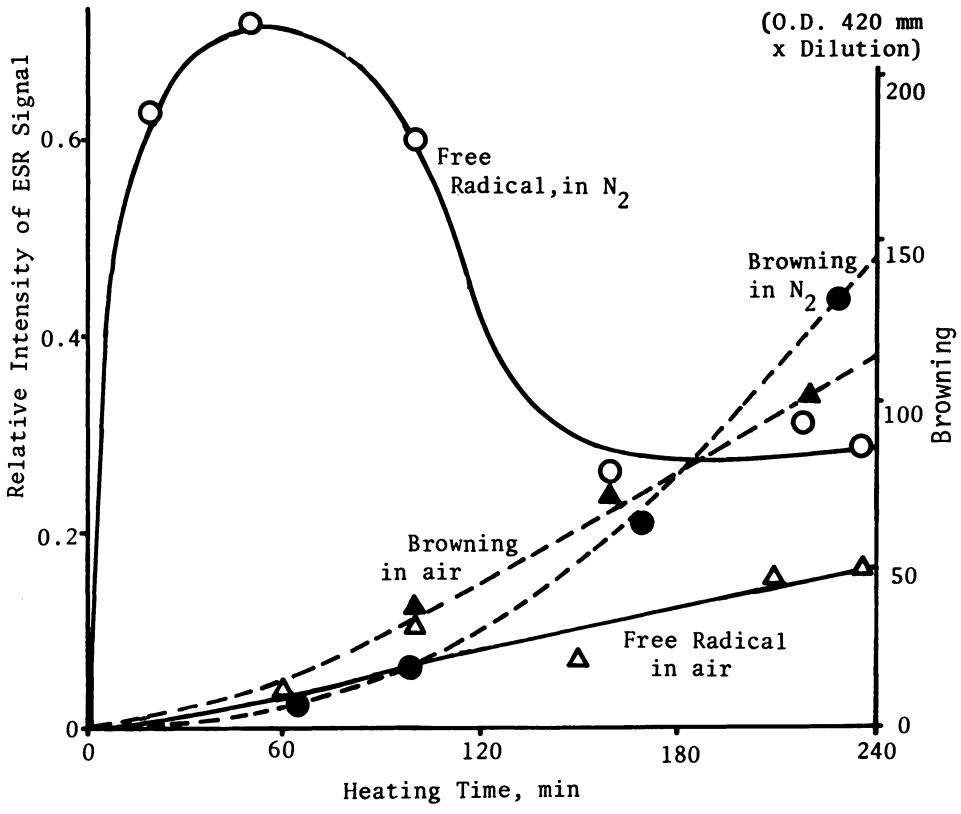


Figure 4. Development of ESR signal and browning in the reaction of D-glucose with β-alanine (each 1.5 M) heated at 98°C in N_2 or in air. —○— ESR, -●- browning, in N_2 —△— ESR, -▲- browning, in air.

Figure 4 shows two processes of reaction of D-glucose with --alanine, one under bubbling with air and the other with nitrogen. While the development of ESR signal with hyperfine structure was remarkable in the absence of air, it was totally lacking in air bubbling system, suggesting the instability of such radical species towards oxygen. This will be discussed again in a later section.

The ESR spectrum observed at the initial stage of the reaction is shown in Figure 3. The hyperfine splitting was essentially the same as that of the D-arabinose-β-alanine system (Figure 1).

From the results presented hereabove, it seems to say that the novel free radicals formed generally at the initial stage of the amino-carbonyl reaction of sugar with amino acid. The hyperfine splitting of their ESR spectra apparently differed between the reactions of α- and β-alanine with a given sugar.

Structural Effect of the Reactants on the Development of Free Radicals

Sugar and other Carbonyl Compounds. Further ESR studies were done on the systems consisting of various sugars or related carbonyl compounds with α- or β-alanine. Some characteristics of their ESR spectra and the relative intensities of browning are shown in Table 1. In regard to the hyperfine structures of ESR, it is to be noted that all but two of the sugars and related carbonyl compounds gave essentially the same type of ESR spectra with a given amino acid. Glyceraldehyde and dihydroxyacetone, however, gave spectra resembling each other and with more complicated hyperfine structures than others.

Comparison of ESR data, where smaller in the time to a maxmum and larger in the intensity mean superior in the radical formation , lead to the following views; 1) glycolaldehyde is very active and gave the most intense signal , which is noteworthy in connection with the consideration of structure of the radical species and of their formation mechanism. 2) glyceraldehyde and dihydroxyacetone are also very effective and very fast in the radical formation though their spectra are apparently distinct from others. 3) among sugars, the order of the readiness in formation of radicals giving hyperfine structural signal was in the following order; D-ribose>D-arabinose≫D-glucose>D-fructose. 4) although this order showed similarity to that has been found in the browning reaction, they are not necessarily corrected, because furfural and 5-hydroxymethylfurfural that are postulated as precursors to melanoidin, and also crotonaldehyde, all producd intense browning with β-alanine but no formation of radical giving structured signal was observed with them.

Reactions of many other compounds related to sugar with β-alanine or t-butylamine were similarly investigated, and they were tentatively classified into two groups according to their readiness in producing structured ESR signals as in Table 2. Comparison of chemical structures of these groups of sugar-like compounds lead to the supposition that the presence of an endiol group or a potential endiol group (R-HCOH-CHO) in the molecule is a requisite for such radical formation. Glyoxal is an exception for this and,although no explanation can be given, it may conceivably give rise to endiol-like function during reaction, by dimerization, reduction, hydration or dehydration.

Examination of the data for compounds incapable of producing signals with hyperfine structure indicated considerable differences of behavior among them. E.g.methylglyoxal gave a marked ESR signal along with an intense browning but its spectrum was a broad singlet without hyperfine splitting.

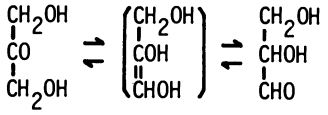
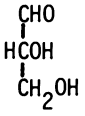
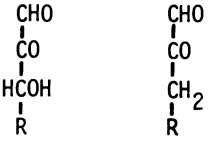
Table 1. ESR Spectral Data of the Free Radicals in the Amino-Carbonyl Reaction of Sugars and Other Carbonyl Compounds with α- or β-Alanine a)

	Max. of ESR signals			
	Line no. of hfs	Time, min	Intensity	Browning
α-Alanine				
D-Glucose	17	20	0.05	+
D-Fructose				+
D-Arabinose	17∿19	∿10	∿0.2	++
D-Xylose	17∿	∿10	∿0.1	++
D-Ribose	17∿	8	0.1	+++
Glycolaldehyde	17∿	3	16	+++
β-Alanine				
D-Glucose	23∿25	15	1.0	++
D-Fructose	23∿	20	0.8	++
D-Arabinose	23∿	5	1.0	++++
D-Xylose	23∿	∿1	∿1.0	++++
D-Ribose	23∿	∿1	∿0.5	++++
Glyceraldehyde	31∿32	0.2	5	+++++
Dihydroxyacetone	31∿32	0.2	5	+++++
Glycolaldehyde	23∿	1	44	+++++
3-Deoxyglucosone	23∿	13	0.8	++++
5-Hydroxymethyl-furfural				++
Furfural				+++++
Glyoxal	23∿	0.2	5	+++++
Crotonaldehyde				++++
Propionaldehyde				+

[a] Aqueous solutions (each 3 *M*) were heated in boiling water bath.

Table 2. Structural Effect of Carbonyl Compounds on the Development of Free Radicals

Group A (ESR spectrum with hyperfine structure)

Glyoxal	Dihydroxyacetone 
Glycolaldehyde	Glyceraldehyde 
Aldose D-Glucose D-Arabinose D-Xylose	Osones Glucosone 3-Deoxyglcosone 
Ketose D-Fructose	

Group B (Broad singlet spectrum or no ESR signal)

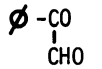
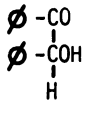
Methylglyoxal		Phenylglyoxal	
Acetoin		Benzoin	
Diacetyl		Formaldehyde	HCOH
Ethyleneglycol			

Table 3. Structural Effect of Amino Compounds on the Development of Free Radicals

Group 1. ESR spectrum with hyperfine structure

Amino acids	
glycine	tryptophan
α-alanine	lysine
β-alanine	glutamic acid
valine	glutamine
leucine	aspartic acid
isoleucine	asparagine
phenylalanine	Amines
histidine	n-butylamine
serine	ethylamine
methionine	methylamine
tyrosine	isopropylamine
arginine	tert-butylamine
threonine	

Group 2. ESR spectrum with broad singlet or no ESR signal

	Amines	Sugars
Quarternary ammonium	tetramethylammonium hydroxide	xylose
Tertiary amine	trimethylamine	xylose
Secondary amines	dimethylamine	xylose,glucose
	imidazole	xylose,arabinose
	indole	xylose,arabinose
	proline	xylose,arabinose
	N-Acetyl Histidine	xylose,arabinose
	N-Acetyl Tryptophan	xylose,arabinose
Primary amines	urea	xylose
	formamide	glucose
	guanidine	xylose,arabinose
	2,4-dinitroaniline	xylose
	aniline	glucose
	2,4-dinitrophenyl-hydrazine	xylose
	ammonium chloride	glucose,glyoxal
	28% ammonia water	glucose
	o-methylhydroxylamine	glucose
	ethylenediamine	glucose,glyoxal
	cysteine	glucose
	cystine	glucose

Amino Compounds. Various amino acids, amines, and other related amino compounds were respectively reacted with D-xylose, D-arabinose or D-glucose, usually suspended mixtures adjusted to pH 8.0-8.5. After heating at 90°C for 10 minutes ESR spectrum was measured.

With respect to the readiness to give the ESR spectrum with hyperfine structure, the amino compounds tested could be classified into two groups as listed in Table 3. The ability to give the characteristic ESR spectrum was only observed in the compounds with a primary amino group , though there were several exceptions as listed in Group 2. Most of these exceptions were for primary amines with special characters. No ESR spectrum was observed with either N-acetyl-histidine and N-acetyl-tryptophan ,though the former gave rise to a weak browning with sugar, and this fact suggest that histidine and tryptophan residues are not responsible in the free radical formation that occurs when protein containing these amino acids were heated with sugar.

Glycine showed somewhat special feature, since free glycine did not exhibit any signal with detectable hyperfine structure in ESR spectrum with common sugars, it could with glycolaldehyde, as will be shown in later (Figure 15). On the other hand, glycine methyl ester gave ESR spectrum with marked hyperfine structure.

Among the compounds listed in group 2, tetramethylammonium hydroxide, imidazole and indol, showed broad singlet ESR signal during the reaction with sugars in alkaline solution.

Effects of Various Reaction Conditions on the Development and Stability of Free Radicals.

pH Effect. The reactions presented above were undertaken at pH range 5-6 in distilled water. Since it has been well known that the browning reaction of sugar with amino acid proceeds faster in alkaline solution (Kato 1956), effects of pH on the development of free radicals and browning were investigated, changing pH of the mixture with NaOH solution.

Figures 5A and 5B show pH dependence of the signal intensity in the reaction systems D-glucose-α-alanine and D-glucose- β-alanine, respectively. In Figure 5A the signal intensities and absorbancies are those taken when the former reached its maximum during each reaction at a given pH.
Whereas in Figure 5B they are those taken uniformly 10 min after the start of the reaction, owing to considerably faster reaction with β-alanine than with α-alanine (Lento 1958).

In both cases it is evident that free radical formation as well as browning were enhanced by higher pH and ,especially, the ESR signal intensity increased remarkably starting at pH 8.0 showing maximum at pH around 10-11, and fell rapidly at still

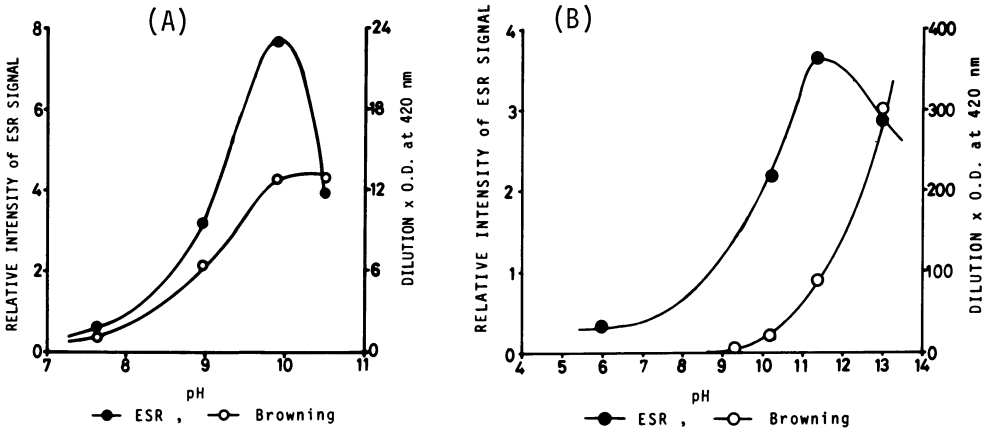


Figure 5. Effect of pH on the development of ESR signal and browning in the reaction mixtures of D-glucose with α-alanine or β-alanine (each 1.5 M) heated at 90°C.

(A) D-glucose-α-alanine (B) D-glucose-β-alanine

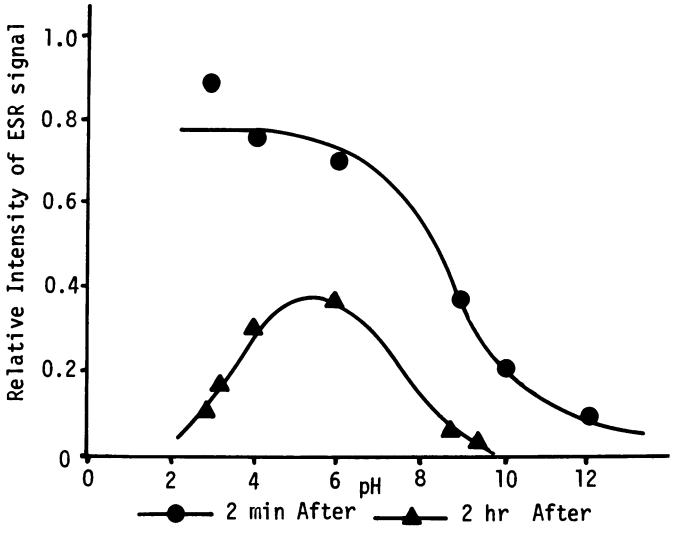


Figure 6. Effect of pH on the stability of free radicals at room temperature.

higher pH values. The reason for these is not yet clear but the decrease might be caused by the lability of the free radical products at higher pH values.

Here it is to be recalled that the reducing sugars as glucose have been known to give free radicals at highly alkaline solutions (Lagercrantz, 1964). Test was therefore made to know whether the free radicals observed in the sugar-amino acid systems involve the radicals originated from sugar alone in alkaline solution. The D-glucose solutions prepared with NaOH more concentrated than 1 M were heated and ESR spectra were measured in a similar way. It was demonstrated that the free radicals originated from sugar alone developed markedly only at pH 12 or above and the ESR spectral pattern was apparently differed from these presented above.

pH Effect on the Stability of the Free Radicals. After the mixture of D-glucose and β-alanine in distilled water was heated at 95°C for 20 min, pH was adjusted at a given value ranging from 2 to 11 by addition of HCl or NaOH solutions. The relative intensity of ESR signal of each mixture was measured at 2 min and 2 hr after standing at room temperature. As shown in Figure 6, the ESR signal disappeared rapidly in alkaline solution, especially those above pH 10, while it was more stable on acidic side, but it disappeared completely after 2 hrs at pH 2 or below. Thus the free radicals were known to unstable both in strong acid and especially in alkaline solutions , while being fairly stable in the mixture at pH around 4-5.

In an another experiment the mixture of D-glucose and t-butyl amine, each 1.0 M in distilled water was adjusted to pH 11.3 and heated for 5 min at 90°C. The reaction mixture was divided into several portions and each was adjusted to different pH values ranging 0.3 to 11.7. The mixtures were left standing at room temperature and the ESR signal intensity was measured at a given time intervals.

Here, it was also demonstrated that the free radical products were considerably stable at pH around 4-5, where the half-decay time of ESR signal was about 44 hr.

Effect of Molar Ratio of the Reactants. The free radical development and browning were measured the mixtures of D-glucose and β-alanine in different molar ratios. The results shown in Figure 7 indicate that the radical formation and browning as well was favored by 2:1 amino acid-sugar ratio. However, in the case of D-glucose and t-butylamine, the development of the free radical was reached to a constant level at 1:1 sugar-amino acid ratio.The reason of this difference has not been explained.

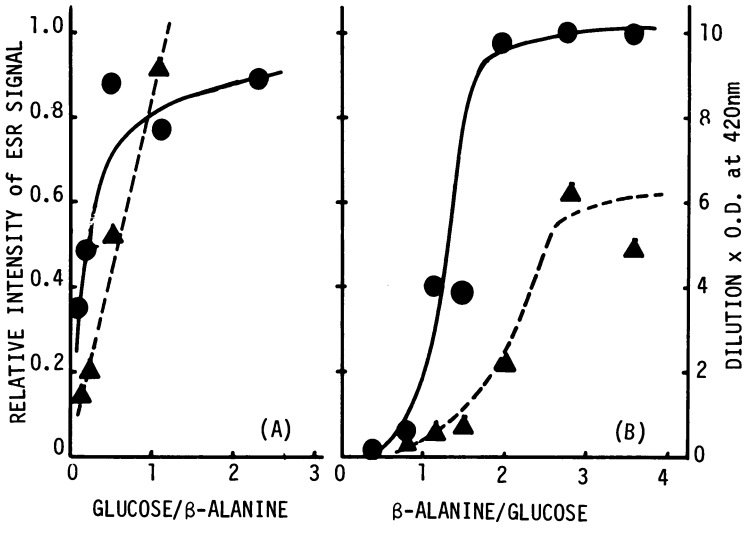


Figure 7. Effects of molar ratio on the formation of free radicals and browning in the reaction of D-glucose with β-alanine: (A) β-alanine 2.3 M, heated at 98°C for 10 min; (B)D-glucose 1.4 M,heated at 98°C for 6 min: (●) ESR signal; (-▲-) browning.

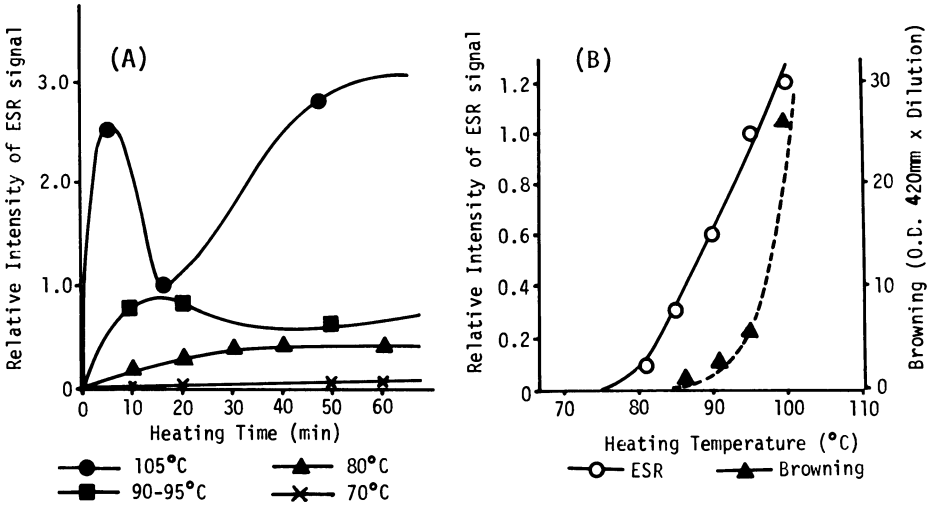


Figure 8. Effects of heating temperature on the formation of free radicals and browning in the reaction of D-glucose with β-alanine (each 3 M in distilled water) , see text.

Effect of Reaction Temperature. The mixture of D-glucose and β-alanine (each 3 M) in distilled water was heated in an oil or water bath for 10 min at different temperatures. Figure 8-A shows changes in the relative intensities of ESR signal during the reaction and Figure 8-B indicates the ESR signal intensity and absorbancy due to browning at 10 min.

Remarkable development of ESR signal was observed by reaction at 80°C , and the rate increased rapidly with temperature. Enhancement of the radical formation by higher temperature was more significant than that of browning, especially at around 80-90°C.

These results indicate that the free radical products formed in the early stage of the reaction are fairly stable for the heating though they are not so stable as those in melanoidin and easily changed to the later type, and that the radical formation greatly enhanced by the heating. So the enhanced rate of formation might be superior to that of the decomposition and resulted in a marked relative intensity of the ESR signal at the higher reaction temperatures. While, the fact that no detectable ESR signal was observed with the heating below 70°C is not necessarily means the radical formation was absent at such heating temperature but it may be caused by the processes of a weak radical formation followed by the decomposition by air or any other factors.

Effect of Oxygen. As is known , oxygen plays an important role in free radical reaction, usually by reacting rapidly with radical product to form peroxide leading to its destruction. In a reaction of 4-NQNO, however, a slight amount of dissolved oxygen (10^{-4} mm) is required to give the free radical product (Kataoka et al., 1967).

Then, the effect of oxygen on the development of free radicals in the reaction of sugar with amino acid as well as on its stability was examined.

The results of foregoing experiments indicate the following; the free radical formation in the systems being studied is actually affected by oxygen and is abolished by bubbling of air(see Figure 3), but does not seem to be as sensitively affected as the radical reactions usually encountered because most of the reaction has been carried out in open test tubes without exclusion of atomospheric oxygen. In order to examine the possibility of the mechanism similar to the radical formation from 4-NQNO requiring trace of dissolved oxygen, the reaction of D-glucose with β-alanine was carried out in a sealed tube evacuated below 10^{-4} mmHg. As seen in Figure 9 the ESR signal at the end of the reaction did not differ significantly from that by the pallalel run reacted in an open tube and,morover, the intensity of signal after 30 min standing at room temperature was nearly equal. These results seemed to suggest the absence of essential role of oxygen in radical formation in this system.

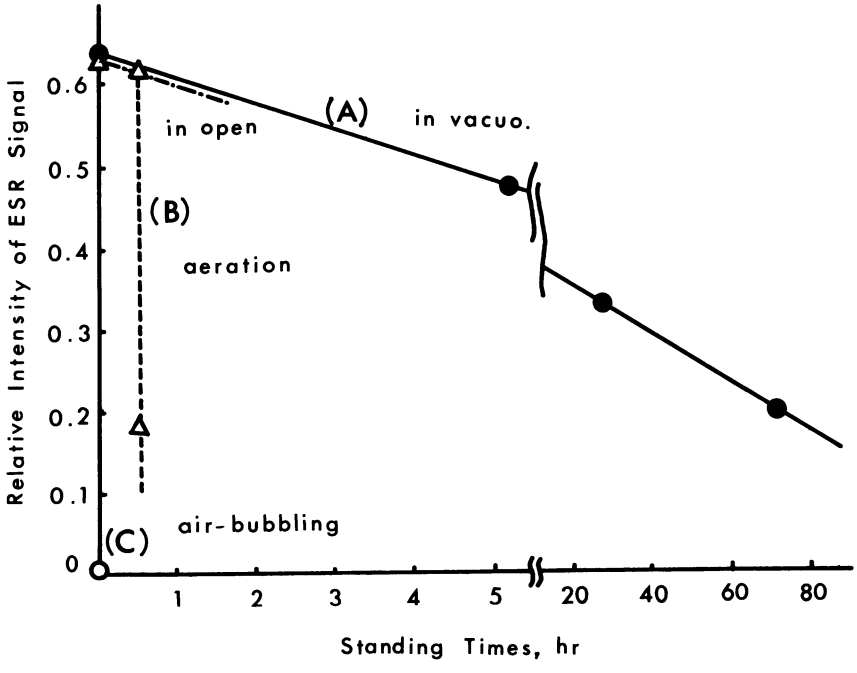


Figure 9. Effect of oxygen on the formation and stability of free radicals in the reaction of D-glucose with β-alanine (each 1.5M), heated at 98°C for 10 min and then kept at room temperature: (A) the reaction done in vacuo and kept intact; (B) the reaction done in an open test tube and after standing for 30 min was aerated for 2 min; (C) the reaction done under air bubbing.

Analyses of Hyperfine Structures of the ESR Spectra

To elucidate the structures of the free radical products, analyses of the hyperfine structures were made mainly on the spectra observed in the mixtures of glycolaldehyde, D-arabinose, or D-glucose with several amino compounds, because the type of hyperfine structures were known to be determined mainly by the structural differences of the amino compounds.

Amines with Sugars or Glycolaldehyde. The analysis was first made on the spectrum of t-butylamine and glycolaldehyde system, because this amine has no α-proton so that influence of protons in the amino compound residue on the hyperfine structure would be neglibibly small and, moreover, the reactions of t-butylamine with sugars and related compounds gave essentially the same spectral pattern, including the three -carbon aldehydes as glyceraldehyde and dihydroxyacetone. As shown in Figure 10, the hyperfine structure could be resolved into the 2.74 G quartet and the 8.74 G doublet responsible for four equivalent protons and two equivalent nitrogens, respectively, as indicated by the stick diagram in the figure. Here, it is to be noted that no spectral change was observed by the reaction employed in heavy water, suggesting no exchangeable proton in neighbor of the free radical.

Figure 11 shows the ESR spectrum of the reaction mixture of methylamine and D-glucose,each 2 M in distilled water, heated at 90°C for 20 min. The hyperfine structure could be resolved into the 8.35 G quintet, the 7.98 G septet and the 2.83 G quintet due to two equivalent nitrogens, six equivalent protons and four equivalent protons.

When methylamine was replaced by methyl-d_3-amine(CD_3NH_2) the hyperfine structure was changed as shown in Figure 11-b, which is resolved into the 8.38 G doublet, the 2.86 G quintet and the 1.25 G septet responsible for two equivalent nitrogens, four equivalent protons and six equivalent deutrons. The change in the splitting constant of the septet from 7.98 G to 1.25 G is due apparently to the replacement of CH_3 to CD_3 and thus it was demonstrated that the equivalent six protons belong to a couple of methyl protons. This fact and the presence of two equivalent nitrogens suggest the involvemnet of two molecules of methylamine in one free radical product molecule.

A similar experiment with ethylamine and D-glucose gave the spectrum shown in Figure 12, which could also be assigned to the contributions of two equivalent nitrogens, four equivalent protons, and four equivalent protons.

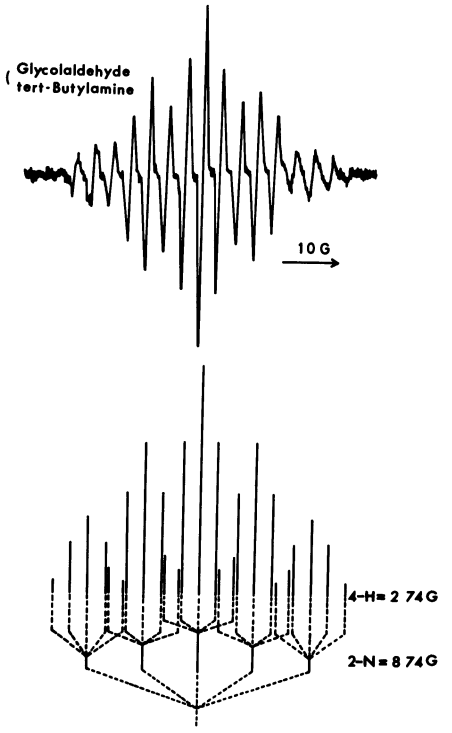


Fig. 10

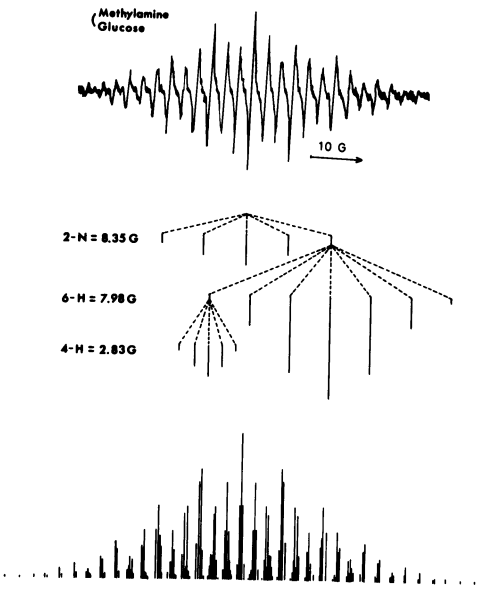
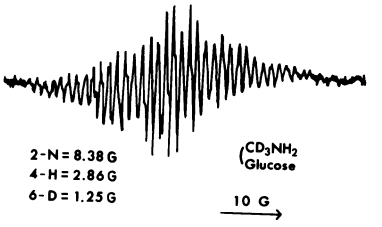


Fig. 11a

Fig. 11b



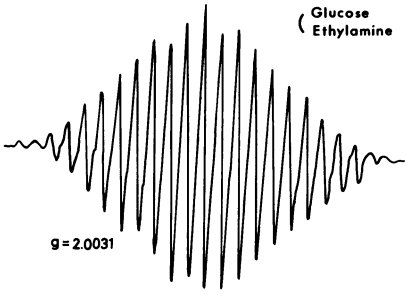


Fig. 12a

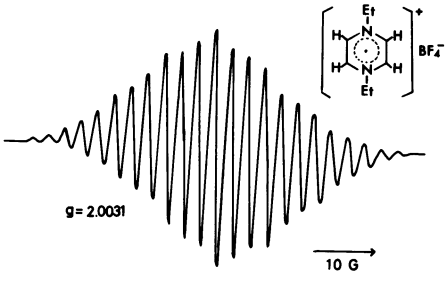


Fig. 12b

Figs. 10-12. ESR spectra of the reaction mixtures of amines with sugar or glycolaldehyde. Fig. 10. t-Butylamine with glycolaldehyde; Fig. 11a. Methylamine with D-glucose; Fig. 11b. Methyl-D_3-amine with D-glucose; Fig. 12a. Ethylamine with D-glucose; Fig. 12b. Synthesized N,N'-diethyl pyrazine cation radical.

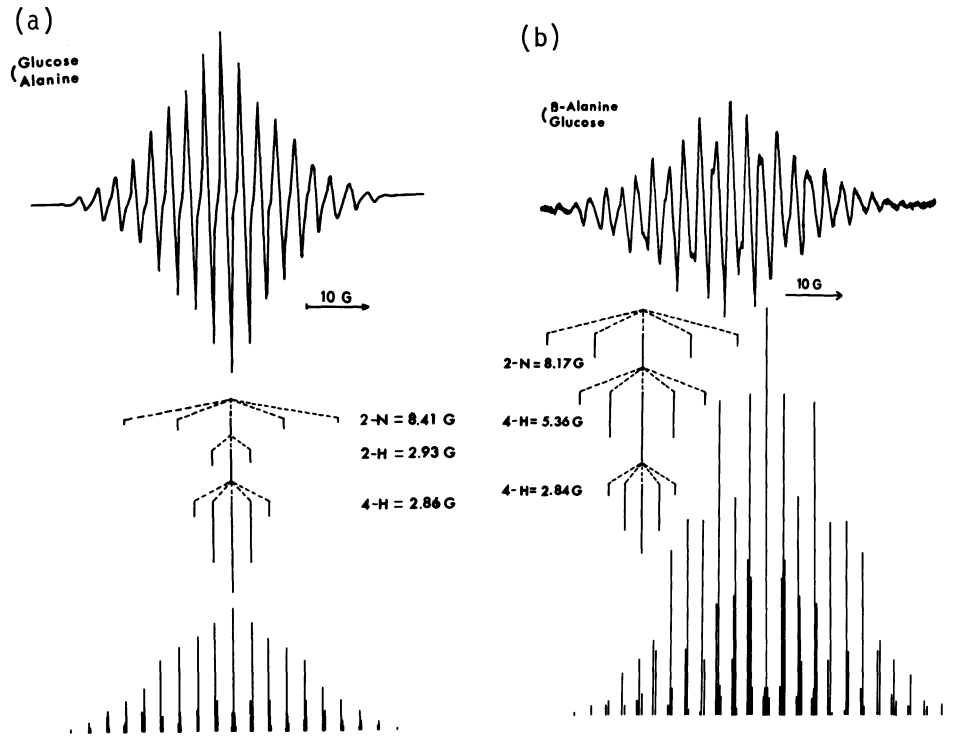


Figure 13. ESR spectra of the reaction mixtures of (a) D-glucose α-alanie and (b) D-glucose-β-alanie.

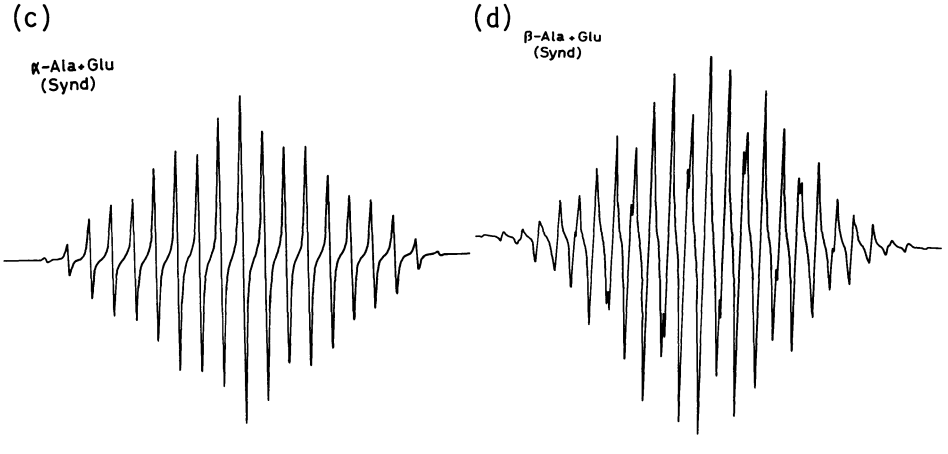


Figure 13-b. Synthesized ESR spectra based on the analytical data of the hyperfine structures.
(c) D-glucose-α-alanine and (d) D-glucose-β-alanine.

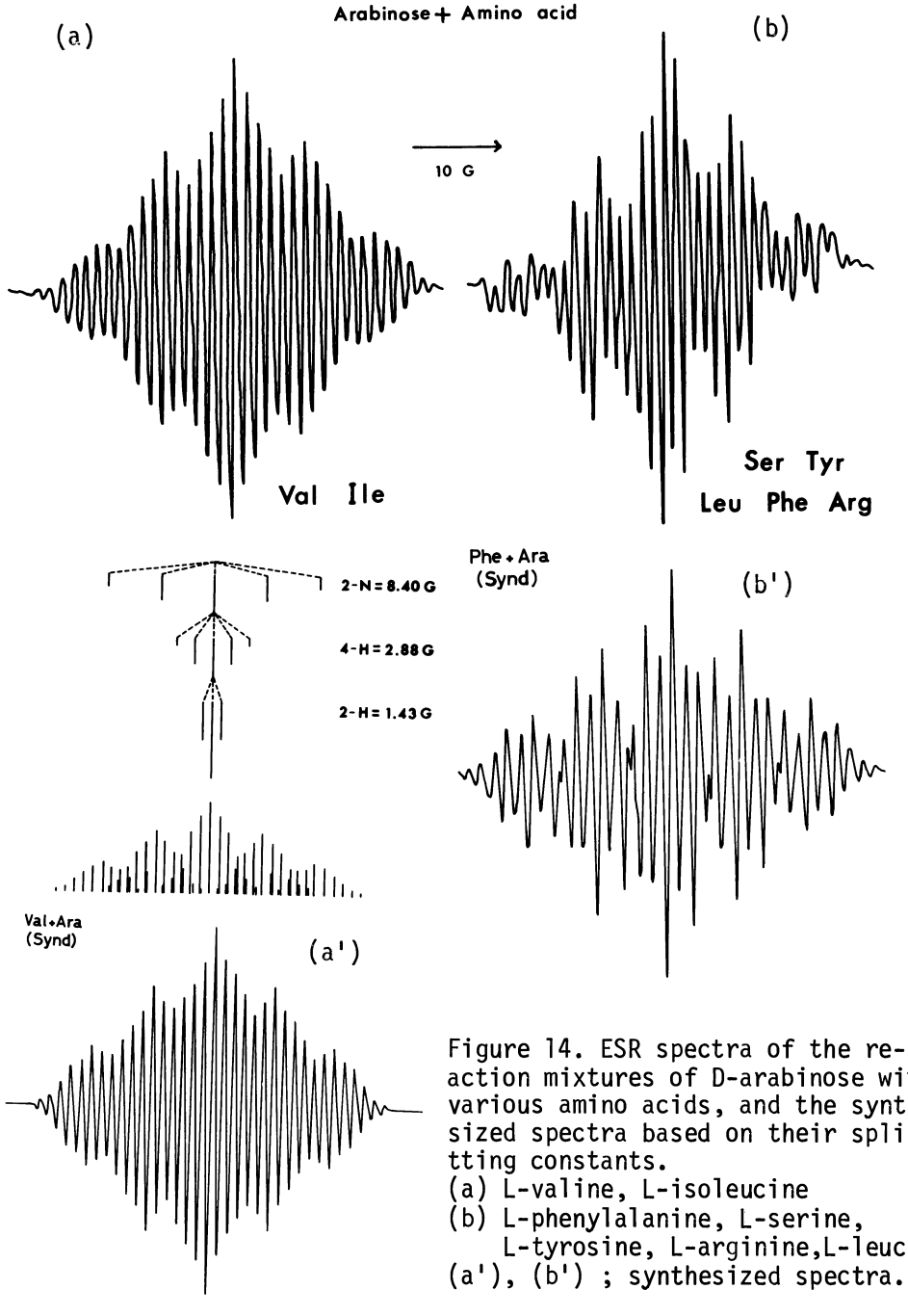


Figure 14. ESR spectra of the reaction mixtures of D-arabinose with various amino acids, and the synthesized spectra based on their splitting constants.
(a) L-valine, L-isoleucine
(b) L-phenylalanine, L-serine, L-tyrosine, L-arginine, L-leucine
(a'), (b') ; synthesized spectra.

Table-4. Analyses of the hyperfine structures of ESR spectra

Amino acid or Amine	Splitting constants (G)			
	(α-H)		(2-N)	(4-H)
Glycine	4.69	(4H)	8.15	3.04
L-α-Alanine	2.93	(2H)	8.41	2.86
L-β-Alanine	5.36	(4H)	8.15	2.84
L-Valine	1.43	(2H)	8.40	2.88
L-Phenylalanine	1.31	(2H)	7.99	3.03
Other amino acids*	1.5±0.1	(2H)	8.3±0.15	2.9±0.1
tert-Butylamine			8.74	2.74
Methylamine	7.98	(6H)	8.35	2.83
Methyl-d_3-amine	1.25	(6D)	8.38	2.86
Ethylamine	5.37	(4H)	8.35	2.85
N,N'-Diethyl-pyrazinium salt	5.33	(4H)	8.37	2.82

Sugars; D-glucose, D-arabinose, D-xylose or glycolaldehyde.
*Other amino acids; L-serine, L-methionine, L-leucine, L-isoleucine, L-tyrosine, L-arginine, etc.

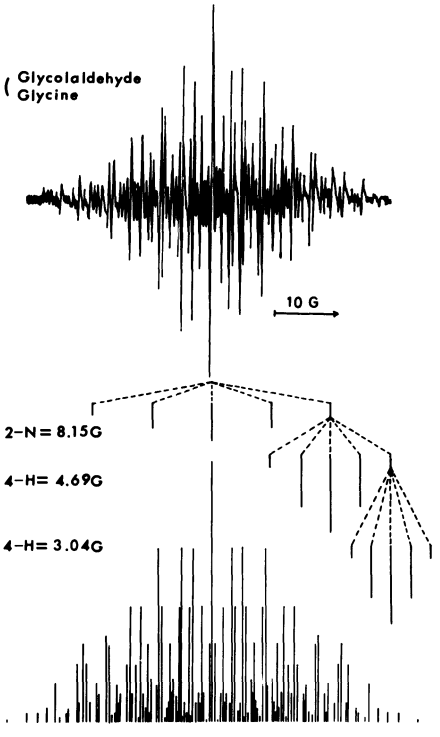


Figure 15. ESR spectrum of the reaction mixture of glycine with glycolaldehyde and analysis of its hyperfine structure.

The analyzed data of the hyperfine structures of ESR spectra observed in various amino acids or amines with arabinose or glucose are summarized in Table 4.

ESR Spectra of Three Carbon Aldehydes with Amino Compounds. As has been mentioned in Table 1, the three carbon aldehydes, glyceraldehyde and dihydroxyacetone, gave exceptionaly the ESR spectra of different type from other carbonyl compounds in the reactions with amino compound.

As can be seen in the representative spectra shown in Figure 16, glyceraldehyde and dihydroxyacetone with a given amino acid gave quite similar ESR spectra in their hyperfine splitting and both spectra has an overlapped broad singlet even in the early stage of the reaction. The fact that dihydroxyacetone showed behavior similar to glyceraldehyde in the radical formation might be due to its isomeric conversion to glyceraldehyde through endiol structure, as the scheme shown in Table 2.

Analysis of the hyperfine structure of these spectra seemed rather difficult, so the structure of radical product formed from these three carbon aldehydes remained obscure. However, in connection with the assumption that the radical products formed by the reaction of glycolaldehyde with amines are N,N'-disub - stituted pyrazine cation radicals,the radical product from three carbon aldehyde is expected to be 2,5-disubstituted, probably CH_2OH or CH_3 group, derivative of the above pyrazine radical. Then, 2,5-dimethyl-N,N'-diethyl pyrazine cation radical was prepared in a similar way as above from 2,5-dimethylpyrazine and the ESR spectrum is shown in Figure 17. In comparison this spectrum with those observed in the case of three carbon aldehyde, we can observed some corresponding signal between them ,but the spectrum of synthetic radical has more complicated hyperfine splitting, so assignment of the radical product from glyceraldehyde with ethyl amine to the 2,5-dimethyl derivative of pyrazine radical seemed unsuccessful and the structure remains to be identified.

Here an interesting fact is that the reactions of these three carbon aldehydes with t-butylamine gave the ESR spectra of apparently different from those observed in the cases with other amino compounds in the splitting and whole pattern of the hyperfine structure, and rather it resembles well with the spectrum observed in the reaction of glycolaldehyde with t-butylamine (see Figure 10).

The reasons why only glyceraldehyde gave different type ESR spectrum among the C-2 to C-6 sugar analogues and, moreover, why it provide the common sugar type spectrum with t-butylamine have not yet been made clear.

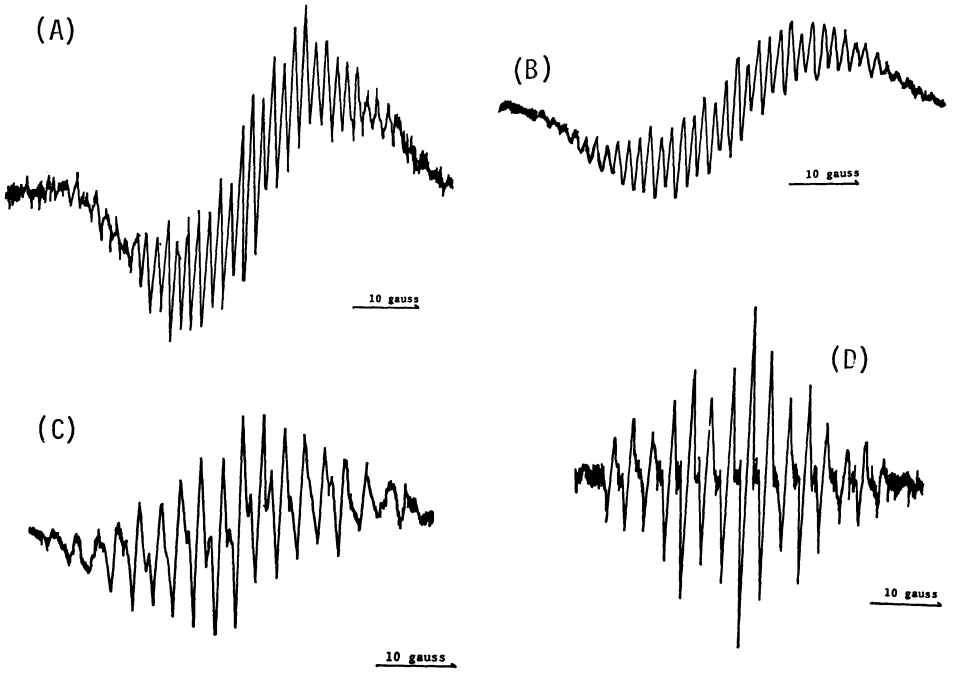


Figure 16. ESR spectra of the reaction mixtures of three carbon aldehydes with amino compounds.
(A) Glyceraldehyde with β-alanine, (B) Dihydroxyacetone with β-alanine, (C) Dihydroxyacetone with α-alanine (D) Glyceraldehyde with t-butylamine

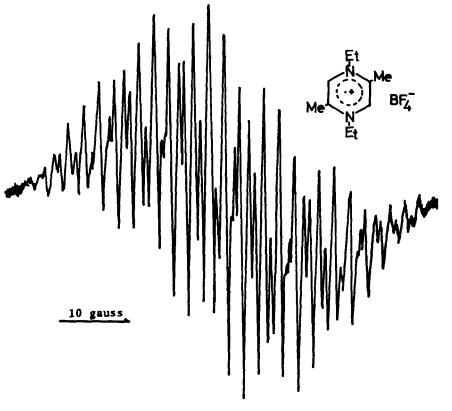
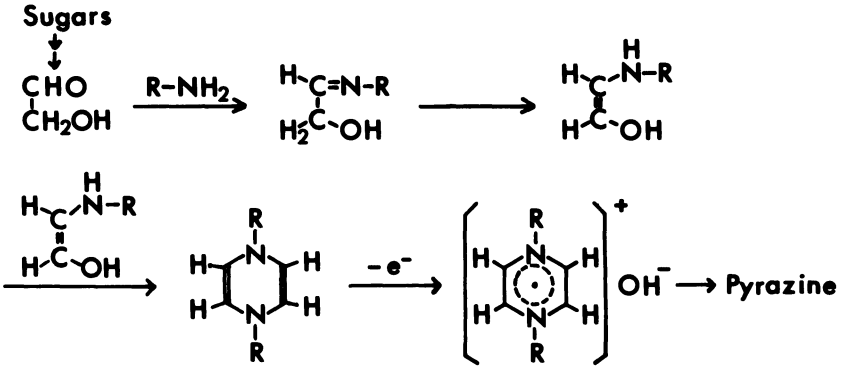


Figure 17. ESR spectrum of N,N'-diethyl-2,5-dimethylpyrazinium fluoroborate cation radical.

Mechanism of Free Radical Formation in the Reaction of Sugars with Amino Acids

From the above spectral analyses, it seems adequate to say that the free radicals which are formed at the initial stage of the reaction of sugar with amino acid and showed characteristic hyperfine structures are probably N,N'-disubstituted pyrazine radical cations. The mechnism of formation of the pyrazine radicals from glycolaldehyde which is the simplest sugar analogoue, may be considered as the following scheme;



Each of the individual steps in this scheme seems adequate from a chemical viewpoint, and it would be supported by the fact that glycolaldehyde is the most effective one among carbonyl compounds in giving the characteristic ESR spectrum and its reaction with ethylamine or others develops intense ESR signal far more rapidly than any other sugar-amino acid system.

However, there are many such problems to be investigated about the mechanism of free radical formation in the reaction of sugars with amino acids. First, if one postulates that radical products could only be formed from glycolaldehyde as an essential compound, adequate amounts of glycoaldehyde must be produced at early stages of the reaction. Although formation of such small carbonyl compounds as glycolaldehyde, glyceraldehyde and others in the sugar-amino acid reaction has been postulated (Hodge 1953), their existence appears not to have been substantiated experimentally. If these aldehydes are actually formed, they should be derived from C-C scission of a reaction intermediate such as a glucosone. Additional studies are, therefore, needed to establish whether enough glycolaldehyde is produced at the initial stage of reaction through quantitative analysis of the reaction intermediates. This is a difficult process because of the high reactivity of the amino acids.

On the other hand, if one considers the formation pathway of the radicals from the various compounds which have been presented as the intermediate products in an early stage of the sugar-amino acid reaction system, the following pathway seems probable; the pyrazine derivative possessing N,N'-diamino acid residues and 2,5-disugar residues would be formed by condensation of two molecules of the enaminol product, e.g. that of N-substituted-1-amino-1-deoxy-ketose in glucose-amino acid system, and subsequent elimination of the subsitutents of sugar residues by C-C sicssion will give the proposed pyrazine radical products.Concerning this postulation, presence of such N,N'-disubstituted pyrazine drivatives as the reaction product is hardly reported and, moreover, the following C-C scission process remains obscure.

To investigate this mechanism, the following experiment was carried out as a preliminary investigation. 1-Alanino-1-deoxy-D-fructose, prepared according to Anet (Anet, 1958), was heated in alkaline solution, with D-glucose in distilled water,or with β-alanine in distilled water, at 90°C for 15 to 30 min. In either case no detectable ESR signal with characteristic hyperfine structure was observed.

In any way, it is not at present possible to decide whether the free radical products formed necessarily from glycolaldehyde or from other unknown pathways as presented above. Further investigation is necessary to explain fully this interesting observation.

Free Radical Formation in the Peptide-Sugar System

The investigation to see the free radical formation in the peptide-sugar reaction system was undertaken by use of some oligopeptides purchased as pure reagents. As mentioned above free glycine gave ESR spectrum with hyperfine structure only with glycolaldehyde, so the reactions of glycine peptides were carried out with glycolaldehyde in distilled water.

It was demonstrated that the oligopeptides so far tested herewith, that is , tetraglycine, triglycine, glycylglycine, and glycyl-L-leucine gave also ESR spectra with hyperfine structure which seemed essentially the same in the splitting to that observed with glycine. The spectrum of triglycine-glycolaldehyde mixture is shown in Figure 18. These results seemed suggestive to consider properties and formation mechanisms of the free radicals observed in the following protein-sugar systems.

Free Radical Formation in Protein-Sugar Systems

It is known that the browning reaction of proteins with sugars causes deterioration in color and nutritional value of foods along with some changes in physicochemical properties of the proteins. It is, therefore, of interest to examine the possibility of formation of novel free radicals during the reaction of proteins with sugars and to note the accompanying changes in the proteins. The following preliminary experiments were carried out: since protein foods are usually used as viscous concentrated solutions or as dried powders, 10% concentrations of protein plus sugar were suspended in distilled water or buffer. In addition, we also employed protein-sugar powders (freeze-dried) which contained 10% protein or more. Purified proteins were obtained from the following sources: bovine albumin, ovalbumin and milk casein from Armour; zein was isolated from corn, protamine sulfate from sperms of herring; hemoglobin from Tokyo Kasei; and crystalline lysozyme from Sigma. Samples were placed in fine Pyrex test tubes and heated in a boiling water bath.

Figure 19 shows the ESR spectra observed with the reaction mixture of D-glucose and fresh egg white or ovalbumin in distilled water heated at 90°C for 30 min. The ESR spectra exhibited only a broad singlet. Heating of each protein alone under similar conditions did not result in any observable signal.

The effect of pH on ESR signal development was studied with the ovalbumin-xylose mixture. As illustrated in Figure 20, signal development hardly took place in acid solutions but were quite marked in alkaline media, especially above pH 8.0. Moreover, at pH 9.2, the ESR signal increased rapidly at the initial stages of heating and then decreased with further heating. The pH effects agree well with those observed in the sugar-amino acid systems.

Figure 21 shows the development of ESR signals in the reaction mixtures of various proteins and xylose in distilled water. Similar free radical formations were noted with bovine albumin, ovalbumin, or dialyzed egg white. However, lysozyme did not give an ESR signal. The intense signal originating from fresh egg white and xylose might be the result of some small molecular-weight amino compounds which are present in small amounts in the material (Sato, 1973). No signal development was observed when the same sample was dialyzed against distilled water.

The development of ESR signals was also observed when freeze-dried powders of protein-sugar mixtures were heated at 90°C. As shown in Fig. 22, the ESR signals increased steadily with increasing heating time with bovine albumin, ovalbumin, dialyzed egg white and gluten, whereas no significant signal was noted with protamin or zein. Exceptionally intense signals observed with fresh egg white might be caused by some minor amino compounds.

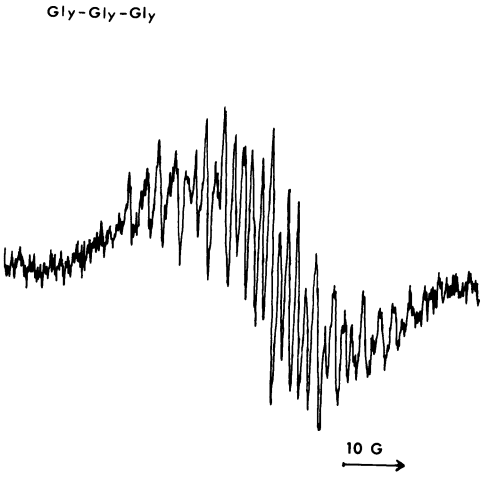


Figure 18. ESR spectrum of the reaction mixture of triglycine with glycolaldehyde.

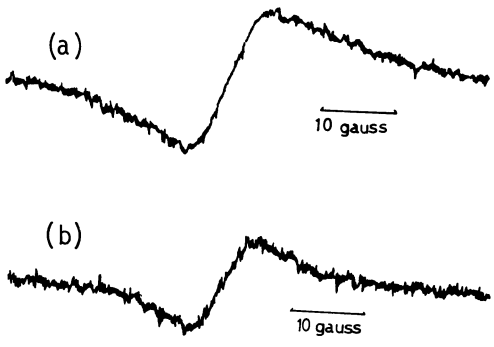
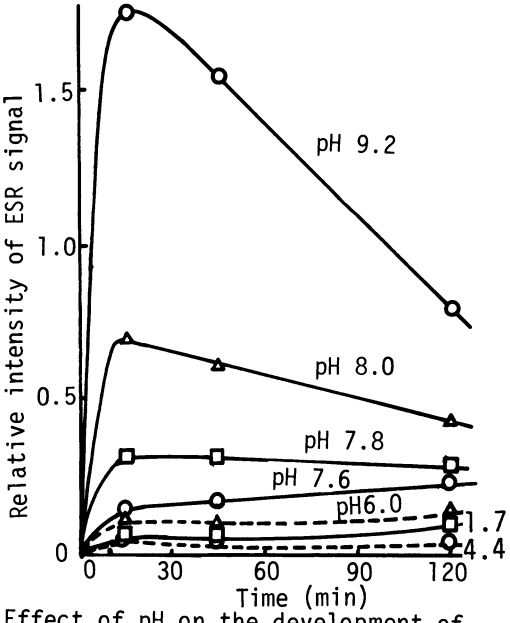
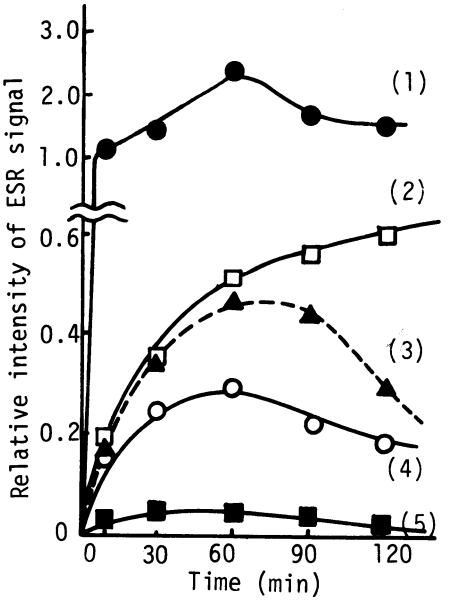


Figure 19. ESR spectra of the reaction mixtures of (a) egg white fresh with D-glucose (b) ovalbumin with D-glucose



Effect of pH on the development of ESR signal in the reaction of ovalbumin and xylose(2:1) in Britton Robinson buffer.

Figure 20.



Development of ESR siganl in the reaction mixture of protein and xylose (solution)

(1)egg white (fresh),(2) bovine albumin,(3)ovalbumin,(4)egg white (dialyzed),(5)lysozyme.

Figure 21.

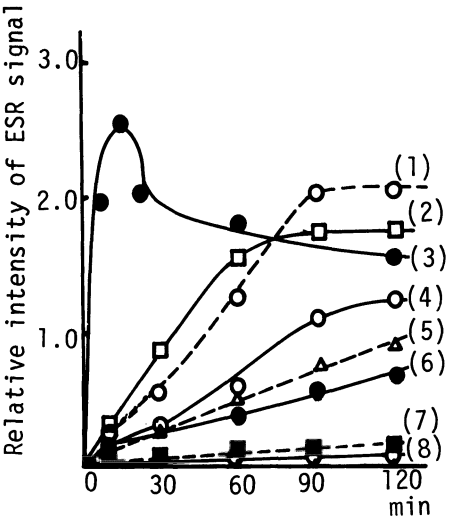


Figure 22. Development of ESR signal in the reaction mixtures of D-glucose with various proteins (freeze-dried powder). (1) egg white (fresh) alone, (2) bovine albumin, (3) egg white (fresh), (4) egg white dialyzed, (5) ovalbumin, (6) gluten, (7) protamin, (8) zein.

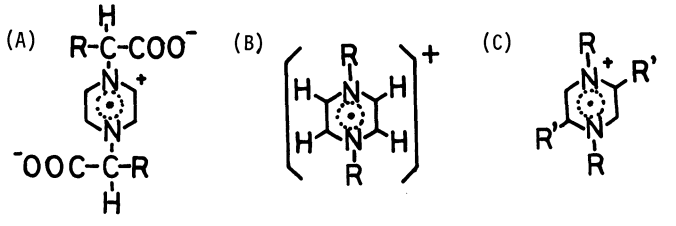
No ESR spectra with hyperfine splitting was observed during heating of either the aqueous mixtures or the freeze-dried powders. However, it cannot be stated that no novel-type free radicals are formed because even if pyrazine-type free radicals were present, the highly restricted configuration of the protein molecules would probably make it impossible to produce the characteristic hyperfine structural spectra.

If pyrazine-type free radicals could be formed, it would suggest the presence of new inter- or intramolecular crosslinks between two amino groups in proteins. However, reaction of proteins with sugars is far more complex than that of an amino acid with a sugar. In addition, heating alone causes significant changes in protein structure. For this reason, no interpretation of the results of protein-sugar reactions can be made at the present time. These results are preliminary in nature and additional studies are needed to clarify the course and mechanisms of these reactions.

CONCLUSIONS

It was clearly shown that novel-type free radical products are formed at the initial stages of amino-carbonyl reactions between sugars or related carbonyl compounds and primary amino compounds.

Based on the analyses of the characteristic hyperfine structures of various ESR spectra and on the confirmatory evidences of synthesized spectra, we proposed the following formula for the novel type free radical products formed in the amino-carbonyl reactions of sugars with amino acids (A) or amines(B) , and tentatively for that formed in the glyceraldehyde with amines (C).



The findings seem to suggest the presence of new reaction processes to produce browning substances in these reaction systems, though there remain many problems to be elucidated.

REFERENCES

Anet,E.F.L.J. and Reynolds, T.M. (1957). Chemistry of nonenzymic browning I. Reactions between amino acids, organic acids, and sugars in freeze-dried appricots and peaches. Aust. J. Chem., 10, 182-192. II. Some crystalline amino acid-deoxy-sugars. ibid. 193-7.

Curphey,T.J. (1965). Heterocyclic diquaternary salts. J. Amer. Chem.Soc., 87, 2063-64.

Hodge, J. E. (1953). Chemistry of browning reactions in model systems. J. Agr. Food Chem., 1, 928-943

Isbel, H.S. and Frush, H.L. (1958). Mutarotation, hydrolysis and rearrengement reactions of glucosyamines. J. Org.Chem., 23 1309-19

Kataoka, N., Imamura, A.,Kawazoe, Y., Chihara, G. and Nagata, C. (1967). The structure of the free radical product from carcinogenic 4-hydroxyaminoquinolone-1-oxide. Bull. Chem. Soc. Japan 40, 62-68

Kato, H. (1956). Studies on browning reactions between sugars and amino compounds. Part I. pH dependency of browning interactions between various kinds of reducing sugars and amino compounds. Bull. Agr. Chem. Soc. Jpn., 20, 273

Lagercrantz, C.,(1964). Formation of stable free radicals in alkali solutions of some monosaccharides. Acta Chem. Scand., 18, 1321

Lento, H. G. Jr., Underwood, J. C. and Willits, C. O. (1958). Browning of sugar solutions. II. Effect of the position of amino group in the acid molecule in dilute glucose solution. Food Res., 23, 68-71

Mitsuda, H., Yasumoto, K. and Yokoyama, K. (1965). Studies on the free radical in amino-carbonyl reaction. Agr. Biol. Chem., 29,751-756

Namiki, M., Hayashi, T., and Kawakishi, S. (1973). Free radicals developed in the amino-carbonyl reaction of sugars with amino acids. Agr. Biol. Chem., 37, 2935-6

Namiki, M.,Yano, M. and Hayashi, T. (1974) Free radical products formed by the reaction of dehydroascorbic acid with amino acids. Chem. Lett., 1974, 125-8

Namiki, M., and Hayashi, T.(1975). Development of novel free radicals during the amino-carbonyl reaction of sugars with amino acids. J.Agr.Food Chem., 23, 487-491

Ohta, Y. Hayashi, T., and Namiki, M., (1976) On the structure of free radical products formed by the reaction of sugar with amino acid. Presented at the Annual Meeting of The Agricultural Chemical Society of Japan., 1976 Apr.2. (Kyoto)

Orr, J. C. (1965). Free radicals derived from amines and amino acids in some reactions of ninhydrin. Nature, 205, 1008-10.

Sato, Y., Watanabe, K. and Ishihara, R., (1973) Further studies on amines and neutral compounds in egg white smell. Jpn. J. Zootech. Sci. 44. 232-240

Yano, M., Hayashi, T. and Namiki,M. (1974) On the structures of the free radical products formed by the reaction of dehydro-ascorbic acid with amino acids. Chem. Lett., 1974. 1193-6.

Yano, M., Hayashi, T. and Namiki,M. (1976-I). On the formation of free radical products by the reaction of dehydroascorbic acid with amines. Agr. Biol. Chem., 40, 1209-15.

Yano, M., Hayashi, T., and Namiki, M. (1976-II). Formation of free radical products by the reaction of dehydroascorbic acid with amino acids. J.Agr. Food Chem., 24 (4). 815

29

SOME CHEMICAL AND NUTRITIONAL PROPERTIES OF FEATHER PROTEIN ISOLATES CONTAINING VARYING HALF-CYSTINE LEVELS

John P. Cherry[1], Kay H. McWatters, Josephine Miller, and A. Lorne Shewfelt
Department of Food Science, University of Georgia College of Agriculture Experiment Stations, Georgia Station, Experiment, Georgia 30212

ABSTRACT

Feather (keratinous) protein isolates containing 2.8 and 7.2% half-cystine were prepared. Solubility of the former increased to 100% between pH 6 and 12, whereas, that of the latter reached only 2.5% at pH 12. Tests showed that mixtures of sodium dodecyl sulfate and 2-mercaptoethanol were needed to completely solubilize the high half-cystine protein, and that sodium dodecyl sulfate alone or in combination with urea and/or 2-mercaptoethanol increased solubilization of the low half-cystine product. The rates of these reactions are further increased by heat. Dry heat denatured the low half-cystine isolate more readily than the high half-cystine product; moist heat denatured both at a similar rate. Gel electrophoretic properties were unique for each keratinous product. Only the low half-cystine isolate had desirable functional properties in that it formed thick, viscous mayonnaise-like emulsions and desirable foams. Functional properties of this isolate were improved dramatically by adjusting the pH from 5.0 to 8.2 or by a two-step change from pH 5.0 to 4.0 to 8.2. Apparent nitrogen digestibility of the two keratinous isolates was greater than 90% as measured by rat growth and by pepsin-HCl digestion.

[1]Present address: Oilseed and Food Laboratory, Southern Regional Research Center, ARS/USDA, P. O. Box 19687, New Orleans, Louisiana 70179.

INTRODUCTION

Keratinous material from feathers is not normally considered to be a source of food-grade protein. In the past 20 years a feather meal feed supplement for poultry has been developed and is produced simply from ground poultry feathers subjected to steam heat under pressure (Routh, 1942; Combs et al., 1958; McCasland and Richardson, 1966; Moran et al., 1966, 1969; Morris and Balloun, 1973a,b; Balloun and Khajareen, 1974). Shortages and rising costs of proteins for human foods and animal feeds have stimulated the development of new technologies to form edible-grade protein from unconventional plant and animal sources (Humphrey, 1970; Hollo and Koch, 1970; Wolf and Cowan, 1971; Rogers et al., 1972; Courts, 1973; Rhee et al., 1973; Kakade, 1974; Basha and Cherry, 1976; Gardner et al., 1976). Many of these fabricated proteins are nutritionally unbalanced because they lack certain essential amino acids or have poor functional properties; and this restricts their use as replacement proteins in foods or feeds. Since unique functional or nutritional properties of one protein may complement those of another (Weisberg, 1972; Horan, 1973; Graham and Baertl, 1974; Anonymous, 1976), research to improve the quality and availability of fabricated proteins should also explore their potential application in foods, feeds, and cosmetics.

Protein isolates with potential food-grade properties were prepared from keratinous material of poultry feathers by Goodwin (1973) and Cherry et al. (1975b). Keratin was extracted from poultry feathers with a refluxing aqueous solution of N,N^1-dimethylformamide (DMF) then precipitated from this extract and washed with water or methylene chloride. Some physical and chemical properties of the isolates were examined to determine the effects of isolating conditions on product quality. While the extracted proteins were similar to those of the raw feathers in that they were consistently low in essential amino acids, they were in fact purified protein isolates rather than partially hydrolyzed feathers. Predominant amino acids in the isolates included serine, glutamic acid, proline, glycine, valine, leucine and arginine. It was observed that the half-cystine content was high when the technique involved mild DMF-extraction.

Studies of the physico-chemical properties of keratin fibers has shown that the stability of the basic structure is due to cross-linking of disulfide bonds between half-cystine residues, hydrogen bonding and salt-like interactions (Woodin, 1954, 1956; Haurowitz, 1963; Harrap and Woods, 1964a,b; Menefee, 1965; Moran et al., 1966; Fraser et al., 1972). The postulated basic polypeptide subunit of keratin has a molecular weight of approximately 10,400. The structure of this subunit appears to have regions that are extensively helical and stabilized by intra-molecular disulfide and

hydrogen bonds. A number of these subunits are thought to aggregate into cylindrical units which in turn combine into rigid cable-like structures. The disulfide bonds of cystine residues tightly stabilize the aggregation of the cylindrical units in these cables and probably contribute to the resistance of keratin molecules to enzymatic proteolysis (Greenwood and Speakman, 1964; McCasland and Richardson, 1966; Roach and Gehrke, 1970; Fraser et al., 1972). Extracting protein components from keratinous tissues requires singly or in combination, such chemical reactions as reduction, alkylylation, oxidative sulfitolysis, alkaline pH and performic acid oxidation (Jones and Mecham, 1943; Ward et al., 1946; O'Donnell and Thompson, 1964; Woods, 1971; McDonagh et al., 1972; Fraser et al., 1972; Baden et al., 1973.

This paper discusses the chemical, functional and nutritional properties of keratinous protein isolates prepared from poultry feathers and hydrolyzed feather meal, and which contained high and low amounts of half-cystine.

MATERIALS AND METHODS

Extraction of Keratinous Protein Isolates

A flow chart depicting the procedures by which the keratinous protein isolates were prepared is shown in Figure 1.

Fresh whole feathers of White Leghorn chickens were collected from a local poultry processing plant, washed to remove all debris, then dried and ground to a small particle size in a hammer mill. The hydrolyzed feather meal was also obtained from a local manufacturer of this material.

A mixture (]:20 w/w) of crushed feathers or hydrolyzed feather meal and 75% N,N^1-dimethylformamide (DMF) was heated and refluxed for 1 hour to dissolve extractable keratinous material. The hot extract was filtered to remove insoluble keratin and the clear brown-colored, viscous filtrate poured into water at room temperature to precipitate the protein. A cream-colored protein curd formed in the water during constant agitation of the mixture for approximately 15 minutes. The protein curd was collected by filtration on Whatman 541 filter paper and washed repeatedly with water until the filtrate was clear. The protein was sequentially washed with 95% ethyl alcohol, acetone and ethyl ether to remove extractable pigments and moisture, then air dried to evaporate residual solvent. The dried cream-colored isolate was passed through a 100-mesh screen to form the final product.

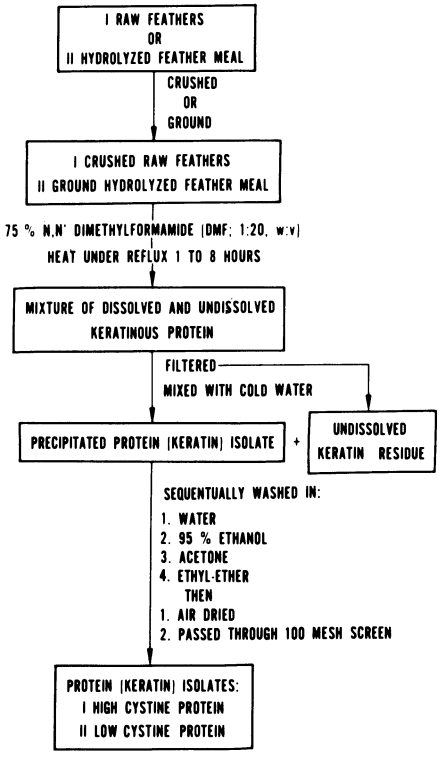


Fig. 1. Preparation of protein isolates from keratinous material.

Proximate Evaluation

Proximate composition of protein isolates was determined by standard methods (AOAC, 1970). A conversion factor of 6.25 was used to calculate protein content from Kjeldahl nitrogen values. The spectrum of minerals in each sample preparation of ash was determined by a Direct Reading Emmissions Spectograph. Isolates were analyzed for DMF by gas chromatography at Greenwood Laboratories Inc., Kennett Square, Pennsylvania. Levels of soluble protein were determined by the method of Lowry et al. (1951). A standard curve was prepared with known amounts of low half-cystine protein.

Protein Solubility

Keratinous isolates were suspended in deionized water, adjusted to pH levels ranging from 1 to 12, centrifuged to remove insoluble materials, and protein solubility of supernatant fractions determined. The effects of different concentrations of urea, sodium

dodecyl sulfate and 2-mercaptoethanol, alone or in various combinations on protein solubility of the isolates were determined also without pH adjustments.

Amino Acid Composition

Total amino acid content of various preparations were determined by hydrolyzing samples in 20 ml of 6N HCl for 22 hours at 110°C, adjusting the mixtures to pH 2.0-2.2 with 12N NaOH and diluting them to 50 ml with water. Quantitative analyses of hydrolysates were performed with a Durrum Model D-500 amino acid analyzer using the ionic-exchange chromatography techniques of Durrum (1972) and Spackman et al. (1958). A column of 1.75 mm (inside diameter) X 48 cm packed with Durrum high-resolution cation exchanger (bead diameter 8 ±1 microns) was used in these experiments. Running time for each sample was 94 minutes including a 20-minute column regeneration period for the analyzer system.

Gel Electrophoretic Techniques

Polyacrylamide gel electrophoresis in the presence and absence of 4M urea and with or without 10mM to 1M 2-mercaptoethanol was performed using a modified procedure of Cherry and Prescott (1974). Ten per cent gels were used instead of standard 7.5% gels as outlined by Canalco (1973).

Heat Denaturation

One-gram samples of high and low half-cystine protein isolates were heated in an oven (dry heat) or steam retort (wet heat) for one hour at various temperatures. The amount of heated protein remaining soluble in aqueous suspensions, either adjusted to pH 12 or containing 6M urea + 0.1M 2-mercaptoethanol, after centrifugation, was used as a solubility index of heat-treated material.

Digestibility Studies

Apparent digestibility of the high and low half-cystine isolates was evaluated using weanling albino rats as the test subjects. Diets contained 12% casein supplemented with arginine, lysine, methionine, threonine and tryptophan to provide adequate essential amino acids and other nutrients required by the growing rat. This basal diet (Control Diet I) was estimated to supply only about 11% of the estimated total 13.3% dietary protein needed by weanling rats for maximum growth. The remainder of the non-essential

nitrogen was added to control Diet II by adding 2.5% of an equal mixture of glycine, alanine and serine. The test diets each contained 2.5% protein supplied by one of the feather isolates. All diets contained the necessary vitamins, minerals and essential fatty acids. Carbohydrates were supplied by an equal mixture of dextrin and sucrose.

In vitro estimations of digestibility were determined using the pepsin-HCl method (AOAC, 1970).

Food Functional Properties

Emulsion capacity and viscosity, and foaming capacity of aqueous protein isolate suspensions were determined by a modified procedure of McWatters and Cherry (1975). Protein suspensions were prepared by blending the isolates with distilled water (4% suspensions, w/v) for 2 min in an Osterizer blender at low speed. The experimental basis for analyzing these functional properties after various pH adjustments including 5.0 to 4.0, 5.0 to 4.0 to 8.2 and 5.0 to 8.2 are discussed by McWatters and Cherry (1975) and McWatters *et al.* (1976). The desired pH was attained by adding 1N HCl or NaOH solution to suspensions with continuous stirring. Oil-in-water emulsions were prepared by adding peanut oil to a 25-ml aliquot of the aqueous protein suspension during blending in an Osterizer blender. Emulsion capacity was the volume of oil (ml) required to effect a sudden change in consistency due to separation of oil and water into two phases. A Brookfield Viscometer (Model RVT) and Helipath Stand (Model C) equipped with a T-B spindle and operated at 2.5 rpm was used to measure emulsion viscosities at 23°C. Foam capacity was determined as the percentage increase in the volume of foam produced after blending 10 ml of the protein isolate suspensions for 1 min with a Brinkman polytron homogenizer at full speed.

RESULTS AND DISCUSSION

Chemical Composition of Keratinous Protein Isolates

Half-cystine content of protein isolates from raw feathers and hydrolyzed feather meal were 7.21 and 2.80 g/100 g of sample, respectively (Table 1). Of all amino acids, half-cystine values showed the greatest difference for the two keratin isolates. Only small quantitative differences occurred in various amino acids such as aspartic acid, glutamic acid, valine, tyrosine, histidine and lysine. There was a large increase in proline in the high half-cystine isolate as compared to raw feathers, and this distinguished the DMF-extracted keratin from the raw feathers. Larger differences

Table 1. Amino acid composition (g/100 g sample) of keratinous material

Amino Acids	Raw Feathers	High Half-Cystine Isolate	Hydrolyzed Feather Meal	Low Half-Cystine Isolate
Aspartic acid	6.48	5.53	6.25	5.47
Threonine	4.67	4.12	4.60	4.47
Serine	14.68	13.36	10.59	12.74
Glutamic acid	10.30	7.50	9.75	8.24
Proline	4.70	15.00	8.16	13.50
Glycine	7.36	7.65	5.31	7.81
Alanine	4.29	4.22	3.97	4.26
Cystine/2	7.72	7.21	5.07	2.80
Valine	8.22	7.10	7.51	7.24
Methionine	0.54	0.74	0.83	0.91
Isoleucine	4.97	4.99	4.28	5.43
Leucine	8.34	8.10	6.72	7.81
Tyrosine	2.71	1.80	4.03	2.21
Phenylalanine	4.98	5.19	7.52	5.14
Histidine	0.45	0.24	2.80	1.00
Lysine	1.21	0.24	2.94	0.91
NH_4	1.00	1.37	1.52	1.21
Arginine	7.41	7.20	7.83	6.46
Unknowns	0	0	0	0.90
TOTALS	100.03	101.52	98.68	98.46

in amino acid content were evident for hydrolyzed feather meal and its keratinous isolate. Pretreatment of feathers to form hydrolyzed feather meal may increase the keratinous fraction available for DMF-extraction and decrease the low half-cystine components. It is possible that keratinous material may be more rapidly solubilized than that from raw feathers so that extended refluxing of the solubilized fractions with DMF may destroy half-cystine. Table 1 shows that the half-cystine content is lower in the hydrolyzed feather meal (5.07 g/100 g sample) than raw feathers (7.72) suggesting some destruction of this amino acid during its preparation. Cherry et al. (1975b) has shown that the acetone filtrate in the final steps of isolate production (Figure 1) contained aspartic acid, serine, glycine, leucine and glutamic acid and this may account for some of the variations in amino acid content. Other variations may be due to the presence of free amino acids or small polypeptide fragments formed during preparation of the isolates and removed during the initial washing of the proteins with water.

The two isolates were high in serine, glutamic acid, proline, glycine, valine, leucine and arginine; intermediate in aspartic acid, threonine, alanine, isoleucine, tyrosine and phenylalanine; and low in methionine, histidine and lysine. DMF-extracted keratin has also been shown to be very low in tryptophan (Goodwin, 1973).

Macro-Kjeldahl analysis showed protein content of the high half-cystine and low half-cystine isolates to be 96.03 and 93.67%, respectively (Table 2). The protein level in the high half-cystine isolate was approximately 6% higher than that of the raw feather, whereas there was no difference between hydrolyzed feather meal and its protein-isolate. Ether extractable oil, crude fiber, ash and nitrogen-free extract (calculated by difference) values were different for feathers and hydrolyzed feather meal. These differences were less than those between the high and low half-cystine isolates.

Levels of potassium, phosphorus, calcium, iron, copper, zinc, aluminum and sodium were higher in hydrolyzed feather meal than in raw feathers. Manganese, iron, zinc, aluminum and strontium either increased or did not change during isolation of the keratinous protein from hydrolyzed feather meal. The other minerals decreased to levels similar to that of the high half-cystine product. In general, most minerals (except iron, zinc, barium, sodium and lead) were relatively low in raw feathers. Only iron and chromium were significantly higher in the high half-cystine isolate than in the raw feathers. Differences in mineral (or ash) content of raw feathers and hydrolyzed feather meal may be due to variations in pretreatment of the source materials; i.e., it is likely that the hydrolyzed feather meal was prepared from raw feathers containing offal material and other debris whereas raw feathers used to prepare high half-cystine protein were washed to remove foreign matter.

Table 2. Chemical composition of keratinous material (dry basis)

Item	Raw Feathers		High Half-Cystine Isolate		Hydrolyzed Feather Meal		Low Half-Cystine Isolate	
	%	ppm	%	ppm	%	ppm	%	ppm
Dry matter	92.63		92.08		94.28		92.50	
Crude protein	90.02		96.03		93.13		93.67	
Ether extract	1.64		0		5.61		0	
Crude fiber	0.44		0.14		0.60		1.00	
Ash	1.55		0.24		3.22		0.37	
N-free extract	6.35		3.59		1.68		5.06	
Potassium	0.04		0.01		0.91		0.05	
Phosphorus	0.20		0.18		0.33		0.10	
Calcium	0		0.01		0.81		0.01	
Magnesium	0		0		0.02		0.01	
Manganese		0		1		0		35
Iron		8.3		36		43.1		208
Boron		3		4		4		3
Copper		9		8		20		9
Zinc		73		35		155		153
Aluminum		1.2		0		5.3		57
Molybdenum		1		1.1		1.1		1
Strontium		4		2		5		112
Barium		27		6		3		2
Sodium		69.4		59		170.3		55
Cadmium		1.2		1.1		0.7		0
Chromium		4.9		6.9		2.4		0
Lead		21		13		7.0		13
N,N[1]-Dimethyl-formamide (DMF)		-		285[1]		-		89

[1]Initial preparation of protein isolates contained 285 and 89 ppm DMF on a weight per weight basis. Subsequent washing of these isolates in water lowered DMF to estimated levels of less than 1 ppm.

The compositional differences of the two products could also account for some of the amino acid variations noted in Table 1.

Residual DMF in the high and low half-cystine protein isolates was estimated to be 385 and 89 ppm, respectively (Table 2). Subsequent washing of the isolates in water lowered the DMF to estimated levels of less than 1 ppm.

Solubility of Keratinous Protein Isolates

Solubility of high half-cystine isolate was less than 1% of total when the water suspension was adjusted to pH levels of 1 to 11 (Figure 2). A small amount (3%) was soluble at pH 12. The low half-cystine isolate produced a solubility curve similar to that of many fabricated proteins (Humphrey, 1970; Hollo and Koch, 1970; Wolf and Cowan, 1971; Rogers et al., 1972; Courts, 1973; Rhee et al., 1973; Kakade, 1974; Basha and Cherry, 1976). There was negligible solubility at pH 2 and 3; and increasing solubility from 2% at pH 5 to 100% at pH 12.

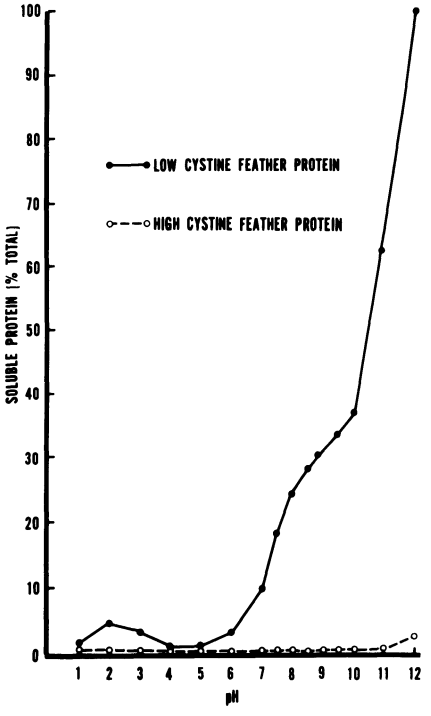


Fig. 2. Effect of pH on the solubility of protein isolates from keratinous material.

These data suggest that the high half-cystine protein may have extensive polypeptide aggregation containing many disulfide bonds. The similarity to native feather keratin is uncertain since during the precipitation and washing steps of the isolation procedure (Figure 1), the polypeptide subunits could randomly reaggregate into various structural forms. Such observations have been noted in other studies where removal of reducing reagents after keratin isolation enabled subunits to reaggregate into various structural components; however, many of these aggregates resembled native keratin (Fraser et al., 1972). The role of disulfide bonding in the high half-cystine protein is emphasized further by the instability of low half-cystine aggregates and ease of solubility of these components under alkaline conditions. Non-covalent or ionic bonding is probably the major form of molecular interaction among extracted polypeptides of low half-cystine protein.

Goodwin (1973) reported that DMF treatment of feathers disrupted the quaternary structure of native keratin and relieved stresses in the long chain molecules. This was based on the observation that native keratin is not hydrolyzed readily by proteolytic enzymes since peptide bonds in the primary structure are inaccessible to hydrolysis. The quaternary structure of keratin is due largely to an extensive network of secondary valance bonding. Breaking these bonding forces by DMF and heat results in loss of the quaternary structure and release of a portion of the keratin subunits which are then susceptible to proteolytic action. The portions of native keratin released by DMF may have disulfide bonds within their structures, the amount of which depends on whether the starting material was raw feathers or hydrolyzed feather meal.

Treatment of Keratinous Isolates with Urea, 2-Mercaptoethanol and/or Sodium Dodecyl Sulfate

The effects of 6M urea and varying levels of 2-mercaptoethanol on the solubility of the two isolates are shown in Figure 3. Increasing the level of 2-mercaptoethanol in the presence of 6M urea increased protein solubility in the high half-cystine isolate; this increase was especially noted at 0.1, 0.375 and 1.0M 2-mercaptoethanol. Approximately 76% of the protein in the low half-cystine isolate was solubilized by 6M urea without 2-mercaptoethanol. Solubility increased to 100% of the total protein in the suspension when increasing amounts of 2-mercaptoethanol were added to the reaction mixture.

Increasing the concentration of 2-mercaptoethanol (0 to 1.0M) in the 6M urea suspension containing high or low half-cystine keratin isolate resulted in substantially different electrophoretic

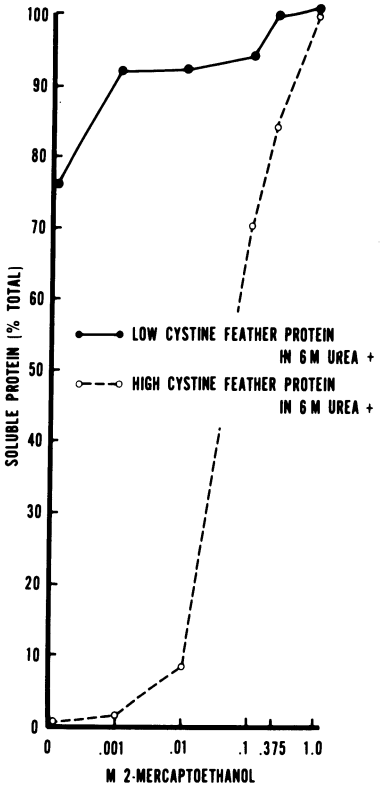


Fig. 3. Effect of varying levels of 2-mercaptoethanol and one concentration of urea on the solubility of protein isolates from keratinous material.

gel patterns (Figure 4). Similar changes were noted in previous studies with keratin isolates and peanuts (Cherry et al., 1975b; Cherry and Ory, 1973). The mobility of proteins in the upper gel region (0 to 2.0 cm) from both keratin isolates increased and unified into two closely migrating components in region 6.5 to 7.0 cm. These changes became notable for both isolates between the levels of 0.1 and 1.0M 2-mercaptoethanol. SDS-gel electrophoresis showed that keratin isolates of feathers contained one band with an approximate molecular weight of 10,000 (Cherry et al., 1975b). Evidently urea in the presence of high amounts of reducing reagent denatures the protein in the two isolates to a basic subunit polypeptide structure of high solubility.

Effects of varying combinations and levels of 2-mercaptoethanol, urea and sodium dodecyl sulfate on solubility are shown in Figures 5 and 6. Sodium dodecyl sulfate has been shown to disrupt secondary, tertiary and quaternary structures of proteins, and at high

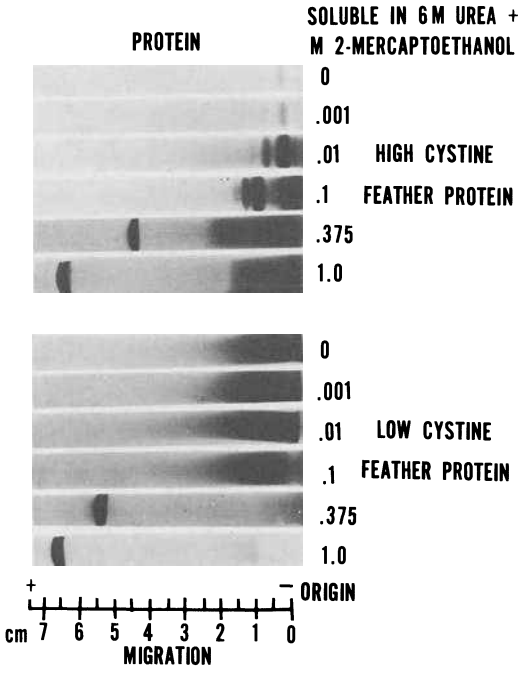


Fig. 4. Polyacrylamide electrophoretic gel patterns of protein isolates from keratinous material in 6M urea and varying levels of 2-mercaptoethanol.

concentrations can reduce disulfide bonds (Shapiro et al., 1967; Fairbanks et al., 1973).

These data show that high levels of 2-mercaptoethanol and sodium dodecyl sulfate with or without urea (.1 to .375M) completely solubilized high half-cystine keratin (Figure 5). Other combinations (urea + 2-mercaptoethanol, urea + sodium dodecyl sulfate, and sodium dodecyl sulfate, 2-mercaptoethanol and urea alone) or low levels of 2-mercaptoethanol and sodium dodecyl sulfate with or without urea solubilized less than 30% of the total protein in this isolate.

Between 70 and 100% of the low half-cystine keratin was solubilized by high levels of urea (6M) and sodium dodecyl sulfate (0.1 to 1.0M) alone or in combination (Figure 6). Addition of 2-mercaptoethanol (0.1 to 0.375M) to these mixtures caused a marked increase in solubility.

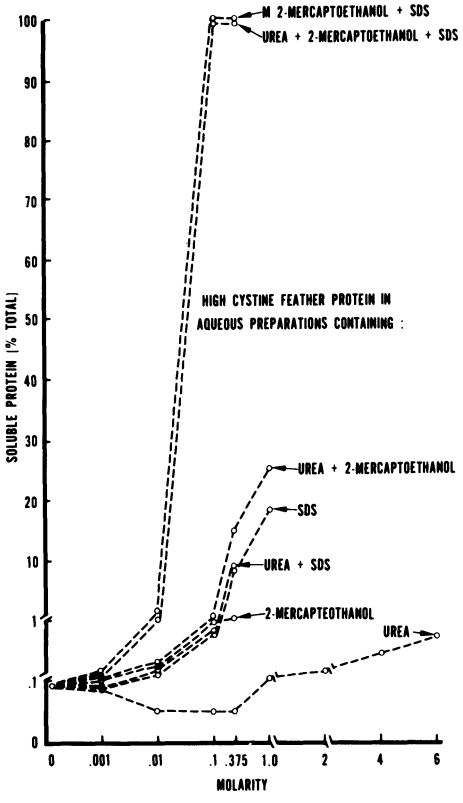


Fig. 5. Effect of varying levels and combinations of sodium dodecyl sulfate, 2-mercaptoethanol and urea on the solubility of high half-cystine keratin isolate.

The solubility of high and low-half-cystine proteins in 0.1M each of sodium dodecyl sulfate, 2-mercaptoethanol and urea alone or in combination after heating the suspensions to boiling are shown in Tables 3 and 4. Various descriptions of the transitional changes observed during heating of the suspension mixtures are presented. In general, high half-cystine protein was solubilized during heating when the mixture contained either 2-mercaptoethanol + sodium dodecyl sulfate or this combination plus urea. These suspensions went through three phases of changes including cloudy to applesauce-like to total solubility or a clear solution. Sodium dodecyl sulfate alone or in combination with urea caused the keratin suspension to go to the second or applesauce-like phase, while addition of 2-mercaptoethanol with or without urea caused the keratin to aggregate into a gum-like structure.

On the other hand, addition of sodium dodecyl sulfate alone or in the presence of urea or 2-mercaptoethanol followed by heating

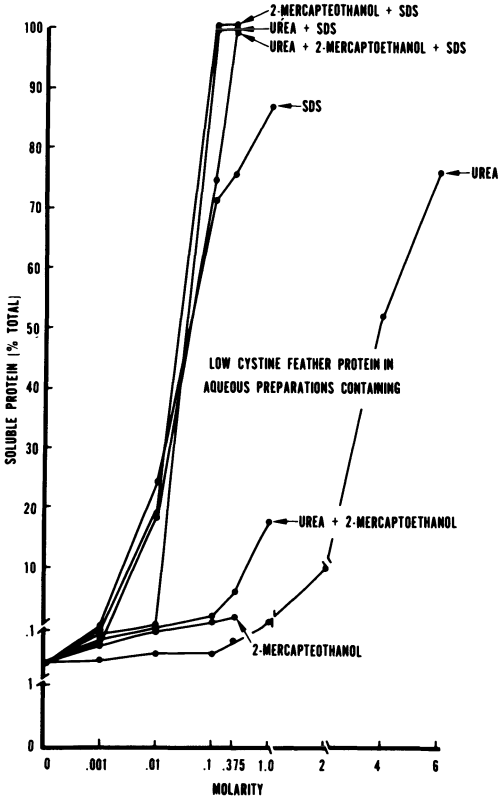


Fig. 6. Effect of varying levels and combinations of sodium dodecyl sulfate, 2-mercaptoethanol and urea on the solubility of low half-cystine keratin isolate.

totally solubilized the low half-cystine protein (Table 4). Combinations without sodium dodecyl sulfate had little effect on solubility.

These data confirm earlier discussions by Goodwin (1973) and Cherry et al. (1975b), that aqueous DMF and heat under reflux disrupts quaternary interactions in the raw feather keratin causing a release of subunits. In the high half-cystine protein, much more of the cystine and hence disulfide bonds remain than in the low half-cystine keratin. The keratin precipitated after DMF extraction probably forms some randomly aggregated polypeptide subunits bound by disulfide and ionic bonding. This is supported by the following two observations: (1) In the high half-cystine isolate, low levels (0 to 0.1M) of denaturant alone or in combination solubilize less than 1% of the total protein. Combinations of urea + 2-mercaptoethanol + sodium dodecyl sulfate are needed to denature helical structures and break disulfide bonds that lead to complete

Table 3. Solubility of high half-cystine keratin isolate heated in aqueous .1M SDS, .1M urea, and .1M 2-mercaptoethanol alone or in combination

Treatment (Heat+)	High Half-Cystine Protein Soluble protein (% of total)	Description of suspension changes during heating
Urea	0.16	None
SDS	33.00	Applesauce-like
2-Mercaptoethanol	1.30	Gum-like
Urea + SDS	52.90	Applesauce-like
Urea + 2-Mercaptoethanol + SDS	100.00	Total solubility[1]
Urea + 2-Mercaptoethanol	1.55	Gum-like
2-Mercaptoethanol + SDS	100.00	Total solubility[1]
Water	0.11	None

[1]Suspension goes through three phases of change including: cloudy to applesauce-like to total solubility or a clear solution.

Table 4. Solubility of low half-cystine keratin isolate heated in aqueous .1M SDS, .1M Urea, and .1M 2-mercaptoethanol alone or in combination

Treatment (Heat+)	Low Half-Cystine Protein Soluble protein (% of total)	Description of suspension changes during heating
Urea	3.9	None
SDS	100.0	Cloudy[1]
2-Mercaptoethanol	5.7	None
Urea + SDS	100.0	Cloudy[1]
Urea + 2-Mercaptoethanol + SDS	100.0	Cloudy[1]
Urea + 2-Mercaptoethanol	6.5	None
2-Mercaptoethanol + SDS	100.0	Cloudy[1]
Water	1.7	None

[1]Protein mostly solubilized, however solution remains cloudy.

solubility. (2) High levels of urea (4 to 6M) and sodium dodecyl sulfate (0.1 to 1.0M) alone or in combination and with or without 2-mercaptoethanol (0.1 to 0.375M) solubilize most of the low half-cystine keratin isolate. Aggregation of keratin in this latter isolate is probably through ionic interaction.

Heat Treatment of Keratinous Protein Isolates

The effects of wet and dry heat on the solubility of high and low half-cystine proteins suspended in aqueous solutions are presented in Figures 7 and 8. The decline in solubility of both isolates when heated above 20°C was more rapid for wet than for dry heat. Both wet and dry heat had little effect on the solubility of the high half-cystine protein in aqueous solution adjusted to pH 12. This protein showed a slight increase in solubility at 130°C after wet heating and between 140 to 240°C after dry heating. Between 20 and 120°C, wet heat rapidly denatured low half-cystine protein to a form which had low solubility at pH 12; a slight increase in solubility was noted by the preparation heated at 130°C. Both the high and low half-cystine proteins heated at 20 to 40°C showed a decline in solubility in 6M urea + 0.1M 2-mercaptoethanol which leveled off to approximately 70% of the total protein after they were heated at 40 to 120°C. These isolates showed approximately 50% solubility in the presence of these denaturants after being heated at 130°C.

Dry heating the isolates up to 180°C produced little change in solubility in the urea + 2-mercaptoethanol mixture. The low half-cystine protein suspended in pH 12 solution showed little change in solubility when dry heated at 20 to 160°C. The solubility profile of all protein suspensions declined rapidly when the temperature was increased to 220°C and either leveled off or increased slightly at 240°C.

Changes in gel electrophoretic patterns of the isolates could be correlated to the effects of wet and dry heat treatments (Figures 9 and 10). As protein solubility declined with increasing heat treatments, the protein components in each isolate became diffuse and indistinguishable. High half-cystine samples showed qualitative changes in gel patterns when heated at 120 to 180°C. A gradual decrease in protein solubility was noted in this temperature range. At 240°C, keratin solubility decreased rapidly while gel patterns showed fewer protein components. The low half-cystine isolate did not show any major changes in gel patterns until heated at 180 to 240°C.

These data suggest that increased unfolding of protein structures, subunit reaggregation and insolubility is probably heat-

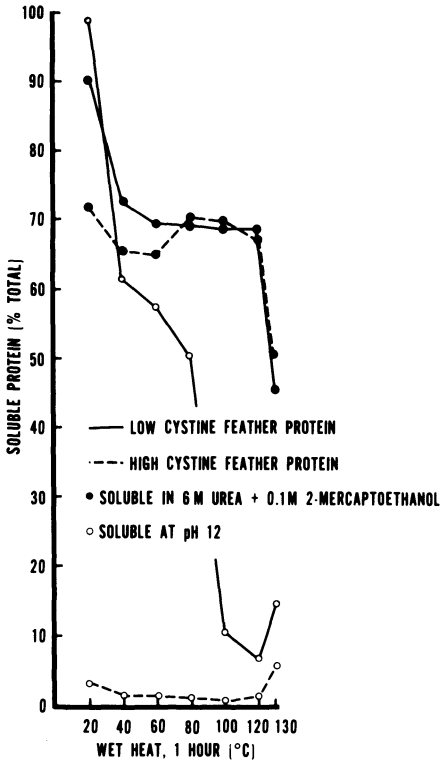


Fig. 7. Low and high half-cystine keratin isolates solubilized in 6M urea + 0.1M 2-mercaptoethanol or at pH 12 after treatment with wet heat at various temperatures for 1 hour.

related. This sequence of changes has been suggested in studies of wet and dry heat denaturation of various other keratin isolates (Cherry et al., 1975b). In addition, peanut and soybean proteins heated at various temperature and time intervals showed that the major storage globulins were sequentially denatured first to subunit forms, then to aggregates and finally to insoluble components (Wolf and Cowan, 1971; Cherry et al., 1975a; Cherry and McWatters, 1975). In certain cases, more extensive heating converted some of the insoluble proteins to aggregated soluble forms.

High and low half-cystine proteins did not show as rapid a decline in solubility in mixtures of urea + 2-mercaptoethanol as they did when placed in aqueous solution adjusted to pH 12. The denaturants may have disrupted insoluble aggregates formed during heating and resulted in soluble subunit components. High temperatures are needed to irreversibly denature these proteins to this effect of urea + 2-mercaptoethanol.

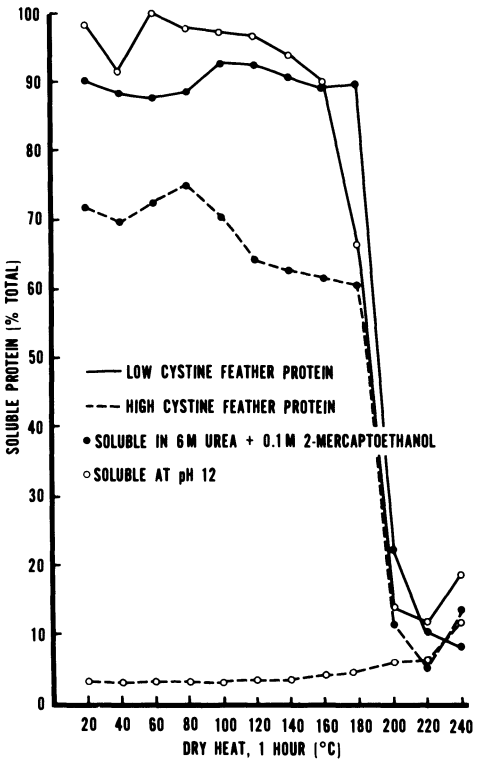


Fig. 8. Low and high half-cystine keratin isolates solubilized in 6M urea + 0.1M 2-mercaptoethanol or at pH 12 after treatment with dry heat at various temperatures for 1 hour.

Apparent Digestibility of Keratinous Protein Isolates

In studies to determine the apparent digestibility of high and low half-cystine isolates, differences in weight gain and feed efficiency were not significant for the three diets. Apparent digestibility of the low half-cystine isolate (92.4%) was lower than that of the control (93.6%) and digestibility of the high half-cystine isolate (90.5%) was lowest of the three. The differences between the isolates and the control were due primarily to increased excretion of fecal nitrogen by animals fed the isolates (Table 5).

Adult rats, fed high half-cystine isolate as 44% of a diet adequate in all other nutrients, continued to lose weight throughout the five-week feeding trial. Both the high and low half-cystine isolates were deficient in several essential amino acids, and therefore limited in their nutritional attributes. However, they are purified protein isolates which may be valuable as functional ingredients in food product systems.

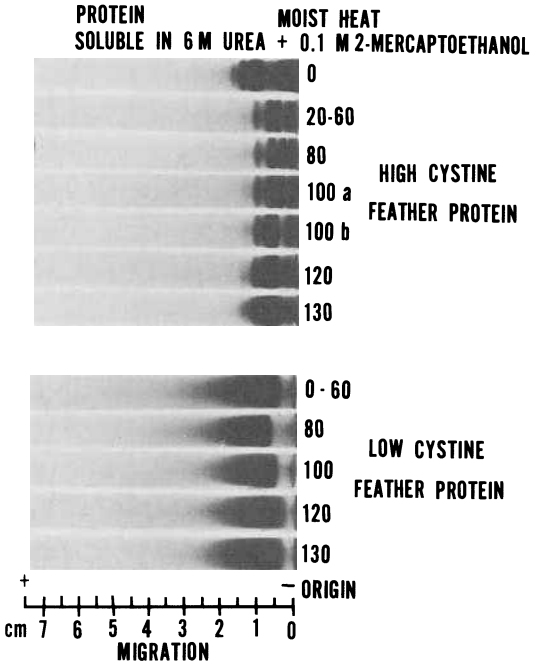


Fig. 9. Effect of moist heat on protein isolates from keratinous material shown by gel electrophoresis.

Nitrogen solubilities of the high and low half-cystine products analyzed by pepsin-HCl digestion were approximately 100%. Nutritional studies using feathers as a source protein in animal feeding trials have been conducted in other laboratories (Routh, 1942; Combs et al., 1958; McCasland and Richardson, 1966). Animals fed only raw feather meal as a protein source lost weight and eventually died. A 75% mortality rate was obtained with raw feather meal supplemented with deficient amino acids. The digestibility value for this product was only 7.7%. On the other hand, 80 to 82% digestibility values were obtained with hydrolyzed feather meal. Rats fed on a diet containing only hydrolyzed feather meal as a protein source lost weight but none died. When this diet was supplemented with the deficient amino acids, the rats were similar to the control group fed a soybean meal.

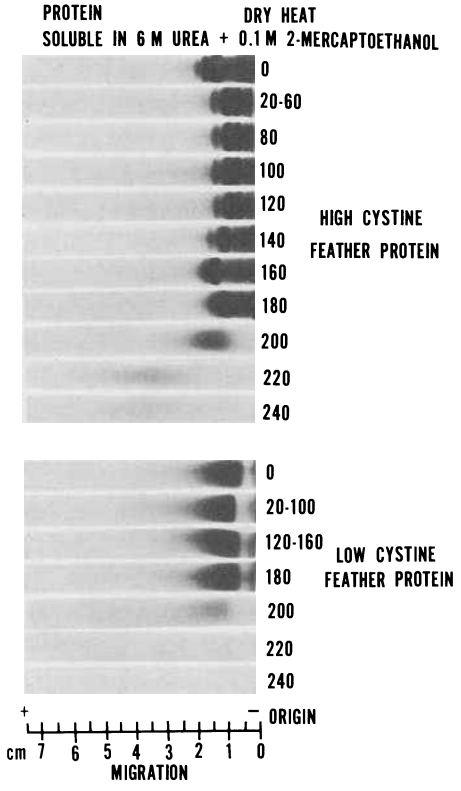


Fig. 10. Effects of dry heat on protein isolates from keratinous material shown by gel electrophoresis.

Food Functional Properties

The high half-cystine isolate exhibited poor functional properties when used to prepare emulsions and foams. Various pH adjustments did little to improve its ability to bind oil or trap air (Table 6). However, suspensions of low half-cystine isolate adjusted to pH 8.2 by the single or two-step adjustment exhibited excellent emulsifying properties and produced thick, viscous mayonnaise-like products resembling those made with peanut meal. Suspensions (4%) of this isolate at pH 8.2 emulsified more oil (112.7, 114.6 ml) than 8% peanut meal suspensions (77.4, 89.7 ml oil) adjusted to the same pH level. Also, it produced thick egg white-like foams which were much firmer than those made with peanut meal. These data show that the low half-cystine protein equaled or surpassed peanut meal in certain functional attributes, requiring half the amount of material and less soluble protein. In studies with peanuts (McWatters and Cherry, 1975; McWatters et al., 1976),

these investigators suggested that protein quality and solubility are two major factors which determine functionality characteristics of protein products in certain food systems, particularly those with emulsions or foams. The functionality tests with the two keratin isolates suggest that protein solubility is an important variable.

Table 5. Nitrogen intake, fecal excretion, and apparent digestibility in rats fed feather keratin isolates as a source of non-essential dietary nitrogen

Diets	Nitrogen Intake[1,3]	Fecal Nitrogen[1,3]	Apparent N Digestibility[2,3]
Basal (Control I)	5.88[b]	0.495[b]	91.6[b]
Basal + gly, ala, ser (Control II)	7.62[a]	0.489[b]	93.6[a]
Basal + High half-cystine isolate	7.57[a]	0.714[a]	90.5[c]
Basal + Low half-cystine isolate	7.58[a]	0.580[b]	92.4[b]

[1]Total for 16 days.

[2](Nitrogen intake - fecal nitrogen) divided by nitrogen intake.

[3]Values in a column not followed by the same superscript are significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

Table 6. Functional properties of keratin isolates compared to peanut flour

Sample	Treatment (pH)	Soluble Protein (mg/25 ml)	Emulsions			Soluble Protein (mg/10 ml)	Foams	
			Capacity (ml of oil)	Viscosity (cps)	Type Emulsion		Percentage Increase	Type Foam
High half-cystine	5.0	0.7	No emulsion formed			0.3	15	Thin
Feather protein	5.0→4.0	0.8	"			0.3	10	"
(4% suspension)	5.0→4.0→8.2	2.0	"			0.8	20	"
	5.0→8.2	2.0	"			0.8	10	"
Low half-cystine	5.0	8.3	No emulsion formed			3.3	20	Thin-sticky
Feather protein	5.0→4.0	9.5	"			3.8	25	Thin
(4% suspension)	5.0→4.0→8.2	272.5	114.6	65,280	Mayonnaise-like	109.0	Beyond capacity	Very thick
	5.0→8.2	272.5	112.7	64,640	Mayonnaise-like	109.0	110	Thick, egg-white like
Peanut meal (8% suspension)	6.7	675.0	78.0	5,760	Pourable suspension	270.0	100	Thick, egg-white like
	6.7→4.5	147.5	No emulsion formed			59.0	100	Medium
	6.7→4.0→8.2	920.0	89.7	70,880	Mayonnaise-like	368.0	120	Medium
	6.7→8.2	1112.5	77.4	17,280	Thick salad dressing like	445.0	100	Medium

REFERENCES

Anonymous. (1976). Rice-textured soy blend has 18% protein content, PER of 2.5+. Food Proc. 37, 60-61.

AOAC. (1970). "Official Methods of Analysis of The Association of Official Analytical Chemists," Ed. Horwitz, W., Chichilo, P., and Reynolds, H. Assoc. Official Analytical Chemists, Washington, D. C.

Baden, H. P., Goldsmith, L. A., and Fleming, B. (1973). The polypeptide composition of epidermal prekeratin. Biochim. et Biophys. Acta 317, 303-311.

Balloun, S. L., and Khajareen, J. K. (1974). The effects of whey and yeast on digestibility of nutrients in feather meal. Poultry Sci. 53, 1084-1095.

Basha, S. M. M., and Cherry, J. P. (1976). Composition, solubility, and gel electrophoretic properties of proteins isolated from Florunner (Arachis hypogaea L.) peanut seeds. J. Agric. Food Chem. 24, 359-365.

Canalco Catalog. (1973). Disc electrophoresis. Canalco, Rockville, Md.

Cherry, J. P., and Ory, R. L. (1973). Gel electrophoretic analyses of peanut proteins and enzymes. 2. Effects of thiol reagents and frozen storage. J. Agric. Food Chem. 21, 656-660.

Cherry, J. P., and Prescott, J. M. (1974). Electrophoretic evaluation of various procedures for solubilizing erythrocyte membranes. Proc. Soc. Exp. Biol. & Med. 147, 418-424.

Cherry, J. P., McWatters, K. H., and Holmes, M. R. (1975a). Effect of moist heat on solubility and structural components of peanut proteins. J. Food Sci. 40, 1199-1204.

Cherry, J. P., Young, C. T., and Shewfelt, A. L. (1975b). Characterization of protein isolates from keratinous material of poultry feathers. J. Food Sci. 40, 331-335.

Cherry, J. P., and McWatters, K. H. (1975). Solubility properties of proteins relative to environmental effects and moist heat treatment of full-fat peanuts. J. Food Sci. 40, 1257-1259.

Combs, G. E., Alsmeyer, W. L., and Wallace, H. D. (1958). Feather meal as a source of protein for growing finishing swine. J. Animal Sci. 17, 468-472.

Courts, A. (1973). Recent advances in protein production. Process Biochem. 8(2), 31-33.

Durrum Model D-500 Amino Acid Analyzer Operation and Maintenance Manual. (1972). Durrum Instrument Corporation, Palo Alto, Calif.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1973). Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10, 2606-2617.

Fraser, R. D. B., MacRae, T. P., and Rogers, G. E. (1972). "Keratins, Their Composition, Structure and Biosynthesis." Charles C. Thomas, Springfield, Illinois.

Gardner, H. K., Hron, R. J., and Vix, H. E. L. (1976). Removal of pigment glands (Gossypol) from cottonseed. Cereal Chem. 53, 549-560.

Goodwin, W. D. (1973). Nutrient protein from keratinaceous material by extraction with dimmethylformamide. U. S. Patent 3,773,745.

Graham, G. G., and Baertl, J. M. (1974). Nutritional effectiveness of soy cereal foods in undernourished infants. J. Amer. Oil Chem. Soc. 51, 152A-155A.

Greenwood, B. D., and Speakman, P. T. (1964). Use of proteolytic enzymes in structural investigations of wool keratin. Nature 204, 144-146.

Harrap, B. S., and Woods, E. F. (1964a). Soluble derivatives of feather keratin. L. Isolation, fractionation and amino acid composition. Biochem. J. 92, 8-18.

Harrap, B. S., and Woods, E. F. (1964b). Soluble derivatives of feather keratin. 2. Molecular weight and conformation. Biochem. J. 92, 19-26.

Haurowitz, F. (1963). Structural proteins (scleroproteins and the muscle proteins)i Chapter IX in "The Chemistry and Function of Proteins." Academic Press, New York, N. Y.

Hollo, J., and Koch, L. (1970). Protein from green matter. Process Biochem. 5(10), 37-39.

Horan, F. E. (1973). Wheat-soy blends. High quality protein products. Cereal Sci. Today 18, 11-14.

Humphrey, A. E. (1970). Microbial protein from petroleum. Process Biochem. 5(6), 19-22.

Jones, C. B., and Mecham, D. K. (1943). The dispersion of keratins. II. Studies on the dispersion of keratins by reduction in neutral salt solutions of protein denaturants. Arch. Biochem. 3, 193-202.

Kakade, M. L. (1974). Biochemical basis for the differences in plant protein utilization. J. Agric. Food Chem. 22, 550-555.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

McCasland, W. E., and Richardson, L. R. (1966). Methods for determining the nutritive value of feather meals. Poul. Sci. 45, 1231-1236.

McDonagh, J., Messel, H., McDonagh, R. P., Murano, G., and Blomback, B. (1972). Molecular weight analysis of fibrinogen and fibrin chains by an improved sodium dodecyl sulfate gel electrophoresis method. Biochim. Biophys. Acta 257, 135-142.

McWatters, K. H., and Cherry, J. P. (1975). Functional properties of peanut paste as affected by moist heat treatment of full-fat peanuts. J. Food Sci. 40, 1205-1209.

McWatters, K. H., Cherry, J. P., and Holmes, M. R. (1976). Influence of suspension medium and pH on functional and protein properties of defatted peanut meal. J. Agric. Food Chem. 24: 517-523.

Menefee, E. (1965). Cross linking in keratin. II. Theory for sulfur disproportionation after chain scission and crosslink cleavage. J. Appl. Polym. Sci. 9, 2829-2834.

Moran, E. T., Summers, J. D., and Slinger, S. J. (1966). Keratin as a source of protein for the growing chick. 1. Amino acid imbalance as the cause for inferior performance of feather meal and the implication of disulfide bonding in raw feathers as the reason for poor digestibility. Poultry Sci. 45, 1257-1266.

Moran, E. T., Pepper, W. F., and Summers, J. D. (1969). Processed feather and hog hair meals as sources of dietary protein for the laying hen with emphasis on their needs in meeting maintenance needs. Poultry Sci. 48, 1245-1251.

Morris, W. C., and Balloun, S. L. (1973a). Effect of processing methods on utilization of feather meal by broiler chicks. Poul. Sci. 52, 858-866.

Morris, W. C., and Balloun, S. L. (1973b). Evaluation of five differently processed feather meals by nitrogen retention, net protein values, xanthine dehydrogenase activity and chemical analysis. Poul. Sci. 52, 1075-1084.

O'Donnell, I. J., and Thompson, E. O. P. (1964). Studies on reduced wool. IV. The isolation of a major component. Aust. J. Biol. Sci. 17, 973-989.

Rhee, K. C., Cater, C. M., and Mattil, K. F. (1973). Aqueous process for pilot plant-scale production of peanut protein concentrate. J. Food Sci. 38, 126-128.

Roach, D., and Gehrke, C. W. (1970). Hydrolysis of protein. J. Chrom. 52, 393-404.

Rogers, C. J., Coleman, E., Spino, D. F., Purcell, T. C., and Scarpino, P. V. (1972). Production of fungal protein from cellulose and waste cellulosics. Environ. Sci. Tech. 6, 715-719.

Routh, J. I. (1942). Nutritional studies on powdered chicken feathers. J. Nutr. 24, 399-404.

Shapiro, A. L., Vinuela, D., and Maizel, J. V., Jr. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. Biophys. Res. Comm. 28, 815-820.

Spackman, D. H., Stein, W. H., and Moore, S. (1958). Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30, 1190-1206.

Ward, W. H., High, L. M., and Lundgren, H. P. (1946). Physicochemical characterization of dispersed chicken feather keratin. J. Polymer Res. 1, 22-36.

Weisberg, S. M. (1972). Developing and marketing low-cost protein foods in developing countries. Food Tech. 26, 60-68.

Wolf, W. J., and Cowan, J. C. (1971). Soybeans as a food source. CRC Critical Rev. Food Tech. 2, 81-158.

Woodin, A. M. (1954). Molecular size, shape and aggregation of soluble feather keratin. Biochem. J. 57, 99-109.

Woodin, A. M. (1956). Structure and composition of soluble feather keratin. Biochem. J. 63, 576-581.

Woods, E. F. (1971). Chromatography of the soluble proteins from feathers. Comp. Biochem. Physiol. 39A, 325-331.

ACKNOWLEDGMENTS

Dr. William D. Goodwin is given special acknowledgment for his gift of low half-cysteine feather isolate and valuable expertise in discussions while this research was conducted. Special appreciation is given to Dr. Clyde T. Young for analysis of amino acids, Mrs. Linda P. Garrison for conscientious technical assistance, Alton F. Fayette and Jack J. Berquist for photographing the figures, and Mrs. Ann Autry for skillfully organizing and typing the manuscript into final publication form.

30

EFFECT OF PROCESSING ON PROTEIN UTILIZATION BY RUMINANTS[1]

Glen A. Broderick

Department of Animal Science
Texas A&M University and Texas Agricultural Experiment Station, College Station, Texas 77843

ABSTRACT

Processing with heat or chemical reagents will reduce protein digestibility by reducing protein solubility in digestion media, due in part to crosslinking of peptide chains. This effect will have adverse effects on protein utilization by monogastric animals. However, processing may actually improve ruminant protein utilization by reducing ruminal microbial degradation, thereby increasing the proportion of dietary protein escaping to the small intestine for digestion there. Chemical treatments, particularly formaldehyde-treatment, have been used to obtain ruminal protein protection. Results with formaldehyde however, have not yielded consistent improvements in ruminant protein utilization, probably because proteins have been over-protected. Heat-treatments, such as those used during oil extraction from oilseed meals, will also increase ruminal protein escape or by-pass. Experimental results illustrating these effects were obtained with samples of "cold-extracted" cottonseed meal (CSM) which were subjected to graded levels of heat-processing by autoclaving for various lengths of time. Reaction of lysine ε-amino groups with carbohydrate and other compounds was assessed by ninhydrin assay on intact CSM samples. Also measured were N-solubilities in .02N NaOH, free gossypol content, *in vitro* ruminal degradation rate and estimated ruminal by-pass. Heat-treatment of CSM proteins was found to reduce solubility and ruminal degradation, with corresponding increases in estimated ruminal protein by-pass. Gossypol binding appeared to account for much of the loss of protein amino groups and possibly acted as a crosslinking agent, reducing ruminal solu-

[1]T. A. 13136 from the Texas Agricultural Experiment Station.

bility and therefore degradation. Other aspects of processing and ruminant protein nutrition are also discussed

INTRODUCTION

All feed consumed by ruminants is subjected to an extensive microbial fermentation in the rumen, the first compartment of the ruminant stomach. During this fermentation, most feed protein is degraded to amino acids and finally to NH_3, which serves as the principle nitrogen (N) source for protein resynthesis by the ruminal microbes. Tissue amino acid requirements of the ruminant host are met primarily by microbial protein and secondarily by feed protein which has by-passed ruminal degradation. Although the synthetic activity of the ruminal microbes allows dietary non-protein nitrogen (NPN) compounds, such as urea, to be converted to good quality protein, the microbial degradative activity wastes dietary proteins. Frequently, ruminal degradation of high quality proteins results in production of more NH_3 than is utilized by growing bacteria, with the excess NH_3 being absorbed from the rumen and eventually lost as urinary urea (Chalmers *et al.*, 1954). For more thorough discussions on the quantitative aspects of ruminant protein nutrition, the reader is referred to several recent reviews (Broderick, 1975; Clark, 1975; Hogan, 1975).

Ruminal protein degradation to NH_3 appears to be limited by microbial proteolysis to amino acids, a process which in turn is thought to be primarily a function of the protein's solubility in ruminal liquor (Henderick and Martin, 1963). Processing of proteins, particularly with heat, results in peptide chain crosslinking (Hurrell *et al.*, 1976) decreasing the protein's solubility and, hence, its degree of microbial degradation in the rumen. It is well known that crosslinking of proteins during processing will reduce their nutritional value to monogastric animals (Hurrell and Carpenter, 1977). However, crosslinking will yield greater amounts of protein by-passing the rumen and reaching the small intestine. Provided crosslinking has not been too great, protein processing may actually result in greater quantities of intestinally absorbed amino acids because intestinal digestibility, although reduced, will not be decreased nearly so much as ruminal degradation. This is illustrated by the theoretical curves shown in figure 1. Processing results in an immediate reduction in protein solubility from the natural state at harvest, thereby reducing monogastric protein digestibility and N-utilization. In ruminants, although overall protein digestibility may be reduced, processing may actually increase N-utilization, before it declines with over-processing. Some overall protein digestibility can be sacrificed to give greater ruminal protein by-pass. Increased protein by-pass will give enhanced intestinal digestion to amino acids, rather than ruminal digestion to NH_3 and resultant urinary excretion as urea.

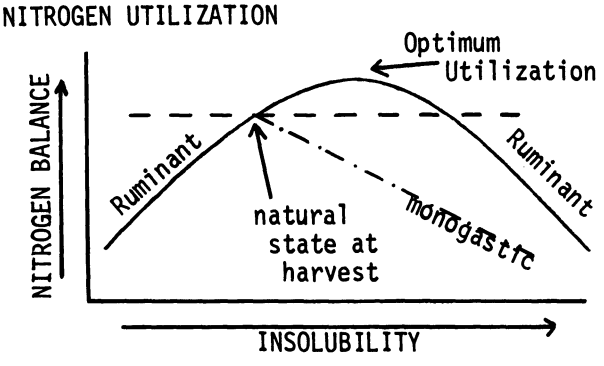


Fig. 1. Theoretical relationship between dietary N-utilization (N-balance) and insolubility of dietary protein. Figure illustrates how processing that reduces protein solubility decreases monogastric utilization, but may actually improve ruminant protein utilization. Figure taken from Goering and Waldo (1974) by permission of the authors.

Before processing steps which increase post-ruminal protein digestion can be thought to be beneficial to ruminants, proof must be obtained showing that amino acid supply from microbial synthesis and normal dietary protein by-pass is inadequate. Several lines of evidence indicate that this is indeed the case for ruminants in a number of productive situations. Infusion of both high-quality proteins and sulfur-amino acids into the abomasum, the stomach compartment immediately preceeding the small intestine, increased wool growth (Reis and Schinckel, 1964) and N-retention (Schelling and Hatfield, 1968; Schelling *et al.*, 1973) in sheep. Ørskov and coworkers (1969, 1970) found significant improvements in lamb N-retention by using esophageal groove closure to obtain ruminal by-pass of high-quality proteins. Abomasal infusion of casein, casein hydrolysate or mixtures of all 10 essential amino acids (EAA) also markedly improved N-balance of rapidly growing beef steers (Chalupa *et al.*, 1972, 1973). Responses of 10-13% in milk protein secretion were obtained by several groups when casein was abomasally infused to supplement diets previously believed adequate in protein (Broderick *et al.*, 1970; Derrig *et al.*, 1974; Vik-Mo *et al.*, 1974).

Although there is strong evidence that post-ruminal supplements of methionine or cystine will give both wool growth and weight gain responses in sheep, similar evidence is lacking for

an obviously first-limiting EAA in beef and dairy cattle. Chalupa and coworkers were unable to obtain consistent N-balance response to abomasal infusion of methionine alone (1972), or other individual EAA (1973). Feeding studies with encapsulated methionine (a product which prevents ruminal degradation of the methionine, but still allows its intestinal absorption) have also given inconsistent growth responses in beef cattle (Mowat and Deelstra, 1972), and had no effect on milk production (Broderick *et al.*, 1970). Although Schwab and coworkers (1976) observed increased milk protein production when cows fed a low protein diet were abomasally supplemented with lysine plus methionine, the increase was only 40% of that obtained with abomasally infused casein. These results suggest that rather than limitations of specific EAA, there is a general protein shortage in beef and dairy cattle, and greatest production responses are to be obtained when feed processing results in increased ruminal by-pass of high-quality proteins.

PROCESSING TECHNIQUES WHICH INCREASE RUMINAL PROTEIN BY-PASS

Chemical modification and heat-processing techniques have been used to increase ruminal protein by-pass, as described in several recent reviews (Broderick, 1975; Chalupa, 1975). Aldehydes, particularly formaldehyde (Ferguson *et al.*, 1967), have gained the widest prominence as chemical modifiers. The probable mode of formaldehyde protection is through crosslinking of proteins, resulting in reduced microbial degradation, due to decreased ruminal solubility (Henderickx and Martin, 1963). Peter *et al.* (1971), found that glyoxal and formaldehyde treatments both reduced ruminal protein degradation, as shown by lowered *in vitro* NH_3 release rates (figure 2). The aldehyde treatments also reduced buffer solubility and enhanced lamb gains and feed efficiencies (table 1). However, positive effects with formaldehyde protection, have not been uniformly obtained. The most frequent findings obtained with common feed proteins, illustrated here by the data of Faichney and Davies (1972), have been decreased N-digestibility with little change in animal performance (table 2). The lack of consistent response with formaldehyde-treatments has led to studies of other chemical agents to ruminally protect proteins (Friedman and Broderick, 1977).

For ruminant feeding, processing of casein or proteins similarly high in solubility will usually result in more protein being intestinally digested to amino acids. But most feed proteins have already been subjected to some heat-processing, and may require little, if any, further modification to reach optimum ruminal protection and utilization (figure 1). Therefore, experiments were done to obtain baseline information regarding heat-processing effects on the ruminal properties of cottonseed meal

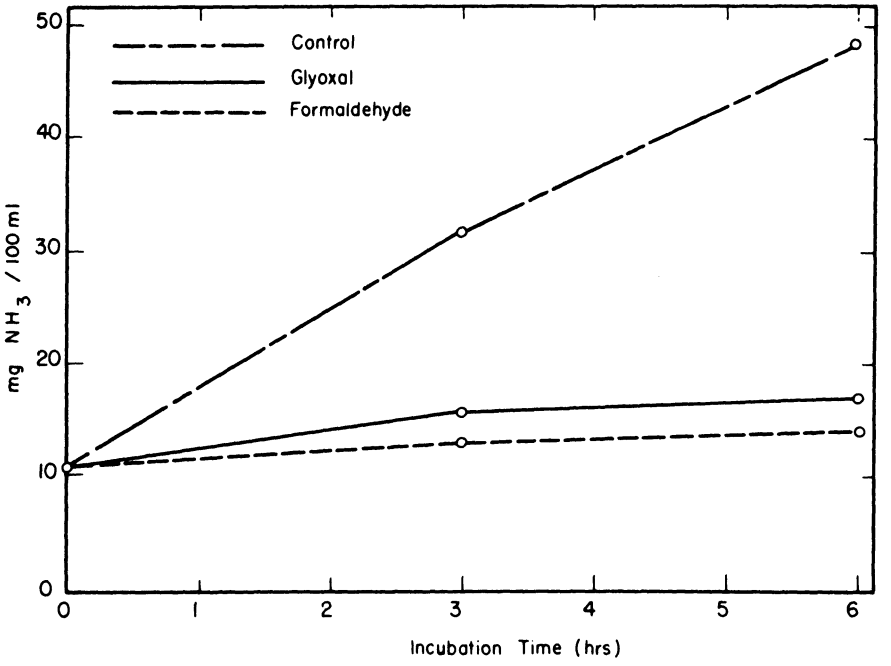


Fig. 2. Effect of aldehyde-treatment on ruminal *in vitro* degradation of soybean meal (SBM) protein. Relative to the control (untreated SBM), there was little change in NH_3 release, the index of ruminal degradation, from either the glyoxal or formaldehyde-treated SBM after incubation for 6 hr. with ruminal contents. Figure taken from Peter *et al.*, (1971) by permission of the Journal of Nutrition.

(CSM), the major protein supplement fed ruminants in the Southwest. "Cold-extracted" CSM was prepared by extracting with hexane and desolventizing at room temperature. Samples of this CSM were heat-treated by autoclaving at 121°C and 15 psi for time periods ranging from 5 to 240 min. N-solubilities in .02 N NaOH (Lyman *et al.*, 1953) and free gossypol contents (method Ba 7-58, A.O.C.S, 1970) of CSM preparations were measured using standard procedures. Ninhydrin reactivity of intact CSM samples was determined by a modification of the procedure of Beckwith and coworkers (1975). Ninhydrin color yield should be inversely related to the degree of reaction of lysine ε-amino groups with other amino acid residues, gossypol and/or carbohydrates. Ruminal degradation rate and proportion ruminal by-pass were estimated for CSM preparations using the kinetic procedure of Broderick (1976). This technique is based on release of amino acids and NH_3, in the presence of hydrazine (an inhibitor of amino acid and NH_3 uptake by ruminal microbes), when limited amounts of protein are incubated *in vitro* with ruminal fluid.

Table 1

Effect of Aldehyde Treatment of Soybean Meal (SBM) on Protein Solubility and Lamb Growth and Feed Efficiency[1]

SBM Treatment	Protein Solubility[2] (%)	Growth Rate (kg/day)	Gain/Feed Ratio
Control	73.5	0.246	0.201
Glyoxal	16.3[a]	0.280[b]	0.249[b]
Formaldehyde	3.9[a]	0.290[b]	0.239[b]

[1]Data taken from Peter *et al.*, (1971).

[2]Solubility of crude protein (Nx6.25) in buffered medium similar to ruminal fluid.

[a]Significantly different from control ($P < .01$).

[b]Significantly different from control ($P < .05$).

Table 2

Effect of Formaldehyde Treatment of Peanut Meal on Growth Rate, Feed Efficiency and N-Digestibility in Calves[1]

Dietary Protein Content[2] (%)	Growth Rate (Kg/day)	Feed Efficiency (Gain/Feed)	N-Digestibility (%)
13U	.61	.278	67.3
13T	.62	.294	60.0
20U	.73	.334	79.8
20T	.74	.337	73.4

[1]Data taken from Faichney and Davies (1972).

[2]Diets contained either 13 or 20% crude protein equivalent. U indicates dietary protein was untreated; T indicates peanut meal added to diet was formaldehyde-treated.

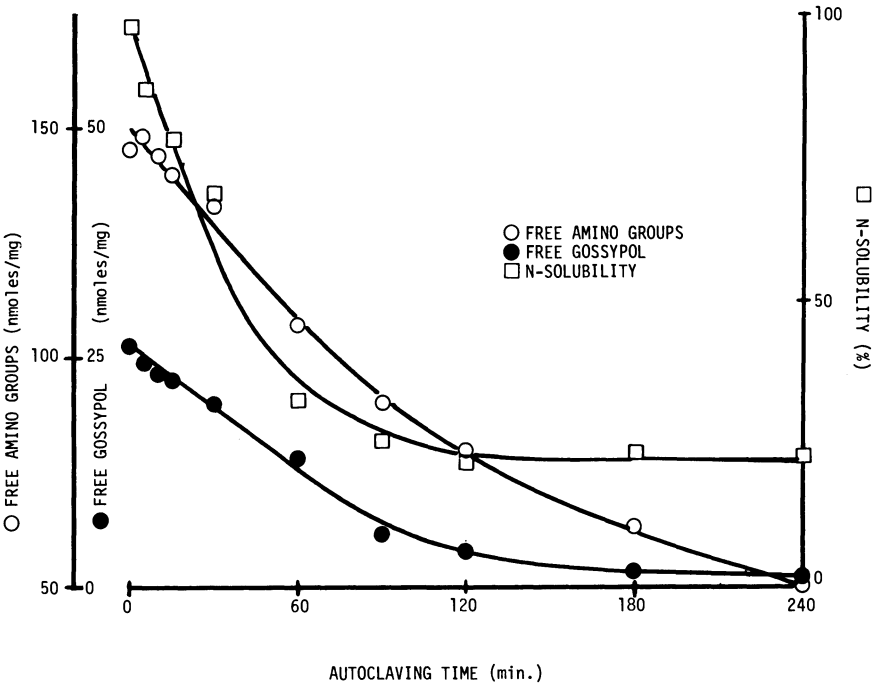


Fig. 3. Effects of cottonseed meal (CSM) heat-treatment (autoclaving time) on free protein amino groups (determined by ninhydrin assay on the intact protein), free gossypol content and N-solubility in .02 N NaOH.

The relationships between CSM heat-treatment (autoclaving time) and N-solubility, free gossypol content and free protein amino groups (calculated from ninhydrin reactivity) are shown in figure 3. There was a sharp, almost linear drop in N-solubility between 0 and 90 min. of heat-treatment, before it plateaued at about 25% after 90 min. The decline of both free amino groups and free gossypol content was also approximately linear through 90 min. of heat-treatment, although these slopes were not nearly so steep as that of N-solubility. Gossypol is a bifunctional compound with 2 carbonyls per molecule. Therefore, it can potentially crosslink CSM proteins by reacting with lysine ε-amino groups from different peptide chains. Assuming both gossypol carbonyls reacted with lysine residues, and the ninhydrin molar response for protein-bound amino groups was comparable to that of free leucine (which was used for the ninhydrin standard curve), gossypol binding could have accounted for as much as 64% of the loss of CSM free amino groups through 90 min of autoclaving. From 90 to 240 min, free amino groups continued

Table 3

Effect of CSM Heat-Treatment on Ruminal *in vitro* Degradation Rates (k_d) and Estimated % By-Pass Values

Protein	Autoclaving Time (min)	k_d (%/hr)	Estimated Ruminal By-Pass[1]
Casein	-	21.3	16
CSM	0	15.7	20
	5	13.9	22
	10	12.6	24
	15	13.9	22
	30	11.1	26
	60	5.2	43
	90	4.2	49
	120	3.4	54
	180	3.2	56
	240	2.5	62

[1]% By-Pass = $[k_r/(k_r+k_d)] \times 100$, k_r set = 4% hr.

to fall, while free gossypol appeared to plateau. At 240 min, gossypol binding may have accounted for about 50% of the total loss of CSM free amino groups.

Ruminal *in vitro* degradation rates, k_d (%/hr), and estimated proportion ruminal by-pass (%) of casein and the CSM preparations are shown in table 3. The degradation rate of 21.3%/hr obtained for casein was typical of previous *in vitro* results, but somewhat lower than the expected *in vivo* value of about 35%/hr (Broderick, 1976). The degradation curve for each CSM preparation was treated as if it represented a protein of homogeneous degradation rate, although the curves obtained with some CSM preparations appreared to resolve into two rather than one curve. Nevertheless, it was felt that the relative trends in k_d obtained using this "single-curve assumption" for all CSM preparations were still valid. These results show clearly that ruminal protein degradation rates decline with increasing heat-treatment, and result in corresponding increases in estimated protein by-pass. There appeared to be a sharp "break-point" in degradation rate between 30 and 60 min of CSM autoclaving time. Heating from 0 to 30 min resulted in only slight increase and decrease in k_d and by-pass, respectively. But k_d found for the 60 min CSM sample was less than half, and the estimated by-pass nearly twice that

observed for the 30 min sample. Autoclaving from 60 to 240 min yielded only gradual decline in k_d and increase in estimated by-pass.

The probable mode by which heat-treatment reduced ruminal degradation was through reduced protein solubility. There was a very high correlation (r = .994) between observed k_d values and N-solubilities in .02 N NaOH. N-solubility was also effective in identifying the sharp drop in degradation rate observed between 30 and 60 min of heat-treatment. Gossypol, a bifunctional compound present in CSM, may act as a naturally-occurring crosslinking agent if both carbonyls react with lysine ε-amino groups of different peptide chains. Between 0 and 90 min of heat-treatment, 64% of the loss in protein free amino groups may be accounted for by gossypol binding, indicating that crosslinking of this type was probably acting to reduce ruminal solubility and degradation. Lyman and coworkers (1959) found, using ultra centrifugation sedimentation, that increasing concentrations of gossypol crosslinked bovine plasma albumin, with progressive formation of dimers, trimers and higher order polymers (figure 4). Lyman's group also showed that gossypol binding accounts for a large proportion of the available lysine loss occuring with heat-treatment of CSM (Baliga and Lyman, 1957; Lyman *et al.*, 1959). However, agreement on this point is not complete. Gossypol is released from pigment glands during processing but it may not have opportunity to react with much CSM protein because most protein is compartmentalized in the cottonseed aleurones or protein bodies (Martinez *et al.*, 1967).

No measurements were made that would relate to post-ruminal (intestinal) protein digestibility, which would be expected to fall with increasing heat-treatment. Ideally, a protein should be modified to the least extent necessary to achieve nearly maximal ruminal protection, sacrificing as little post-ruminal digestibility as possible. In the present experiment, CSM heat-treated for 60 min may best fit this criterion - least amount of heating giving substantial ruminal protection. Sherrod and Tillman (1964) obtained results with lambs, shown in figure 5, which were nearly identical to the theoretical curve (figure 1) relating heat-treatment and ruminant N-utilization. Sherrod and Tillman found that autoclaving cold-extracted CSM for 60 min significantly reduced urinary-N excretion and increased N-retention in growing lambs, although fecal excretion also increased (suggesting decreased intestinal protein digestion). These results indicate that ruminal degradation was reduced and by-pass increased, resulting in greater intestinal protein digestion to amino acids. Beyond 60 min heat-treatment, N-retention began to fall because the large increases in fecal-N loss were not off-set by further reductions in urinary-N. Various other workers have

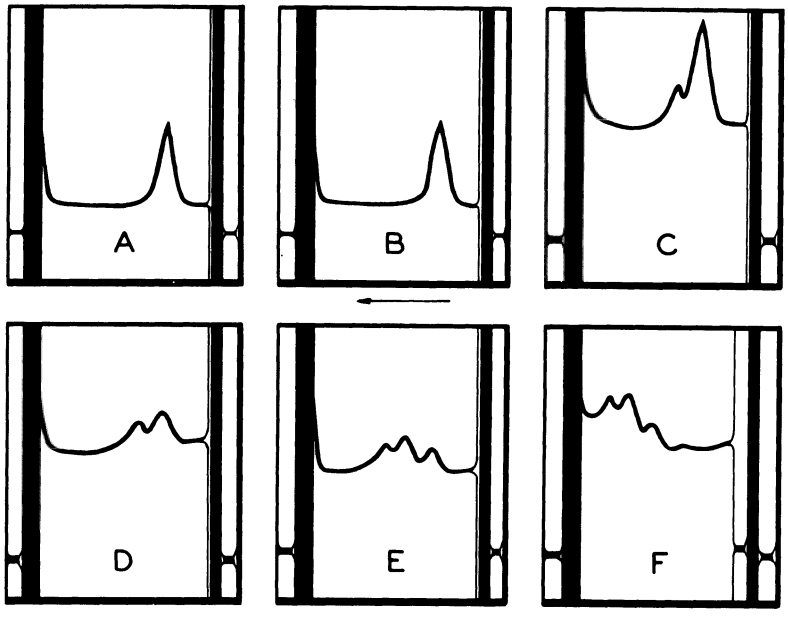


Fig. 4. Effect of increasing gossypol concentrations on bovine plasma albumin (BPA) ultracentifugation sedimentation patterns. BPA migrated from left to right. Increasing gossypol concentration resulted in crosslinking of BPA to form dimers (C and D), trimers (E) and higher multiples of BPA (F). Gossypol concentrations were: A, 0(control); B, 1.0%; C, 4.6%; D, 6.6%; E, 7.6%; F, 17.2%. Figure taken from Lyman *et al.* (1959) by permission of the Archives of Biochemistry and Biophysics.

also found that protein heat-treatment improved N-retention in growing lambs (Tagari *et al.*, 1962; Glimp *et al.*, 1967) and calves (Whitelaw *et al.*, 1961 and 1963).

The oil in commercial CSM is removed by solvent extraction or by screw-pressing. These two general processes result in CSM which is either of high (solvent-extracted) or low (screw-pressed) protein solubility. Preliminary results with commercial CSM samples indicated that at least 50% more protein from screw-pressed CSM will by-pass the rumen than from solvent-extracted CSM. Because the gossypol present in CSM is toxic to monogastrics, ruminant feeding represents the major use of CSM protein in animal nutrition. It is suggested that the CSM industry consider adopting conditions for oil removal that would be conducive to maximal ruminant protein utilization -- i.e., optimal ruminal protection without too large a sacrifice of intestinal protein digestibility.

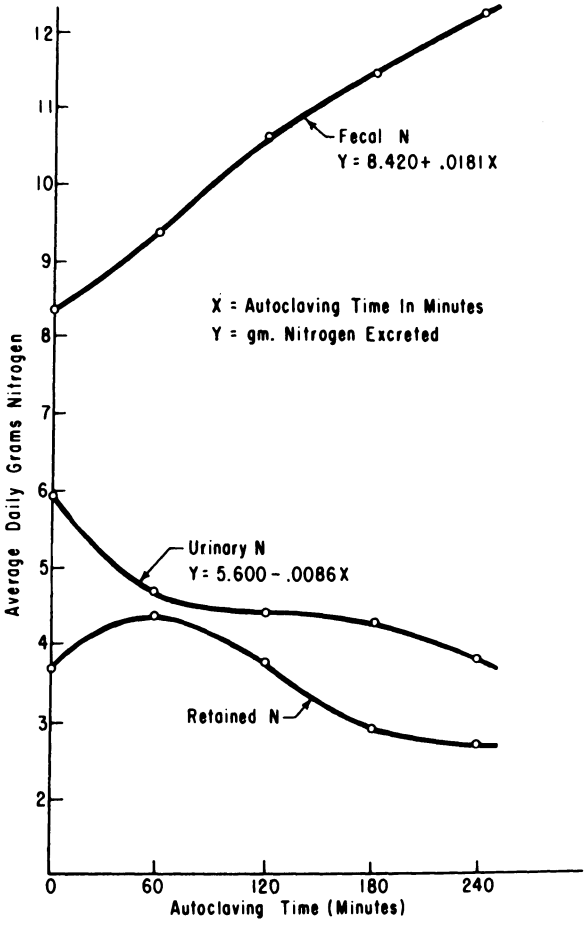


Fig. 5. Effects of cottonseed meal (CSM) heat-treatment (autoclaving time) for various lengths of time on N-excretion and N-retention in growing lambs. Figure taken from Sherrod and Tillman (1964) by permission of the American Society of Animal Science.

ACKNOWLEDGEMENTS

The author wishes to thank Mrs. Karolyn K. Smith and Mr. Mike Craig for excellent technical assistance.

REFERENCES

A.O.C.S. (1970). Official and tentative methods of the American Oil Chemists Society (3rd Ed). Amer. Oil Chem. Soc., Champaign, Ill.

Baliga, B. P. and Lyman, C. M. (1957). Preliminary report on the nutritional significance of bound gossypol in cottonseed meal. J. Amer. Oil Chem. Soc. 34:21.

Beckwith, A. C., Paulis, J. W. and Wall, J. S. (1975). Direct estimation of lysine in corn meals by the ninhydrin color reaction. J. Agr. Food Chem. 23:194.

Broderick, G. A. (1975). Factors affecting ruminant responses to protected amino acids and proteins. In M. Friedman (Ed.) Protein Nutritional Quality of Foods and Feeds. Vol. 1, Part 2. Marcel Dekker, Inc., New York.

Broderick, G. A. (1976). Modification of an *in vitro* procedure for estimating ruminal protein by-pass. Fed. Proc. 35:442 (Abstr.).

Broderick, G. A., Kowalczyk, T., and Satter, L. D. (1970). Milk production response to supplementation with encapsulated methionine per os or casein per abomasum. J. Dairy Sci., 53:1714.

Chalmers, M. I., Cuthbertson, D. P., and Synge, R. L. M. (1954). Ruminal ammonia formation in relation to the protein requirement of sheep. I. Duodenal administration and heat processing as factors influencing fate of casein supplements. J. Agr. Sci., 44:254.

Chalupa, W. (1975). Rumen bypass and protection of proteins and amino acids. J. Dairy Sci. 58:1198.

Chalupa, W., Chandler, J. E. and Brown, R. E. (1972). Amino acid nutrition of growing cattle. Fed. Proc., 31:681 (Abstr.).

Chalupa, W., Chandler, J. E. and Brown, R. E. (1973). Abomasal infusion of mixtures of amino acids to growing cattle. J. Anim. Sci., 37:339 (Abstr.).

Clark, J. H. (1975). Nitrogen metabolism in ruminants: Protein solubility and rumen bypass of protein and amino acids. In M. Friedman (Ed.) Protein Nutritional Quality of Foods and Feeds. Vol. 1, Part 2. Marcel Dekker, Inc., New York.

Derrig, R. G., Clark, J. H. and Davis, C. L. (1974). Effect of abomasal infusion of sodium caseinate on milk yield, nitrogen utilization and amino acid nutrition of the dairy cow. J. Nutr., 104:151.

Faichney, G. J. and Davies, H. L. (1972). The effect of formaldehyde treatment of peanut meal in concentrate diets on the performance of calves. Australian J. Agr. Res., 23:167.

Ferguson, K. A., Hemsley, J. A. and Reis, P. J. (1967). The effect of protecting dietary protein from microbial degradation in the rumen. Australian J. Sci., 30:215.

Friedman, M. and Broderick, G. A. (1977). Protected proteins in ruminant nutrition: *In vitro* evaluation of casein derivatives. This volume.

Glimp, H. A., Karr, M. R., Little, C. O., Woolfolk, P. G., Mitchell, Jr., G. E. and Hudson, L. W. (1967). Effect of reducing soybean protein solubility by dry heat on the protein utilization of young lambs. J. Anim. Sci., 26:858.

Goering, H. K. and Waldo, D. R. (1974). Processing effects on protein utilization by ruminants. Proc. Cornell Nutr. Conf. p. 25.

Henderickx, H. and Martin, J. (1963). *In vitro* study of nitrogen metabolism in the rumen. Compt. Rend. Rech., Inst. Rech. Sci. Ind. Agr., Bruxelles, 31:7.

Hogan, J. P. (1975). Quantitive aspects of nitrogen utilization in ruminants. J. Dairy Sci., 58:1164.

Hurrell, R. F. and Carpenter, K. J. (1977). Nutritional significance of cross-link formation during food processing. This volume.

Hurrell, R. F., Carpenter, K. J., Sinclair, W. J., Otterburn, M. S. and Asquith, R. S. (1976). Mechanisms of heat damage in proteins. 7. The significance of lysine-containing isopeptides and of lanthionine in heated proteins. Brit. J. Nutr. 35:383.

Lyman, C. M., Baliga, B. P., Slay, M. W. (1959). Reactions of proteins with gossypol. Arch. Biochem. Biophys., 84:486.

Lyman, C. M., Chang, W. Y., and Couch, J. R. (1953). Evaluation of protein quality in cottonseed meals by chick growth and by a chemical index method. J. Nutr., 49:679.

Martinez, W. H., Berardi, L. C., Frampton, V. L., Wilcke, H. L., Greene,D. E., and Teichman, R. (1967). Importance of cellular constituents to cottonseed meal protein quality. J. Agr. Food Chem., 15:427.

Mowat, D. N. and Deelstra, K. (1972). Encapsulated methionine supplement for growing-finishing lambs. J. Anim. Sci., 34:332.

Ørskov, E. R. and Fraser, C. (1969). The effect on nitrogen retention in lambs of feeding protein supplements direct to the abomasum. Comparison of liquid and dry feeding and of various sources of protein. J. Agr. Sci., 73:469.

Ørskov, E. R., Fraser, C., and Corse, E. L. (1970). The effect on protein utilization of feeding different protein supplements via the rumen or via the abomasum in young growing sheep. Brit. J. Nutr., 24:803.

Peter, A. P., Hatfield, E. E., Owens, F. N. and Garrigus, U. S. (1971). Effects of aldehyde treatments of soybean meal on *in vitro* ammonia release, solubility and lamb performance. J. Nutr. 101:605.

Reis, P. J., Schinckel, P. G. (1964). The growth and composition of wool. II. The effect of casein, gelatin and sulphur-containing amino acids given per abomasum. Aust. J. Biol. Sci., 17:532.

Schelling, G. T., Chandler, J. E. and Scott, G. C. (1973). Post-ruminal supplemental methionine infusion to sheep fed high quality diets. J. Anim. Sci., 37:1034.

Schelling, G. T. and Hatfield, E. E. (1968). Effect of abomasally infused nitrogen sources on nitrogen retention of growing lambs. J. Nutr. 96:319.

Schwab, C. G., Satter, L. D. and Clay, A. B. (1976). Response of lactating dairy cows to abomasal infusion of amino acids. J. Dairy Sci. 59:1254.

Tagari, H., Ascarelli, I. and Bondi, A. (1962). The influence of heating on the nutritive value of soya-bean meal for ruminants. Brit. J. Nutr., 16:237.

Vik-Mo, L., Emery, R. S. and Huber, J. T. (1974). Milk protein production in cows abomasally infused with casein or glucose. J. Dairy Sci., 57:869.

Whitelaw, F. G. and Preston, T. R. (1963). The nutrition of the early-weaned calf. III. Protein solubility and amino acid composition as factors affecting protein utilization. Anim. Prod., 5:131.

Whitelaw, F. G., Preston, T. R. and Dawson, G. S. (1961). The nutrition of the early-weaned calf. II. A comparison of commercial groundnut meal, heat-treated groundnut meal and fish meal as the major protein source in the diet. Anim. Prod., 3:127.

31

PROTECTED PROTEINS IN RUMINANT NUTRITION. IN VITRO EVALUATION OF CASEIN DERIVATIVES

Mendel Friedman[1] and Glen A. Broderick[2]

[1]Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California 94710

[2]Department of Animal Science, Texas A&M University and Texas Agricultural Experiment Station, College Station, Texas 77843

ABSTRACT

Chemical treatment of proteins in feeds can, by crosslinking protein chains or other chemical effects, decrease their solubility and microbial degradation in the rumen. A need exists to modify proteins systematically under well-defined conditions with inexpensive reagents and to evaluate the treated products nutritionally for possible beneficial effects on wool growth and quality as well as production of meat and milk. As a first step toward this goal we evaluated many derivatives of casein treated at pH 9-10 with various acylating and alkylating agents. Initial tests indicate that all treatments decreased protein digestion by rumen microorganisms. Potentially crosslinking reagents are usually more effective than similar ones that cannot crosslink. A few treatments gave ruminal protection that approached or exceeded that obtained with formaldehyde. This result shows that systematic evaluation of ruminal in vitro digestibility of protein derivatives may disclose new products deserving tests of post-ruminal digestibility and practical nutritive value.

INTRODUCTION

Protecting proteins for ruminant feeds shows great promise for increasing cattle and sheep production. Extensive studies (Broderick, 1975; Clark, 1975) suggest that feeding sheep protected proteins could double wool production. Under

experimental conditions more than 20% increase in weight gain is possible in meat animals. Such increases could amount to 4 billion pounds worth about 2 billion dollars annually in the United States alone. Milk protein production could be increased by more than 10%, or by at least 375 million pounds annually. An added benefit is that the more efficient utilization of protected proteins would decrease livestock use of oilseed or grain proteins that are also needed for human food.

Feed protein consumed by ruminants is largely degraded to NH_3 by ruminal organisms. This process wastes amino acids because the NH_3, although used by microbes in part to resynthesize protein assimilated by the animal, is partly excreted as urea. This loss has stimulated interest in chemical treatment to reduce ruminal protein degradation and thereby increase the net intestinal absorption of amino acids (Asplund, 1975; Broderick, 1975, 1977; Clark, 1975; Miller, 1973; Phillipson, 1972; Dinius et al., 1974).

Because results of protecting proteins with formaldehyde and other reagents are not always consistent, in terms of reproducibility and in terms of observed increases in meat, milk, and wool production, a need exists to relate the chemical properties of the modifying reagents and conditions of treatment to their effectiveness in protecting proteins from ruminal degradation without impairing their later digestion. In this paper we report attempts to develop such structure-reactivity correlations by comparing digestibilities of various casein derivatives by ruminal microorganisms in vitro.

EXPERIMENTAL

PROTEIN MODIFICATIONS

Anhydrides. Commercial casein (130 g) and 30 g of succinic anhydride are suspended in 1000 ml H_2O and 300 ml pH 9.2 sodium borate buffer (0.05 M). The suspension is adjusted to pH 8.0 with NaOH, mixed for 2 hr at room temperature, dialyzed against distilled water, and lyophilized. Other anhydrides are used similarly.

Formaldehyde. Casein (130 g) is suspended in 1000 ml H_2O and 300 ml pH 9.2 borate buffer (0.05 M). The suspension is adjusted to pH 7.0 with NaOH and 50 ml of 37% formaldehyde solution added. The reaction mixture is stirred for 24 hr, dialyzed against water and lyophilized.

Epoxides. Ethylene oxide (60 ml) is added to 60 g casein in 150 ml pH 9.1 borate buffer (0.05 M) and 500 ml water. The reaction mixture is stirred. Aliquots are removed at various

time intervals, dialyzed, and lyophilized. The other epoxides listed in Table 3 are used similarly.

Divinyl sulfone. Divinyl sulfone (30 ml) (Aldrich) is added to 130 g casein suspended in 1000 ml H_2O and 300 ml pH 9.8 borate buffer (0.05 M). Aliquots are removed from the stirred mixture at various time intervals, dialyzed, and lyophilized.

N-Methylolacrylamide. To a suspension of 50 g of casein in 1000 ml H_2O and 300 ml pH 9.1 borate buffer is added 120 ml of 60% aqueous N-methylolacrylamide (Cyanamid). The pH is adjusted to 9.5. Aliquots were removed from the stirred reaction mixture at various time intervals, dialyzed against distilled water, and lyophilized.

Methyl acrylate, hydroxethyl acrylate, acrylamide, and N-vinylpyrrolidone are used similarly except that with these reagents, the ratio of casein to vinyl compound is about two to one and the reaction was carried out at pH 9.1.

In Vitro DETERMINATION OF RUMINAL DEGRADATION RATE

In vitro incubation. Samples containing about 180 mg casein are weighed exactly into 50 ml polyethylene centrifuge tubes. Five (5) ml of McDougall's buffer (McDougall, 1948) is added to each. Protein sources plus buffer are allowed to soak overnight at 40°C. The next morning the tubes are put in a water bath to warm to 39°C, and ten (10) ml of Incubation Mixture (IM) are added to each tube; tubes are rapidly capped with stoppers with Bunsen valves, incubated for 2 hr at 39°C in a shaker water bath. Digestion is stopped by adding 1 ml 50% trichloroacetic acid (TCA) to each tube.

The Incubation Mixture contains 50% v/v McDougall's buffer, 50% v/v strained rumen liquor (SRL), 1% w/v maltose, 1.5 mM dithithreitol (DTT). In most experiments 1.5 mM hydrazine sulfate (HS) was also added to inhibit microbial removal of NH_3 and amino acids released during protein degradation (Broderick, 1976). Maltose and HS are dissolved in an appropriate amount of McDougalls buffer that has been freshly saturated with CO_2 gas. This mixture is warmed to 39°C with continued CO_2 gassing until the SRL is brought to the laboratory. Just before adding the appropriate volume of SRL, the DTT is weighed and dissolved in the warmed, gassed McDougalls solution. SRL is mixed with the buffer and the Incubation Mixture is rapidly dispensed to the incubation tubes. Final concentration in incubation tubes: 15 ml total (10 ml buffer + 5 ml SRL), 1 mM DTT and 1 mM HS; 0.67% w/v maltose (0.10 g maltose/tube).

Each incubation experiment consisted of 2 blank tubes, 2

tubes with each casein preparation, and 2 special blank tubes which are stopped with exactly 1 ml of 50% w/v TCA solution containing 32 mM leucine (amino acid standard) and 32 mM NH_3 (ammonia standard added as $(NH_4)_2SO_4$). This gave added leucine and ammonia concentrations of 2.0 μM/ml. Ammonia and corrected amino acid (non-NH_3 ninhydrin positive material) concentrations are determined using a Technicon Auto Analyzer System. Net NH_3 and net amino acid release are calculated by subtracting concentrations of NH_3 and amino acids in blank tubes from those in tubes receiving casein preparations.

Net release values are adjusted according to recoveries of added NH_3 and leucine in the special blank tubes. Finally, degradation rates are reported as the sum of adjusted net NH_3 plus amino acid release in μM/SRL.

Determination of NH_3 release. This procedure is an adaptation to the Auto Analyzer of McCullough's (1967) phenol-formaldehyde method for determining NH_3 in whole blood. Sodium nitroprusside is added to the phenol reagent to enhance color development. The reaction temperature is 95°C rather than 37°C. The higher temperature does not reduce sensitivity to NH_3, but may reduce color yield from amino acids. Molar response is at least 80 times greater for NH_3 than for leucine with this method. Similar procedures were used by Munro and Fleck (1969) for Kjeldahl digests and by Streeter et al. (1970).

Determination of amino acid release. This procedure is adapted from the "Air-Stable Ninhydrin" method of Technicon (1968). No hydrindantin is added to the ninhydrin reagent in this procedure, so the ninhydrin reagent is stable at room temperature in air. Hydrindantin is formed when ninhydrin mixes with 0.002 M hydrazine in the analytical system. The color yield with this method is about 75% of that with the normal Technicon ninhydrin reagent containing hydrindantin. Use of 0.004 M hydrazine did not appreciably increase color yield. The principle of this procedure is to first measure the NH_3 concentration (using phenol-hypochlorite method), then the relative ninhydrin-color yield of NH_3 and leucine standards, and finally the total ninhydrin-positive material ("gross amino acid concentration") in the ruminal *in vitro* samples. The contribution of NH_3 to the total ninhydrin color is determined and the corrected amino acid concentration calculated by difference.

NINHYDRIN PROCEDURE FOR MODIFIED PROTEINS

The procedure was adapted from Beckwith et al. (1975) and Friedman and Williams (1973, 1974). Reagents: 0.125 M sodium metabisulfite ($Na_2S_2O_5$); 2.375 g/100 ml H_2O; DMSO/H_2O (4:1

v/v); 3% w/v ninhydrin -3 g; ninhydrin/25 ml 4 N NaAcetate pH 5.5 buffer/50 ml DMSO/25 ml H_2O. Just before use, 13 mg hydrazine sulfate is dissolved in 10 ml H_2O and mixed with 50 ml ninhydrin solution.

Procedure: Weigh 3.0 mg samples of casein preparation into 16 x 100 mm test tubes. Add 3.8 ml DMSO/H_2O and 0.2 ml H_2O (blank) or 0.2 ml of leucine standards (1-8 μM/ml) to test tubes to be used for standard curve. Add 0.2 ml of 0.125 M $Na_2S_2O_5$ and 3.8 ml to DMSO/H_2O to each tube with casein preparations. Do not to blank or leucine standards. Mix casein samples using vortex mixer for 30 sec. Add 1.2 ml ninhydrin HS reagent to each tube. Mix briefly with vortex, cap with marbels. Heat tubes in boiling water bath 30 min then cool rapidly by partial immersion in cold water. Centrifuge tubes 5 min at 900-1000 g. Transfer 1 ml aliquot from each tube to cuvettes (10 mm path length) and dilute with 5 ml DMSO/H_2O. Mix with vortex and read at 580 nm.

RESULTS AND DISCUSSION

As already noted, formaldehyde treatment of casein fed to sheep has been found to increase their weight gain and wool growth. This result is ascribed to temporary crosslinking that protects the protein while it is in the rumen. Since results with formaldehyde have not been reproducible when evaluated by different investigators (Wright, 1971; Barry, 1972; Hemsley et al., 1973; Faichney, 1974; Clark, 1975; Broderick, 1975), we have modified casein with several kinds of acid anhydrides, vinyl compounds, and epoxides, including examples with one and two reactive sites. Treated materials were evaluated for resistance to ruminal degradation in vitro as described in the experimental section. The results are summarized in Tables 1-3.

Data in Table 1 show that although acid anhydrides give some protection, the small degree observed does not encourage further feeding studies. The effectiveness of the anhydrides as protecting agents appears unrelated to the degree of modification of the amino groups as measured by the ninhydrin reaction (Figure 1 and Table 1, % reacted column).

Evaluation of a series of vinyl compounds (Figure 2) strikingly illustrates that bifunctional vinyl compounds such as divinyl sulfone or potentially bifunctional ones such as N-methylolacrylamide are more effective than the monofunctional ones such as methyl acrylate, hydroxyethyl acrylate, acrylamide, and N-vinyl-2-pyrrolidone. The greater protective effects of difunctional reagents is not surprising, since the ability of formaldehyde to protect the protein against ruminal

Table 1

Ruminal In Vitro Degradation of Casein (Anhydride) Derivatives

Treated with	Average relative release rate[a] (%)	Ninhydrin Analyses of Proteins: Average μmole Leu equiv. / mg N	Ninhydrin Analyses of Proteins: % Reacted	% Protein from Kjeldahl analyses[b] (NX6.25)
Control	100.0	3.50	0.0	87.3
Formaldehyde-1 hr	4.1-21.6	2.64	24.6	74.52
Citraconic anhydride -2.5 hr	71.9	2.79	20.3	77.28
Glutaric anhydride -2.5 hr	58.6	0.302	91.4	79.97
Acetic anhydride	75.2	1.57	55.1	86.24
Succinic anhydride	55.5	0.365	89.6	79.24

[a] Protein degradation to NH_3 plus amino acids during in vitro incubation with ruminal digesta. Release from untreated casein control = 100%.

[b] On air-dry basis.

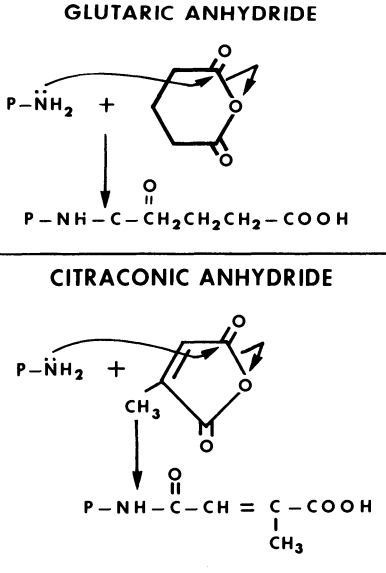


Fig. 1. Reaction of protein amino groups with anhydrides.

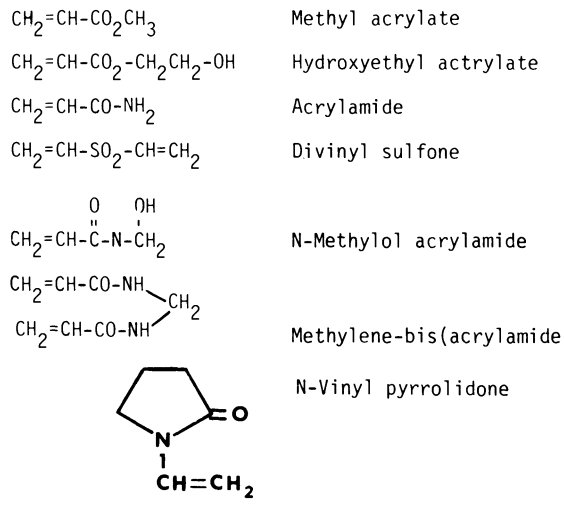


Fig. 2. Structures of vinyl compounds

degradation is thought to be due to greater chemical or physical resistance of the presumably crosslinked protein (Figure 3) to attack by microbial proteolytic enzymes in the ruminal fluid. Divinyl sulfone can crosslink a protein analogously to formaldehyde as shown in Figure 4. The striking effectiveness of N-methylolacrylamide can also be plausibly explained as due to its ability to crosslink protein functional groups as illustrated in Figure 5. A structurally similar compound, hydroxyethyl acrylate, in which the O-hydroxyethyl moiety is not known to be reactive towards protein functional groups, in contrast to the known reactivity of the N-methylol group (-N-CH_2-OH) of N-methylolacrylamide, was much less effective than the latter compound.

Results of analogous comparisons of mono- and difuctional epoxides (Figure 6, Table 3) again strikingly demonstrate the same point. Although ethylene and propylene oxides as well as glycidol and allyl glycidyl ether exhibited moderate effectiveness, epichlorohydrin and epibromohydrin were highly effective. Their effectiveness is, again, presumably due to their ability to crosslink protein as illustrated in Figure 7.

The effectiveness of styrene oxide, a monofunctional epoxide may be due to two factors: The ability of the aromatic benzene ring to interact with the protein, the net result of which is that styrene oxide behaves as a pseudo-crosslinking agent; or to other steric or hydrophobic effects that the aromatic side chains of the casein-styrene oxide adduct exert in hindering interaction with the proteolytic enzymes in ruminal fluid. This aspect of

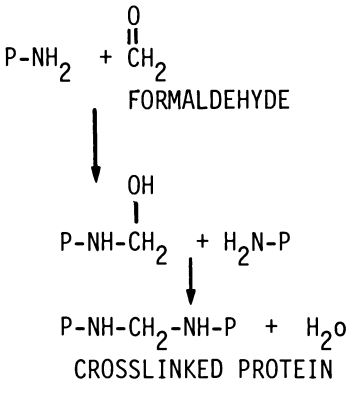


Figure 3. Crosslinking of a protein by formaldehyde.

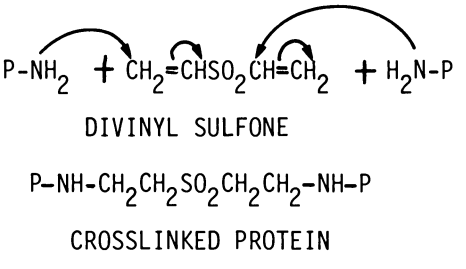


Figure 4. Crosslinking of a protein by divinyl sulfone.

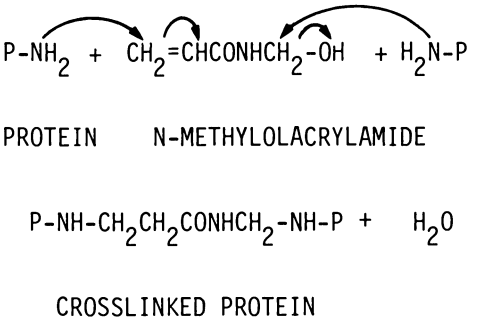


Figure 5. Crosslinking of a protein by N-methylolacrylamide.

Table 2

Ruminal In Vitro Degradation of Casein Vinyl Adducts

Treated with	Average relative release rate[a] (%)	Ninhydrin Analyses of Proteins: Average μmole Leu equiv. / mg N	Ninhydrin Analyses of Proteins: % Reacted	% Protein from Kjeldahl analyses[b] (NX6.25)
Control	100.00	3.50	0.0	87.03
Formaldehyde-1 hr	4.1-21.6	2.64	24.6	74.52
N-Methylolacryl-amide				
-15 min	10.1	2.32	33.7	80.57
-60 min	11.6	2.27	35.1	80.68
-3 hr	10.3	2.20	37.1	81.10
-6 hr	17.2	2.25	35.7	80.20
Divinyl sulfone				
-5 min	21.6	0.126	96.4	74.60
-20 min	10.6	0.108	96.9	73.35
-40 min	5.9	0.101	97.1	73.62
-60 min	11.7	0.081	97.7	73.55
-2 hr	7.3	1.06	97.0	73.06
N-Methylene-bis-acrylamide				
-15 min	56.5	1.52	56.6	81.85
-2 hr	44.4	1.02	70.9	82.47
-6 hr	34.7	0.628	82.1	82.81
Hydroxyethyl acrylate	48.9	1.01	71.1	73.91
N-vinyl-2-pyrrolidone	57.6	2.62	25.1	81.56
Acrylamide	61.4	1.14	67.4	87.79
Methyl acrylate	62.5	0.747	78.7	74.20

[a] Protein degradation to NH_3 plus amino acids durin in vitro incubation with ruminal digesta. Release from untreated casein control = 100%.

[b] On air-dry basis.

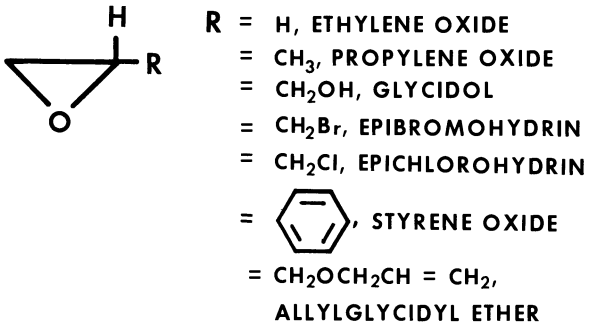


Fig. 6. Structures of epoxides.

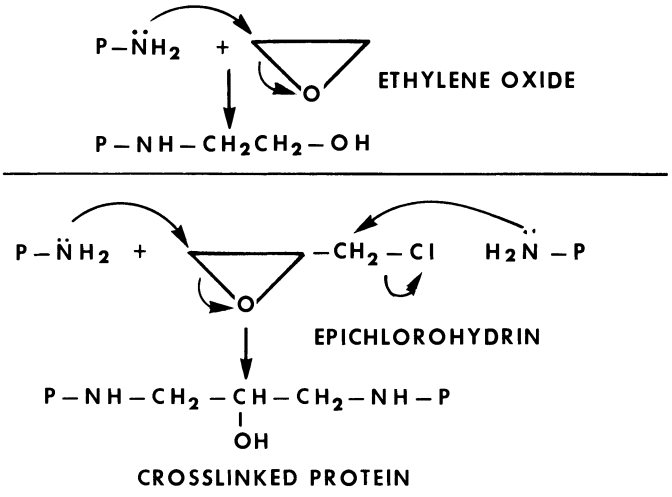


Fig. 7. Modification of a protein by monofunctional (ethylene oxide) and bifunctional (epichlorhydrin) epoxides.

the problem merits further investigation with substituted aromatic epoxides (and aldehydes).

It is noteworthy that the extent of modification of amino groups by vinyl compounds or epoxides does not appear to be related to the observed effectives of these reagents. Thus, the highly effective N-methylolacrylamide modified only about one-third of the amino groups (Table 2) whereas the much less effective methyl acrylate modified nearly 80%.

Table 3

Ruminal In Vitro Degradation of Casein (Epoxide) Derivatives

Treated with	Average relative release rate[a] (%)	Ninhydrin Analyses of Proteins: Average µmole Leu equiv. / mg N	Ninhydrin Analyses of Proteins: % Reacted	% Protein from Kjeldahl analyses[b] (NX6.25)
Control	100.00	3.50	0.0	87.03
Formaldehyde	4.1-21.6	2.64	24.6	74.52
Ethylene oxide				
-1 hr	85.5	1.93	44.9	80.19
-4 hr	78.5	1.05	70.0	76.55
-24 hr	70.1	0.454	87.0	77.00
-48 hr	62.4	0.198	94.3	78.62
Propylene oxide				
-24 hr	68.7	0.743	78.8	76.17
-48 hr	65.9	0.500	85.7	75.53
Glycidol				
-2 hr	78.3	2.09	40.3	72.11
-24 hr	60.0	0.208	94.1	77.83
Allyl glycidyl ether				
-48 hr	45.2	0.472	86.5	70.50
Styrene oxide				
-24 hr	26.1	1.62	53.7	69.03
-48 hr	13.4	1.37	60.9	67.91
Epichlorohydrin				
-26 hr	8.1	0.146	95.8	70.79
Epibromohydrin				
-24 hr	5.1	0.291	91.7	74.76
-72 hr	3.7	0.156	95.5	70.75

[a] Protein degradation to NH_3 plus amino acids during in vitro incubation with ruminal digesta. Release from untreated casein control = 100%.

[b] On air-dry basis

CONCLUSIONS

Chemical treatment of feed proteins, can, by crosslinking and modifying amino acid and other functional groups, decrease their solubility and microbial degradation in the rumen. Feeds in which proteins have been reliably modified with inexpensive reagents under readily controlled conditions need to be evaluated for possible beneficial effects on meat, milk, and wool production. The described screening technique for evaluating potential new reagents as protein protectants against ruminal degradation disclosed that N-methylolacrylamide has possible advantages that may favor its commercial use if it can be shown to be nutritionally efficient and nontoxic. These include: (a) Low cost -- The compound is produced commercially in large amounts for use in plastics and is therefore relatively cheap. (b) High effectiveness -- N-methylolacrylamide gave at least as good protection as formaldehyde. Moreover, it is effective with only about one-third of the available amino groups alkylated, as measured by the ninhydrin reaction. (c) Possible additional nitrogen for ruminal microbial utilization -- Chemical modification with N-methylolacrylamide introduces one nitrogen atom per mole of NMA. The greater nitrogen-content of the NMA-modified casein samples compared to those treated with other reagents is shown in the last column of Table 2. It is possible that this nonprotein nitrogen may be transformed to ruminal ammonia and then used for the biosynthesis of amino acids, although expectations are that if N-methylol nitrogen is effective in ruminal protection, it is probably unavailable nutritionally. The nutritional quality in terms of nitrogen availability of proteins modified with NMA, though not yet tested may, therefore, be expected to be better than for proteins treated with non-nitrogenous reagents. Finally, we wish to emphasize that the nutritional value and safety of these modified proteins both to the animal and to rumen organisms (Dinius et al., 1974) is still to be determined and that part of the crosslinking may be due to NMA-liberated formaldehyde.

ACKNOWLEDGEMENTS

We thank Mr. Eddie G. Marshall and Mrs. Karolyn K. Smith for excellent technical assistance.

REFERENCES

Asplund, J. M. (1975). The determination and significance of biological values of proteins for ruminants. In "Protein Nutritional Quality of Foods and Feeds," Part 1, M. Friedman (Editor), Dekker, New York, pp. 37-49.

Barry, T. N. (1972). The effect of feeding formaldehyde treated casein to sheep on nitrogen retention and wool growth. New Zealand J. Agr. Res., 15, 107-116.

Beckwith, A. C., Paulis, J. W. and Wall, J. S. (1975). Direct estimation of lysine in corn meals by the ninhydrin color reaction. J. Ag. Food Chem., 23, 194-196.

Broderick, G. A. (1976). Modification of an in vitro procedure for estimating ruminal protein by-pass. Fed. Proc., 35, 442 (Abstract).

Broderick, G. A. (1977). Effect of processing on protein utilization by ruminants. This volume.

Broderick, G. A. (1975). Factors affecting ruminant responses to protected amino acids and proteins. In "Protein Nutritional Quality of Foods and Feeds," Part 2, M. Friedman (Editor), Dekker, New York, pp. 211-259 and references cited there.

Clark, J. H. (1975). Nitrogen metabolism in ruminants: protein solubility and rumen bypass of protein and amino acids. In "Protein Nutritional Quality of Foods and Feeds," Part 2, M. Friedman (Editor), Dekker, New York, pp. 261-304 and references cited there.

Dinius, D. A., Lyon, C. K. and Walker, H. G., Jr. (1974). In vitro evaluations of protein and protein-safflower oil complexes treated with formaldehyde. J. Anim. Sci., 38, 467-474.

Faichney, G. J. (1974). The effect of formaldehyde treatment of a casein supplement on urea excretion and on digesta composition in sheep. Aust. J. Agr. Res., 25, 599-612.

Friedman, M. and Williams, L. D. (1974). Stoichimetry of formation of Ruhemann's purple in the ninhydrin reaction. Bioorganic Chem., 3, 267-280.

Friedman, M. and Williams, L. D. (1973). The reaction of ninhydrin with keratin proteins. Anal. Biochem., 54, 333-345.

Hemsley, J. A., Reis, P. J. and Downes, A. M. (1973). Influence of various formaldehyde treatments on the nutritional value of casein for wool growth. Aust. J. Biol. Sci., 26, 961-972.

McCullough, H. (1967). The determination of ammonia in whole blood by a direct colorimetric method. Clin. Chim. Acta, 17, 297-304.

McDougall, E. I. (1948). Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem. J., 43, 99-109.

Miller, E. L. (1973). Evaluation of foods as sources of nitrogen and amino acids. Proc. Nutr. Soc., 32, 79-84.

Munro, H. N. and Fleck, A. (1969). Analysis of tissues and body fluids for nitrogenous constituents. In "Mammalian Protein Metabolism," Vol. III, H. N. Munro (Editor), Academic Press, pp. 423-525.

Phillipson, A. T. (1972). The protection of dietary components from rumen fermentations. Proc. Nutr. Soc., 31, 159-164.

Streeter, C. L., Little, C. O. and Mitchell, G. E., Jr. (1970). Automated analysis of NH_3 in ruminal fluid. J. Animal Sci., 31, 255 (Abstract).

Technicon. (1968). A new air-stable ninhydrin reagent for use with standard Technicon amino acid analyzers. Technicon Research Bulletin No. 20.

Wright, P. L. (1971). Body weight gain and wool growth response to formaldehyde treated casein and sulfur amino acids. J. Animal Sci., 33, 137-141.

32

PROTEIN INTERRELATIONSHIPS IN ROUGHAGES AS AFFECTING RUMINANT DIETARY PROTEIN ADEQUACY

J.E. Knipfel

Research Station

Research Branch, Agriculture Canada

Swift Current, Saskatchewan. S9H 3X2

INTRODUCTION

Roughages have been defined (Troelsen, 1967) as feedstuffs which are sufficiently fibrous that their intakes and rates of passage through the ruminant may be limited by the rate of physical and/or chemical breakdown in the reticulorumen. Within these terms, roughages would include such feedstuffs as pasturage, hays, crop residues, silages, forestry and/or other byproducts, and would represent the basic feed for the herbivorous livestock of the world. A common characteristic of roughages is the presence of a complex carbohydrate fraction composed largely of cellulose and hemicellulose (Pigden, 1955). Mammalian species do not secrete cellulases in their digestive juices (Pigden, 1955; Lewis, 1961a; Phillipson, 1964); therefore, cellulosic materials are essentially indigestible by the monogastric animal, and other nutrients trapped within the fibres may also be unavailable.

Through the processes of evolution the ruminant has become well adapted to the utilization of highly fibrous feeds through the development of the forestomachs into specialized fermentation chambers in which extremely active microbial populations breakdown the complex carbohydrates of roughages to provide mainly short chain fatty acids which can be used as energy sources by the ruminant itself. Concommitant with cellulolytic activity, other components of the roughage are also fermented, and the material passing into the lower digestive tract for mammalian enzymatic digestion may differ considerably from that initially ingested. This

phenomenon is of particular concern in regard to the protein adequacy of the diet of the ruminant.

THE PROTEIN REQUIREMENTS OF RUMINANTS

The term "protein requirement" is of particularly doubtful accuracy when dealing with the ruminant. The ruminant animal itself is suggested to have amino acid requirements which qualitatively are similar to those of monogastric animals (Black et al., 1952; Downes, 1961). The rumen microflora, on the other hand, appear to fulfill a major proportion of their N requirements from NH_3 or other simple nitrogenous compounds. Since the ruminant derives much of its amino acid supply from the digestion of rumen microbes, in considering the adequacy of dietary protein for the ruminant one must consider the extent to which both the microbial protein and the ingested feed protein are utilized to meet the animal's requirements.

Nitrogen Metabolism in the Rumen

Before the turn of the 20th century a number of studies had suggested that ruminants might satisfy at least part of their protein requirements from simple nitrogenous compounds. Pearson and Smith (1943) observed protein anabolism and catabolism in the rumen. In 1948 the investigations of McDonald revealed several basic concepts of ruminant nitrogen metabolism, which were summarized by Lewis (1961b) as follows:

1. The amount of NH_3 present in the rumen depends upon the type of protein and carbohydrate materials in the rumen contents.

2. A considerable amount of NH_3 is absorbed directly into the ruminal veins and passes to the liver.

3. Some of the absorbed NH_3 re-enters the rumen as urea, through the saliva.

From these observations has evolved the concept of the nitrogen cycle in the ruminant, as represented in Fig. 1.

While many rumen microflora have been proposed to require only NH_3 as an N-source for protein synthesis (Bryant and Robinson, 1962), significant improvements in N utilization have resulted from inclusion of either amino acids (Bunn et al., 1968) or the α-keto analogs of specific amino acids (Cline et al., 1966). These data suggest that availabilities of carbon skeletons of at least some amino acids may be more critical than the ability of the bacteria to incorporate N through transamination reactions. In a large

number of roughages a significant proportion of the N is present as nonprotein nitrogen (NPN, Synge, 1968). The degree and efficiency of utilization of such compounds may depend upon the intensity of transamination activity in the rumen, which in turn would be as dependent upon the presence of α-keto analogs as upon the supply of N.

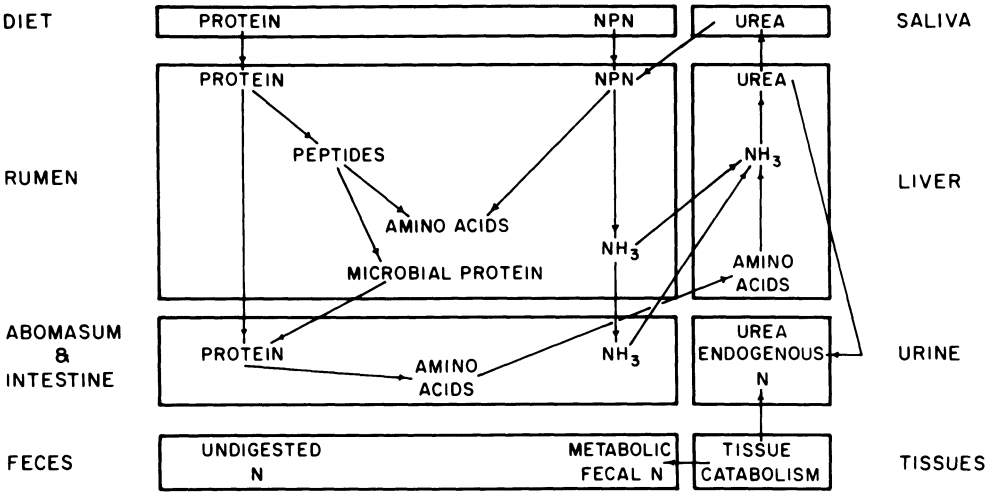


Fig. 1. The nitrogen cycle of the ruminant (from Lewis, 1961b).

The degradative and synthetic activities of the rumen microbial population may be altered by a variety of factors, both natural and man-induced. Considerable effort has been directed toward reducing the effects of rumen degradation upon high quality protein. This topic lies beyond the scope of the present discussion and for further information the reader is referred to recent reviews by Broderick (1975) and Clark (1975).

The Amino Acid Requirements of Ruminants

Extensive studies of the essential amino acid requirements of a variety of monogastric mammals and of poultry have been undertaken for many years, and reasonably accurate data concerning these requirements are available for several species (NAS-NRC series, Nutrient Requirements of Domestic Animals). The presence of the rumen and its intensive metabolic activities toward ingested proteins and amino acids has hampered attempts to estimate the amino acid requirements of the ruminant. Perhaps of equal importance has been the tendency to view the ruminant as "independent" of dietary amino acid sources (Crampton and Lloyd, 1959) as a result of rumen functional activities. Several workers (cited by Chalmers, 1961)

suggested biological values of approximately 80 for proteins from bacteria and protozoa, while more recent estimates (Mason, 1963; Preston, 1970) set the figure at approximately 60 under normal circumstances. Digestibility data for the bacterial and protozoal fractions of the rumen microbial population showed that the protozoal fraction was more highly digestible than was the bacterial fraction (Bergen et al., 1968; McNaught et al., 1954). Bergen et al. (1967) showed wide variations in digestibility and biological value of individual strains of rumen bacteria, and suggested that modifications of the rumen bacterial population might result in changes in amino acid availability to the host animal. Mean digestibility for all bacterial strains was 73.7% which compared well with the 73.3% observed by McNaught et al. (1954).

Purser et al. (1966) as well as earlier workers (Barnett and Reid, 1961) suggest that the average amino acid composition of microbial protein does not vary markedly, although significant differences between individual bacterial strains in amino acid composition were noted by Bergen et al. (1967). Weller (1957) showed only relatively minor changes in amino acid composition in hydrolyzates of bacterial material from the rumens of sheep fed different diets; the amino acid compositions of the bacterial fractions were quite similar to that of grass protein. These observations suggest that under conditions of roughage feeding, the composition of the rumen microflora with respect to amino acids may be secondary in importance to the amounts of microbial and feed protein available as amino acid sources to the animal over a particular time period. Considerable evidence has indicated that sulfur amino acids may be the first limiting amino acids in rumen microbial protein and in roughage proteins. Studies have been undertaken to demonstrate increases in milk production (McCarthy et al., 1970) nitrogen balance (Schelling and Hatfield, 1968), wool production (Reis and Schinckel, 1963; Reis, 1967; Downes et al., 1970) or growth (Wright, 1971) following supplementation of ruminants with sulfur amino acids either by feeding or post ruminal administration. Responses to dietary amino acid supplementation have not been uniform, however, and in several instances (Hatfield, 1970) responses were either lacking or insignificant.

During the past several years the amino acid requirements of the ruminant have been assessed by Burroughs et al. (1974) and Hutton and Annison (1973) using net utilization and deposition of amino acids during growth. Nitrogen Balance and plasma amino acid levels were related to post-ruminal amino acid infusions by Schelling et al. (1973) and Wakeling et al. (1970). Recently Fenderson and Bergen (1976) quantitated the requirements for sulfur amino acids, lysine, threonine and tryptophan in growing steers by using a combination of conventional feeding and post-ruminal infusion techniques. The available evidence suggests that the ruminant animal qualitatively requires the same amino acids as do

monogastric animals. To date, estimates of quantitative amino acid requirements for different physiological states, i.e., growth, maintenance, are lacking. Additional studies such as those described in the foregoing paragraph will greatly expand present knowledge of ruminant amino acid requirements.

INTERACTIONS OF PROTEIN AND SELECTED NONNUTRITIVE CONSTITUENTS OF ROUGHAGES

A large number of forage materials contain compounds which may interact with protein to alter the availability of the protein to the rumen microflora, to the ruminant itself, or to both. Several of these compounds and their effects upon roughage protein adequacy are discussed below.

Tannins

Tannins have been described as water soluble phenolic compounds with molecular weights between 500 to 3000, and which have protein precipitating activity (Swain and Bate-Smith, 1962). Early workers suggested that tannins inactivated amylases in sorghum malt by rendering the enzymes insoluble, while in a more recent study Daiber (1975) demonstrated that the tannins were present in the peripheral nucellar layer of the pericarp, and that separation of tannin containing tissues from the endosperm and embryo of the grain prior to malting removed the enzyme inhibition. Tannins have been observed to reduce the utilization of sorghum grain by both monogastric and ruminant animals. Both dry matter digestibility and protein digestibility were reduced in high tannin strains of sorghum (McGinty, 1968; Stallcup and Davis, 1962). Schaffert et al. (1974) found higher digestibilities of protein and dry matter *in vitro* for low tannin genotypes and suggested that the major factor limiting utilization of high tannin sorghum was availability of protein.

Several studies (Jones and Lyttleton, 1971, Gutek et al., 1974) suggest that forage legumes which did not contain tannins were characteristically higher in bloat potential for the ruminant than were those containing tannins. Bloat potential of legumes has been related to soluble protein (Boda et al., 1957), thus efforts have been made to study the interaction of tannins and proteins in reducing protein solubility. Tannins may reduce the digestibility of forage crops considerably, and thus become undesirable (Donnelly and Anthony, 1969). Sarkar et al. (1976) demonstrated qualitative differences in tannins of a variety of herbaceous legumes and suggested that a portion of the differences in nutritive value among legume species might be attributable to different tannins. These workers proposed that breeding programs to introduce specific

tannins with beneficial bloat-inhibiting properties may be feasible. In studies with crown vetch, Burns and Cope (1974) observed higher concentrations of tannins in leaf tissue than in stems. The protein content of leaf tissue has generally been observed to be higher than that of stem material, thus selection of genotypes for low tannin content may inadvertently result in lower nutritive value of the forage, if a change in morphological composition to favor a lower leaf-stem ratio results.

Tannins may be of value in protecting dietary proteins from ruminal degradation (Broderick, 1975), or in reducing bloat incidence, however, the changes which they induce in availability of protein may alter the adequacy of the protein in roughages to meet the animal's requirements.

Saponins

A number of leguminous roughages such as alfalfa and ladino clover contain saponins, which may be described as the glycosides of steroidal sapogenins (Heftmann, 1965). Although the saponins exhibit womewhat similar chemical structures, their biological reactivities vary widely.

Saponins extracted from different alfalfa strains were shown (Pedersen et al., 1966) to inhibit weight gains of chicks to different extents, and to affect the growth of *Trichoderma* (Pedersen et al. 1967). Kendall and Leath (1976) demonstrated that reduced palatability of alfalfas containing high levels of saponins was an important factor in reduced performance. When selecting alfalfa varieties for low and high saponin contents, Pedersen and Wong (1971) observed a tendency for higher protein levels to be associated with higher saponin contents, and that yields of high saponin alfalfas tended to be lower than those of low saponin strains. These authors suggested that selection for lower saponin content inadvertently selected for a plant with a lower leaf-stem ratio and a lower protein content in some strains, however, these effects were not uniform for all strains tested.

Cyanogenic Glycosides

Cyanogenic compounds, i.e., those compounds which under appropriate physiological conditions may be metabolized to yield hydrogen cyanide, have been observed in a number of forage species.

In ruminants, HCN can be rapidly detoxified by reactions with sulfur compounds such as cysteine or sulfide to produce thiocyanates which can be excreted (Blakely and Coop, 1949). The amounts of sulfur required for detoxification are in the order of 1.2 gS/g HCN

ingested, and thus if the S content of a forage is marginal, the detoxification requirement may cause a further limitation to growth as a result of S deficiency for production (Wheeler et al., 1975). The provision of an S supplement for animals grazing cyanogenic forages has been suggested (Wheeler et al., 1975).

Numerous other compounds have been observed to have properties which affect the feeding value of certain roughages, however, direct evidence of relationships to dietary protein adequacy is scanty. For further information the reader is referred to Barnes and Gustine (1973).

THE ADEQUACY OF ROUGHAGES AS PROTEIN SOURCES FOR RUMINANTS

The definition of the term "roughage" implies that intake and rate of passage through the digestive tract may be critical in terms of the supply of nutrients available to the ruminant. This in turn suggests that a number of nutrient deficiencies may occur simultaneously if intake is restricted, and that when assessing the protein adequacy of roughages for the ruminant, the relative amounts of different nutrients and the interrelationships among nutrients must be considered carefully.

Voluntary Intake and Rate of Passage of Roughages

The present knowledge suggests that at least two mechanisms are responsible for control of voluntary intake. Conrad (1966) suggested two mechanisms:

1. A mechanism sensitive to the absorption of digestible energy by the animal, i.e., chemostatic theory. This mechanism may be similar to that of the monogastric animal. This mechanism may function primarily under conditions of relatively high quality feed ingestion and was suggested to function above dietary energy digestibilities of 70% by Blaxter et al. (1961).

2. A mechanism sensitive to rumen fill and rate of passage. Intakes of most, if not all, roughages would primarily be controlled by this mechanism.

Voluntary feed intake of ruminants increases with increasing nutrient concentration in the feed (Blaxter, 1950), a phenomenon which is contrary to the behavior of monogastric species (Blaxter et al., 1961). Differences in voluntary intake were related to nutritional quality by Crampton (1957). The studies of Troelsen (1967) conclusively demonstrated that a major component of the voluntary intake of roughages was the rate of passage of roughage through the digestive tract. The rate of passage was related to

the time required to reduce particle size of the ingested material sufficiently to pass the reticulo-omasal orifice (Troelsen, 1967). Since cellulosic material represents the main structural component in forage, the cellulolytic activity of the rumen microbial population is undoubtedly of great importance in reducing particle sizes of roughages. A large proportion of the digestible energy in roughages is derived from cellulose fermentation. The rate of physical breakdown would therefore be expected to be related to the availability of digestible energy in the forage. In addition, the release of other nutrients would depend to a large extent upon the rate of breakdown of cellulose cell walls.

An additional factor in the control of voluntary intake of roughages appears to be the concentration of specific nutrients, particularly N, in the roughages. When crude protein contents of roughages fell below 6-7% an acute depression in feed intake occurred (Milford and Minson, 1964; Troelsen, 1963). Elliot and Topps (1963) related N content of the diet to voluntary intakes of dry matter by sheep and concluded that a slow fermentation rate and consequently slow rate of passage through the digestive tract might account for reduced intakes of low protein roughages. Digestibilities of low protein diets were depressed as a result of inadequate N for normal rumen microbial proliferation (Burroughs et al., 1950). Weston (1971) related intake to the ratio of digested crude protein: digested organic matter. Several studies (Campling et al., 1962; Ammerman et al., 1973) have demonstrated that the inclusion of supplemental N in low protein roughage rations has resulted in significant increases in both voluntary intake and digestibility. Egan (1965) suggested that supplemental N increased intake by increasing utilization of energy, however, in a more recent study (Egan, 1972) the level of rumen distention at which intake was limited was a function of N adequacy also.

The provision of additional N to low-protein forages has been shown (Blaxter et al., 1971; Ventura et al., 1972) to have a greater effect upon intake than upon digestibility, leading to the proposal (Moore and Mott, 1973) that forage N adequacy was closely related to intake.

Nutritive Value of Roughages as Related to Maturity

During early stages of growth, plant material which may normally be considered as roughage may in fact equal the nutrient densities of the so-called "concentrate" feeds. As the plant continues growth toward physiological maturity, however, changes in structural and functional aspects of the growth may alter the adequacy of that plant as a dietary protein source.

Morphological Composition as Related to Nutritive Value

Early investigations showed declines in the ratio of leaf to stem (leaf:stem ratio) as a percentage of the dry weight of the whole plant with advancing maturity for several grasses (Fagan and Jones, 1924; Waite and Sastry, 1949), sainfoin (Fagan and Rees, 1930), and for alfalfa (Woodman and Evans, 1935). Kilcher and Troelsen (1973a) observed a decline in the leaf component of oats from nearly 90% of the plant at the early leaf stage to about 14% at the ripened stage. In a subsequent study a decline in the leaf: stem ratio of brome from 0.7 to 0.4 from May until August was observed (Kilcher and Troelsen, 1973b). Kilcher and Heinrichs (1974) observed a decrease in leaf:stem ratio for alfalfa with advancing maturity.

Leaf material has generally been regarded as more nutritious than that from stems, for a variety of species. For alfalfa, leaf protein content over an 8-week period from a very immature stage declined from 30% to 23% by early bloom, while the protein content of stems decreased from initial levels of 18% to approximately 7% by mid-bloom (Kilcher and Heinrichs, 1974).

A similar pattern of decline was apparent for the protein contents of the leaves and stems of bromegrass (Kilcher and Troelsen, 1973b), of oats (Kilcher and Troelsen, 1973a) and of Russian and Altai wild ryegrasses (Kilcher, 1975).

In all studies the leaf component remained considerably higher in protein content than the stem. Coincident with the decreases in protein content were decreases in digestible energy and phosphorus levels in the forage.

Troelsen (1965) observed increased voluntary intake of alfalfa leaves, and that the leaf fraction was more digestible than were the stems. In later studies (Troelsen, 1967) a variety of roughages were studied at various maturity stages. For alfalfa, a decline of 1% in leaf corresponded to a decline of 0.59 g voluntary intake of Digestible Organic Matter (DOM) per kilogram metabolic body size ($W_{kg}^{0.75}$), statistically significant at the 1% probability level. Declines in voluntary intake of DOM/$W_{kg}^{0.75}$ for each 1% decrease in leaf content were 1.07 g for bromegrass and 0.71 g for crested wheatgrass. Troelsen (1967) concluded that leaf percentage might be useful indicators of nutritional value for forages. MacDonald (1946) and Reid et al. (1959) made similar suggestions, although Mowat et al. (1965) were unable to use leaf content as a reliable indicator of digestibility *in vitro*.

It may be concluded that the nutritive value of a roughage may be affected markedly by the relative proportions of leaves and stems in the roughage, with nutrient contents, digestibilities and intakes

all affected. Available data would suggest that the magnitude of changes in these nutritive parameters differs for the different species.

Chemical Composition as Related to Maturity and Nutritive Value

A number of studies of changes in chemical composition occurring during the seasonal development of forages have been reported. The crude protein contents of several range forage species were found to progressively decrease with advancing maturity by a number of investigators (Gordon and Sampson, 1938; Traps and Fudge, 1940), with plant phosphorus levels also declining. Progressive increases in the crude fibre and nitrogen free extract fractions of the forage were also noted.

Clark and Tisdale (1945) published an exhaustive 13-year study of the chemical composition of range forages in Southern Alberta and Saskatchewan. These workers observed highly significant correlations between crude protein content and phosphorus content over the grazing season (+0.79), and between crude protein and crude fibre a highly significant correlation of -0.73.

The lignin content of forages has been shown to increase with advancing maturity (Pigden, 1953), and many studies have suggested that decreases in digestibility with increasing maturity were primarily a result of lignification. Walker et al. (1976) have shown, however, that the lignin content of alfalfa (10.5%) was twice as high as that of rice straw (5.7%) although the digestibility of rice straw is much lower than that of alfalfa. An additional decrease in digestibility of the rice straw, however, may have been due to the presence of a significant quantity of silica (16.5%), which has been suggested to impair digestibility (Van Soest and Jones, 1968).

Pigden (1953) suggested that factors other than the quantity of lignin present in a roughage were involved in a decrease in digestibility with advancing maturity. Drapala et al. (1947) observed that lignification increased steadily with maturation, but that the process was confined to the vascular bundles during early stages. As the lignification process continues, lignin begins to infiltrate and encrust the cell walls of adjacent tissues and may act as a physical barrier to prevent the utilization of cellulose and soluble nutrients within the cell (Pigden, 1953). Bondi and Meyer (1951) suggested that during the processes of digestion in the ruminant that the lignin molecule might be altered profoundly. In a later study Pigden and Stone (1952) obtained evidence that lignin digestion varied from practically zero in two dicotelydonous species to a significant extent in two monocotelydonous grasses.

While lignin chemistry has been the subject of exhaustive research, the interrelationships of lignin with components of nutritive value in roughages remains largely speculative. The "physical barrier to digestion" concept is undoubtedly involved in the reduction in digestibility usually involved with increasing lignification. Other factors may also be of importance.

INTEGRATION OF COMPONENTS OF ROUGHAGE PROTEIN ADEQUACY

An investigation of the components of nutritive value of Altai wild ryegrass (*Elymus angustus* Trin.) and alfalfa (Knipfel and Heinrichs, 1977) revealed that the organic matter digestibilities of these two species were almost identical (57.8% for Altai wild ryegrass, 56% for alfalfa) when fed to sheep. The coefficient of digestibility for crude protein for alfalfa was 75.3% while that of Altai wild ryegrass was 59.2%, while for crude fibre the digestibility coefficients were 51.5% and 69.8%, respectively. Intakes of digestible organic matter and energy tended to be higher, and of digestible crude protein significantly higher for alfalfa than for Altai wild ryegrass. The data indicated that digestibilities of crude protein and crude fibre may differ considerably from the overall digestibility of the organic matter fraction of the roughage. According to the suggested requirements for digestible protein and energy as a percentage of the diet (NAS-NRC, 1975), both Altai wild ryegrass and alfalfa were slightly deficient in digestible energy for optimal growth of lambs, while the digestible crude protein levels were apparently adequate. Examination of levels of intakes of these two forages revealed that the overall intakes of dry matter, of protein and of energy were considerably lower than suggested requirement levels, thus demonstrating the importance of intake measurements along with chemical analyses, in assessing nutritional adequacy of forages.

Average daily gains of cows and calves grazing swards of Russian wild ryegrass plus alfalfa were significantly correlated with daily consumptions of alfalfa (+0.773*) and alfalfa protein (+0.821**), while significant correlations between animal performance and grass consumption were lacking (Knipfel and Heinrichs, 1975) due to a more uniform intake of Russian wild ryegrass for the entire grazing period from June to August. Decreased alfalfa consumption as the grazing season advanced was related to decreased protein level in the alfalfa, which in turn was related to a decrease in leaf:stem ratio. Russian wild ryegrass, on the other hand, did not change markedly in leaf:stem ratio, although the protein content in July was lower than in either June or August. As a result of decreased alfalfa intake during July and August, the overall intakes of energy, protein, and phosphorus were inadequate and resulted in weight losses of the cows and reduced calf performance. Providing that intakes could have been maintained,

the available forage contained sufficient energy and protein for optimum production according to NRC requirement data (NAS-NRC, 1970). Earlier work (Troelsen, 1967) had suggested that voluntary intake of alfalfa would decrease as the leaf:stem ratio and *in vitro* digestibility of organic matter decreased with advancing maturity, but that in Russian wild ryegrass a less acute change in nutritive quality and morphological composition occurred.

In assessing the nutritive value of crested wheatgrass, Russian wild ryegrass, or Altai wild ryegrass with advancing maturity, Knipfel (1977) observed that the ryegrasses retained higher levels of protein and energy than did crested wheatgrass as maturity advanced (Table 1), but by November none of the species appeared to be nutritionally adequate. When these hays were fed to growing wethers, however, the animals fed ryegrasses were able to gain weight for the entire feeding period, while those fed crested wheatgrass lost weight. These differences in performance came about largely as a result of decreased voluntary intake of crested wheatgrass as nutritive quality fell, while intakes of the ryegrasses were much less affected by decreasing nutritive value. While earlier workers (Troelsen, 1963; Melford and Minson, 1964) had found acute feed intake depressions when roughage protein contents fell below 6-7% protein, the present data (Table 1) suggests that this decrease may be characteristic of a forage species rather than a general observation.

A major difference in morphological composition existed between the ryegrasses and crested wheatgrass, with the ryegrasses containing more than twice as much leaf than did crested wheatgrass during later stages of maturity. The differences in voluntary intakes observed in this study suggest that the use of generalized equations to predict voluntary intake of roughages based on laboratory analyses (Minson and Kemp, 1961; Pigden and Heaney, 1962; Troelsen, 1969) may be of limited accuracy when morphological compositions vary widely.

When evaluating the adequacy of roughage as a protein source for the ruminant the overall consideration must be that the animal consumes enough protein to meet its requirements. This consideration suggests that:

1. The protein level and digestibility are sufficiently high that the animal can meet its requirements at a reasonable level of intake.

2. The voluntary intake will be adequate to allow the animal to meet its requirements.

3. Other factors in the diet will not prevent the animal from utilizing the protein to meet its requirements.

TABLE 1

Nutritive Characteristics of Crested Wheatgrass, Russian Wild Ryegrass, and Altai Wild Ryegrass With Advancing Maturity

	June	July	Aug.	Sept.	Oct.	Nov.
Crested wheatgrass						
% CP	12.9	8.3	4.3	7.2	5.2	4.0
% *in vitro* OMD	62.4	50.5	45.7	44.7	40.8	39.7
% P	.254	.148	.089	.119	.076	.066
Feed intake (g/day)	1312	1298	1008	779	572	
Weight gain[1] (g/day)	| +102.9 |		-37.8 |	-67.8	|	
Russian wild ryegrass						
% CP	11.4	7.6	6.3	9.3	7.0	7.7
% *in vitro* OMD	63.3	53.9	50.0	56.2	52.1	51.2
% P	.235	.178	.100	.162	.101	.086
Feed intake (g/day)	1488	1506	1274	1368	1180	
Weight gain (g/day)	| +102.5 |		+8.4 |	+8.8	|	
Altai wild ryegrass						
% CP	14.9	10.0	8.6	8.3	6.5	4.7
% *in vitro* OMD	65.5	56.4	54.6	54.0	49.0	45.2
% P	.270	.186	.140	.162	.103	.069
Feed intake (g/day)	1465	1298	1137	1192	1099	
Weight gain (g/day)	| +80.5 |		+50.4 |	+1.5	|	

[1]Average for the period within brackets

The protein adequacy of roughages for ruminants depends upon a multiplicity of interacting factors, several of which are well documented, and many which are not well understood. Considerable research effort on both plant and animal aspects of this field and their interactions would appear to be necessary to expand our knowledge of roughage protein interrelationships in relation to ruminant dietary protein adequacy.

ACKNOWLEDGEMENTS

The author wishes to acknowledge the valuable advice of fellow scientists of the Forage Production and Utilization Section,

Research Station, Swift Current, Saskatchewan, and particularly that of Dr. J. D. McElgunn.

REFERENCES

Ammerman, C.B., Fick, K.R. and Verde, G.J. 1973. Influence of supplemental nitrogen and energy on voluntary intake and utilization of low-quality forage by ruminants. Down to Earth 29(2): 8.

Barnes, R.F. and Gustine, D.L. 1973. Allelochemistry and Forage Crops. *In* Anti-quality Components of Forages. Metches ed. Crop Sci. Soc. of America, Madison, Wisc. p 1.

Barnett, A.J.G. and Reid, R.L. 1961. The utilization of nitrogenous compounds in the rumen. *In* Reactions in the rumen. Barnett and Reid eds. Edward Arnold Publishers Ltd., London. p 108.

Bergen, W.G., Purser, D.B. and Cline, J.H. 1967. Enzymatic determination of the protein quality of individual rumen bacteria. J. Nutr. 92(3): 357.

Bergen, W.G., Purser, D.B. and Cline, J.H. 1968. Effect of ration on the nutritive quality of rumen microbial protein. J. Anim. Sci. 27(5): 1497.

Black, A.L., Kleiber, M. and Smith, A.H. 1952. Carbonate and fatty acids as precursors of amino acids in casein. J. Biol. Chem. 197: 365.

Blakely, R.L. and Coop, I.E. 1949. The metabolism and toxicity of cyanides and cyanogenetic glucosides in sheep. II. Detoxication of hydrocyanic acid. N.S. J. Sci. Technol. 31 (3): 1.

Blaxter, K.L. 1950. Energy feeding standards for dairy cattle. Nutr. Abs. Rev. 20: 1.

Blaxter, K.L., Wainman, F.W. and Wilson, R.S. 1961. The regulation of food intake by sheep. An. Prod. 3: 51.

Blaxter, K.L., Wainman, F.W., Dewey, P.J.S., Davidson, J., Denerley, H., and Gunn, J.B. 1971. The effects of nitrogenous fertilizer on the nutritive value of artificially dried grass. J. Agric. Sci. 76: 307.

Boda, J.M., Silver, B.S., Calvin, H.W. (Jr.) and Cole, H.H. 1957. Studies on the experimental production and prevention of bloat in cattle. I. Influence of intraruminal administration of fresh egg white on production of bloat. J. Dairy Sci. 40: 759.

Bondi, A. and Meyer, H. 1951. Constitution of green plant lignins and changes undergone in the ruminant body. Twelfth Int. Cong. Pure App. Chem. New York. p 617.

Broderick, G.A. 1975. Factors affecting ruminant responses to protected amino acids and proteins. *In* Protein Nutritional Quality of Foods and Feeds. M. Friedman, ed. Part 2. p 211.

Bryant, M.P. and Robinson, I.M. 1962. Some nutritional characteristics of predominant culturable ruminal bacteria. J. Bact.

84: 605.
Bunn, C.L., McNeill, J.J. and Matrone, G. 1968. Comparison of amino acid and alfalfa supplementation of purified diets for ruminants. J. Nutr. 94: 47.
Burns, J.C. and Cope, W.A. 1974. Nutritive value of crown vetch forage as influenced by structural constituents and phenolic and tannin compounds. Agron. J. 66: 195.
Burroughs, W., Gall, L.S., Gerlaugh, P. and Bethke, R.M. 1950. The influence of casein upon roughage digestion in cattle with rumen bacteriological studies. J. Anim. Sci. 9: 214.
Burroughs, W., Trenkle, A.H. and Vetter, R.L. 1974. A system of protein evaluation for cattle and sheep involving metabolizable protein (amino acids) and urea fermentation potential of feedstuffs. Vet. Med. Small An. Clin. 69: 713.
Campling, R.C., Freer, M. and Balch, C.C. 1962. Factors affecting the voluntary intake of food by cows. III. The effect of urea on the voluntary intake of oat straw. Br. J. Nutr. 16: 115.
Chalmers, M.I. 1961. Protein synthesis in the rumen. *In* Digestive Physiology and Nutrition of the Ruminant. D. Lewis ed. p 210.
Clark, J.H. 1975. Nitrogen metabolism in ruminants: Protein solubility and rumen bypass of protein and amino acids. *In* Protein Nutritional Quality of Foods and Feeds. M. Friedman, ed. Part 2. p 261.
Clark, S.E. and Tisdale, E.W. 1945. The chemical composition of native forage plants of southern Alberta and Saskatchewan in relation to grazing practises. Agr. Canada Tech. Bull. 54.
Cline, T.R., Garrigus, U.S. and Hatfield, E.E. 1966. Addition of brached- and straight-chain volatile fatty acids to purified lamb diets and effects on utilization of certain dietary components. J. Anim. Sci. 25: 734.
Conrad, H.R. 1966. Symposium on factors affecting the voluntary intake of herbage by ruminants: Physiological and physical factors limiting feed intake. J. Anim. Sci. 25: 227.
Crampton, E.W. 1957. Interrelations between digestible nutrients and energy content, voluntary dry matter intake, and the overall feeding value of forages. J. Anim. Sci. 3: 546.
Crampton, E.W. and Lloyd, L.E. 1959. Fundamentals of Nutrition. Freeman Publishers, San Francisco. p 120.
Daiber, K.H. 1975. Enzyme inhibition by polyphenols of sorghum grain and malt. J. Sci. Fd. Agric. 26: 1399.
Donnelly, E.D. and Anthony, W.B. 1969. Relationship of tannin, dry matter digestibility and crude protein in *sericea lespedeza*. Crop Sci. 9: 361.
Downes, A.M. 1961. The amino acids essential for the tissues of the sheep. Aust. J. Biol. Sci. 14: 254.
Downes, A.M., Reis, P.J., Sharry, L.F. and Tunks, D.A. 1970. Metabolic fate of parenterally administered sulfur-containing amino acids in sheep and effects on growth and composition of wool. Aust. J. Biol. Sci. 23: 1077.
Drapala, W.J., Raymond, L.G. and Crampton, E.W. 1947. Pasture

studies. XXVII. The effects of maturity of the plant and its lignification and subsequent digestibility by animals as indicated by methods of plant histology. Sci. Agr. 27: 36.

Egan, A.R. 1965. Nutritional status and intake regulation in sheep. III. The relationship between improvement of nitrogen status and increase in voluntary intake of low protein roughages by sheep. Aust. J. Agr. Res. 16: 463.

Egan, A.R. 1972. Nutritional status and intake regulation in sheep. VII. Control of voluntary intake of three diets and the responses to intraruminal feeding. Aust. J. Agr. Res. 23: 347.

Elliot, R.C. and Topps, J.H. 1963. Voluntary intake of low protein diets by sheep. An. Prod. 5: 269.

Fagan, T.W. and Rees, J. 1930. The effect of cutting sainfoin at different stages on the yield and chemical composition of hay and aftermath. Welsh J. Agric. VI: 231.

Fagan, T.W. and Jones, H.T. 1924. The nutritive value of grasses as shown by their chemical composition. Welsh Plant Breeding Station Bulletins, Series II, 1-7: 85.

Fenderson, C.L. and Bergen, W.G. 1975. An assessment of essential amino acid requirements of growing steers. J. Anim. Sci. 41(6): 1759.

Gordon, A. and Sampson, A.W. 1939. Composition of common California foothill plants as a factor in range management. Calf. Agr. Exp. Sta. Bull. 627.

Gutek, L.H., Goplen, B.P., Howarth, R.E. and McArthur, J.M. 1974. Variation of soluble proteins in alfalfa, sainfoin, and birdsfoot trefoil. Crop Sci. 14: 495.

Hatfield, E.E. 1970. Selected topics related to the amino acid nutrition of the growing ruminant. Fed. Proc. 29(1): 44.

Heftmann, E. 1965. Sapogenins. *In* Plant Biochemistry. Bonner and Varner eds. Academic Press, New York. p 700.

Hutton, K. and Annison, E.F. 1972. Control of nitrogen metabolism in the ruminant. Proc. Nutr. Soc. 31: 151.

Jones, W.T. and Lyttleton, J.W. 1971. Bloat in cattle. XXXIV. A survey of legume forages that do and do not produce bloat. N.Z. J. Agric. Res. 14: 101.

Kendall, W.A. and Leath, K.T. 1976. Effects of saponins on palatability of alfalfa to meadow voles. Agron. J. 68: 473.

Kilcher, M.R. 1975. Contribution of leaves and stems to yield of Russian wild ryegrass and Altai wild ryegrass. Can. J. Plant Sci. 55: 1029.

Kilcher, M.R. and Heinrichs, D.H. 1974. Contribution of stems and leaves to the yield and nutrient level of irrigated alfalfa at different stages of development. Can. J. Plant Sci. 54: 739.

Kilcher, M.R. and Troelsen, J.E. 1973a. Contribution and nutritive value of the major plant components of oats through progressive stages of development. Can. J. Plant Sci. 53: 251.

Kilcher, M.R. and Troelsen, J.E. 1973b. Contribution of stems and

leaves to the composition and nutrient content of irrigated bromegrass at different stages of development. Can. J. Plant Sci. 53: 767.

Knipfel, J.E. 1977. Unpublished data.

Knipfel, J.E. and Heinrichs, D.H. 1975. Nutritive status determination of the grazing cow-calf unit. Can. J. Anim. Sci. 55(4): 796.

Knipfel, J.E. and Heinrichs, D.H. 1977. Altai wild ryegrass (*Elymus angustus*) and alfalfa as ration components for lambs. Can. J. Plant Sci. 57: in press.

Lewis, D. 1961a. Digestive physiology and nutrition of the ruminant. *In* Digestive Physiology and Nutrition of the Ruminant. Lewis ed. Butterworths, London. p VII.

Lewis, D. 1961b. Fate of nitrogenous compounds inthe rumen. *In* Digestive Physiology and Nutrition of the Ruminant. Lewis ed. Butterworths, London. p 127.

MacDonald, H.A. 1946. Factors affecting the nutritional value of forage plants. Agr. Eng. 27: 117.

Mason, V.C. 1963. Amino acids in nutrition. Lic. Agron. Thesis, Royal Veterinary and Agricultural College, Copenhagen, Denmark. p 33.

McCarthy, R.D., Pattan, R.A. and Griel, L.D. (Jr.). 1970. Amino acid nutrition of lactating ruminants. Fed. Proc. 29: 41.

McGinty, D.D. 1968. Factors affecting the digestibility of varieties of sorghum grain by cattle. Ph.D. Thesis, Texas A & M University, College Station, Texas.

McNaught, M., Owen, E.C., Henry, K.M. and Kon, S.K. 1954. The utilization of non-protein nitrogen in the bovine rumen. 8. The nutritive value of the proteins of preparations of dried rumen bacteria, rumen protozoa and brewers yeast for rats. Biochem. J. 56: 151.

Milford, R. and Minson, D.J. 1964. Intake of tropical pasture species. Proc. 9th Int. Grassl. Cong. #236.

Minson, D.J. and Kemp, C.D. 1961. Studies in the digestibility of herbage. IX. Herbage and fecal nitrogen as indicators of herbage organic matter digestibility. J. Gr. Grassl. Soc. 16: 76.

Moore, J.E. and Mott, G.O. 1972. Structural inhibitors of quality in tropical grasses. *In* Anti-Quality Components of Forages. Matches ed. Crop Sci. Soc. of America, Madison, Wisc.

Mowatt, D.N. Sulkerson, R.S., Tossel, W.E. and Winch, J.E. 1965. The *in vitro* digestibility and protein content of leaf and stem portions of forages. Can. J. Plant Sci. 45: 321.

National Academy of Sciences - National Research Council. 1970. Nutrient Requirements of Beef Cattle, Washington, D.C.

National Academy of Sciences - National Research Council. 1975. Nutrient Requirements of Sheep. Washington, D.C.

Pearson, R.M. and Smith, J.A.B. 1943. The utilization of urea in the bovine rumen. 3. The synthesis and breakdown of protein in the rumen ingesta. Biochem. 37: 153.

Pedersen, M.W., Anderson, J.O. and McGuire, C.F. 1966. A comparison of saponins from DuPuits, Lahontan, Langer and Uinta alfalfa. Proc. 10th Intern. Grassl. Congress. Helsinki. p 266.

Pedersen, M.W. and Wang, L.C. 1971. Modification of saponin content of alfalfa through selection. Crop. Sci. 11: 833.

Pedersen, M.W., Zimmer, D.E., McAllister, D.R., Anderson, J.D, Welding, M.D., Taylor, G.A. and McGuire, C.F. 1967. Comparative studies of saponin of several alfalfa varieties using chemical and biochemical assays. Crop Sci. 7: 349.

Phillipson, A.T. 1964. The digestion and absorption of nitrogenous compounds in the ruminant. *In* Mammalian Protein Metabolism. Munro and Allison eds. Vol. 1 p 72.

Pigden, W.J. 1953. The relation of lignin, cellulose, protein, starch and ether extract to the "Curing" of range grasses. Can. J. Agric. Sci. 33: 364.

Pigden, W.J. 1955. A microbiological method for evaluating forage quality. Ph.D. Thesis, Univ. of Sask., Saskatoon, Sask.

Pigden, W.J. and Heaney, D.P. 1962. Relationship between chemical composition and digestibility of forages. Proc. Can. Soc. An. Prod. 73.

Pidgen, W.J. and Stone, J.E. 1952. The effect of ruminant digestion on forage lignin.

Preston, R.L. 1970. Amino acids in ruminant nutrition. Fed. Proc. 29(1): 33.

Purser, D.B. and Buechler, S.M. 1966. Amino acid composition of rumen organisms. J. Dairy Sci. 49: 81.

Rein, J.T., Kennedy, W.K., Turk, K.L., Slack, S.T., Trimberger, G.W. and Murphy, R.P. 1959. Our industry today. Effect of growth stage, chemical composition, and physical properties upon the nutritive value of forages. J. Dairy Sci. 42: 576.

Reis, P.J. 1967. The growth and composition of wool. IV. The differential response of growth and sulfur content of wool to the level of sulfur containing amino acids given per abomasum. Aust. J. Biol. Sci. 20: 809.

Reis, P.J. and Schinckel, P.G. 1963. Some effects of sulfur-containing amino acids on the growth and composition of wool. Aust. J. Biol. Sci. 16: 218.

Sarkar, S.K. Howarth, R.E. and Goplen, B.P. 1976. Condensed tannins in herbaceous legumes. Crop Sci. 16: 543.

Schaffert, R.E., Lechtenberg, V.L., Oswalt, D.L., Axtell, J.D., Pickett, R.C. and Rhykerd, C.L. 1974. Effect of tannin on *in vitro* dry matter and protein disappearance in sorghum grain. Crop Sci. 14: 640.

Schelling, G.T., Chandler, J.E. and Scott, C.C. 1973. Post ruminal supplemental methionine infusion to sheep fed high quality diets. J. Anim. Sci. 37: 1034.

Schelling, G.T. and Hatfield, E.E. 1968. Effect of abomasally infused nitrogen sources on nitrogen retention of growing lambs. J. Nutr. 96: 319.

Stallcup, O.T. and Davis, G.C. 1962. Nutritive value of AKS 614 grain sorghum. Ark. Farm Res. 11(4): 5.

Stanley, E.B. and Hodgson, C.W. 1938. Seasonal changes in the chemical composition of some Arizona range grasses. Ariz. Agr. Exp. Sta. Tech. Bull. 73.

Swain, T. and Bate-Smith, E.C. 1962. Flavonoid compounds. *In* Comparative Biochemistry. Vol. 3. Florkin and Mason eds. Academic Press, New York. p 755.

Synge, R.L.M. 1968. Occurrence in plants of amino acid residues chemically bound otherwise than in proteins. Ann. Rev. Plant Physiol. 19: 113.

Troelsen, J.E. 1963. The effect of pelleting upon intake and digestibility of low quality native hay. Proc. Can. Soc. Anim. Prod. Banff, Alta.

Troelsen, J.E. 1965. Digestibility of the separate leaf and stem fractions of alfalfa hay. Forage Notes, Agr. Canada 11: 51.

Troelsen, J.E. 1967. Systems for estimating the nutritional quality of hay for domestic ruminants. Ph.D. Thesis, Univ. of Sask., Saskatoon, Sask.

Troelsen, J.E. 1969. Outline of procedure for *in vitro* digestion of forage samples. Research Station, Swift Current, Sask.

Traps, G.W. and Fudge, J.F. 1940. The chemical composition of forage grasses of the east Texas timber country. Texas Agr. Exp. Sta. Bull. 582.

Van Soest, P.J. and Jones, L.H.P. 1968. Effect of silica in forages upon digestibility. J. Dairy Sci. 51: 1644.

Ventura, M., Moore, J.E., Franke, D.E. and Ruelke, O.C. 1972. Nutritive evaluation of pangola digitgrass hays. J. Anim. Sci. 35: 234.

Waite, R. and Sastry, K.N.S. 1949. The composition of timothy (*Phleum pratense*) and some other grasses during seasonal growth. Emp. J. Exp. Agric. XVII: 179.

Wakeling, A.E., Annison, E.F. and Lewis, D. 1970. The amino acid requirements of ruminants. Proc. Nutr. Soc. 29: 60.

Walker, H.G. (Jr.), Kohler, G.O., Graham, R.P., Hart, M.R. and Garrett, W.N. 1975. Upgrading cereal straws. *In* Proceedings of the 9th National Conference on Wheat Utilization Research. p 108.

Weller, R.A. 1957. The amino acid composition of hydrolyzates of microbial preparations from the rumen of sheep. Aust. J. Biol. Sci. 10: 384.

Weston, R.H. 1971. Factors limiting the intake of feed by sheep. V. Feed intake and productive performance of the ruminant lamb in relation to the quantity of crude protein digested in the intestines. Aust. J. Agr. Res. 22: 307.

Wheeler, J.L., Hedges, D.A. and Till, A.R. 1975. A possible effect of cyanogenic glucoside in sorghum on animal requirements for sulfur. J. Agric. Sci. Camb. 84: 377.

Woodman, H.E. and Evans, R.E. 1935. Nutritive value of leucerne. IV. The leaf stem ratio. J. Agric. Sci. 25: 578.

Wright, P.L. 1971. Body weight gain and wool growth response to formaldehyde treated casein and sulfur amino acids. J. Anim. Sci. 33: 127-141.

33

SOME THERAPEUTIC IMPLICATIONS OF THE CROSSLINKAGE THEORY OF AGING

Johan Bjorksten

Bjorksten Research Foundation

Madison, Wisconsin 53715

INTRODUCTION

The concept of crosslinkages as the base for aging was suggested by Bjorksten (1941), who in 1942 expressed this theory as follows: "The aging of living organisms I believe is due to the occasional formation by tanning, of bridges between protein molecules, which cannot be broken by the cell enzymes. Such irreparable tanning may be caused by tanning agents foreign to the organism or formed by unusual biological side reactions, or it may be due to the formation of a tanning bridge in some particular position in the protein molecule. In either event, the result is that cumulative tanning of body proteins which we know as old age." If we add the words "and nucleic acids" to "proteins" this still covers the tenets of the crosslinking theory.

Bjorksten (1951) summarized the already then substantial literature on crosslinkages, predicting that their significance in processes of life--and death--would soon become appreciated.

In 1948 Haddow, Kon and Ross noted that substances having more than one alkylating group in the molecule--thus capable of forming crosslinkages--have a dramatically greater cytotoxic action than those having only one such group. Goldacre, Loveless and Ross (1949) proposed that the cytotoxic activity was due to their ability to crosslink. In 1955-1956 F. Verzár presented the same theory independently, in the context of collagen only, and D. Harman (1956) stressed the significance of free radicals, which Charlesby (1953) had proved to be powerful crosslinking agents. In 1957 Zinsser et al. showed the correlation between aging and the build-up of polyvalent metals as potential crosslinkers in aging aorta. Goldacre

et al. (1949) showed that chromosome abnormalities caused by alkylating agents are due to formation of covalent crosslinkages, and Alexander and Lett (1960) showed that crosslinking of DNA occurs in contact with bifunctional nitrogen mustards, an observation later extended to include a majority of carcinogenic compounds. That such reactions would be caused by all crosslinking agents (referred to in the technical and the older literature as "tanning agents") on the genetic matter is obvious in view of the presence in the latter of numerous reactive groups. The alkylating agents were conspicuous biologically only because of their ability to penetrate cell walls. On the other hand, for example, an aldehyde or peroxide formed by a reaction within the cell could be fully as effective as a crosslinker covalently changing DNA or RNA molecules (Bjorksten, 1963, p. 181; Bjorksten & Andrews, 1964, pp. 630-631).

The status of these developments was summarized by Bjorksten in 1962, and again in 1968 and 1971, with fairly comprehensive bibliographies. In 1965 Carpenter published his diffusion theory, based on crosslinkage concepts. It was apparent that the effect of crosslinking coincides with changes taking place in aging, and that no other single reaction known can on a qualitative basis equal its potential destructiveness to macromolecular systems.

More recently Piez, LaBella, Gross, Schmitt, Bensusan, Veis, Gallup, von Hahn and many others have added to our knowledge of the precise nature of the crosslinkages that occur, particularly in collagen. Since all proteins have basically related structures and similar reactive groups, their work has a significance extending much more broadly than to collagen specifically.

The significance of crosslinkage in proteins is its generality and ubiquity. It has long been known that extensively crosslinked proteins become resistant to enzymes (Thomas and Seymour-Jones, 1934; Gustavson, 1942; Lipsitz et al., 1949; Bjorksten and Gottlieb, 1954; Kohn and Rollerson, 1960). Extensively crosslinked protein cannot be broken down biologically. Some protein bound tritium administered at birth was found in rats 809 days after administration (Bjorksten and Ashman, 1970; Bjorksten et al., 1971). One mechanism which would prevent the breakdown by any possible enzyme would be the formation of aggregates of such density that steric hindrance prevents access of any large molecule. Crosslinking is the most obvious and prevalent cause of such steric hindrance.

The significance of crosslinkage in genetic substances as related to aging has been obvious since the work of Haddow, Goldacre and Alexander. That it is a large factor is conclusively proven by Hart and Setlow (1974) who have shown a clear correlation between the specific life span of a species, and its ability to rapidly repair induced damage to genetic molecules. It remains a moot

question which is more detrimental: whether it is the DNA crosslinkage or the more generalized protein-to-any-reactive-large-molecule crosslinkage, as discussed in 1964 (Bjorksten). For every DNA molecule there is a myriad of protein molecules. The DNA molecules are irreplaceable--but if enough of the other molecules accumulate in insoluble aggregates they will preempt space and disrupt transportation so that the damage may be as serious. In both cases, the bottleneck for possible repairs is the steric accessibility for repair of the aggregates. No repair can be made if access of the repair enzymes to the damage site is sterically barred. And the aggregates formed in old animals, which perinatally received radiotracer contain not only peptides, but also nucleotides, pointing to such steric hindrance by repetitive random crosslinkage as the identical limiting factor for repair and scavenging of both genetic and nongenetic immobilized material (Bjorksten et al., 1971).

The repair of damaged genetic molecules, particularly DNA, studied by Howard-Flanders and Boyce (1966), has received a great deal of attention in recent years. Hart et al. (1976) cites 87 references relevant to this work. The establishment by Hart and Setlow (1974) that a good correlation exists between the speed of DNA repair and the life span of various species of mammals confirms strongly the significance of DNA repair speed as an important factor in aging. The postulated repair mechanisms are enzymic. However, any repair of DNA is impossible when the access of repair enzymes is prevented by extensive crosslinking. The aggregate must first be broken down to the extent necessary to permit access of repair enzymes to the DNA. In this manner, and to this extent, crosslinkage is and remains a key mechanism to aging (Bjorksten, 1955), regardless of which factor in the end proves to dominate: the scarcer but more damaging crosslinkage of genetic molecules, or the individually less damaging, but much more frequent, crosslinkage of nongenetic molecules.

COULD VITALITY BE PRESERVED IN A MAJOR WAY?

The lack of progress in countering the destructive impact of the years, which now steadily reduces our ability to maintain health, is apparent from Fig. 1.

In other fields breakthroughs have resulted when enough well directed massive effort was applied.

It should be possible to bend the curve in Fig. 1 into a breakthrough similar to those shown in Fig. 2. It seems a stain on the administration of our science that life expectancy at 60 is still within 2 years of where it was 184 years ago. Where should

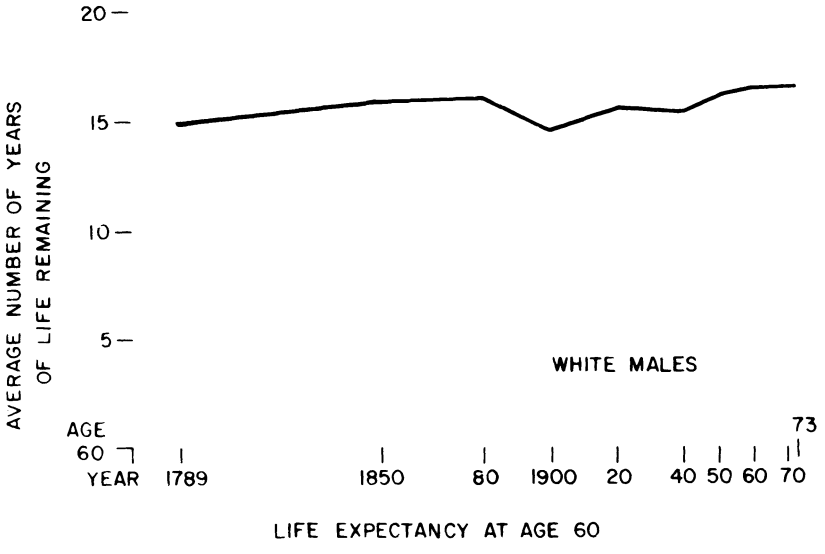


Fig. 1. The life expectancy at age 60 for 1789-1973. (From data of Historical Statistics of the Metropolitan Life Insurance Company, and from Vital Statistics of the United States, 1973).

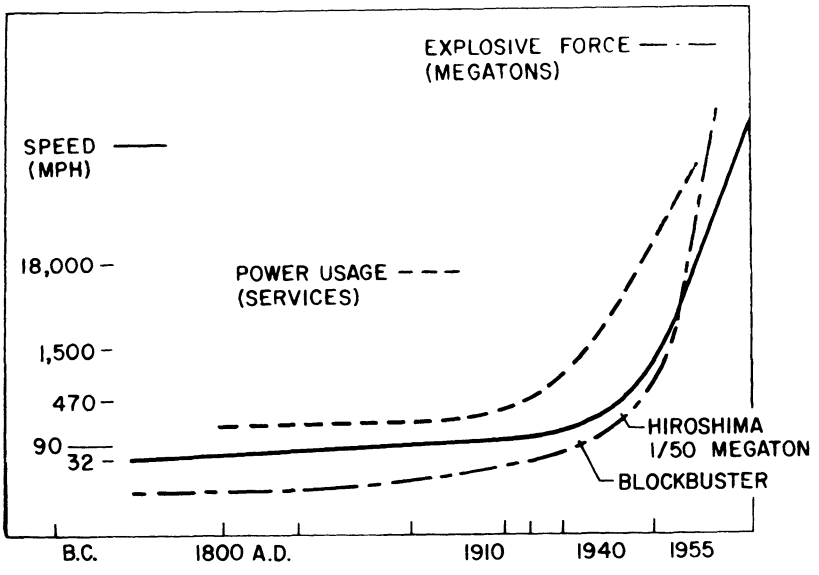


Fig. 2. Other important problems, properly researched and supported, have decisively broken away from past inhibitions. Why not aging? After Powell, J. L., The Chemist 35, 351 (1958).

our efforts be centered? What guidelines do we have? First of all, we need full recognition of the primary cause of aging so that our efforts can be directed by logic as contrasted with convenience or mere chance!

RANDOM CROSSLINKAGE A PRIMARY CAUSE OF AGING

Many theories have been advanced to explain the loss of strength and vitality (Bjorksten, 1969). Of these, only the crosslinkage theory is primary and has answered all objections and met all criteria (Bjorksten, 1971, pp. 33-36). The crosslinkage theory has now withstood the test of time for 35 years; it has been rediscovered independently at least 4 times; there has been enough testing and evaluating. Since it is basic, I shall again briefly restate the way random crosslinking can initiate aging on a molecular scale. The following illustration (Figures 3, 4, 5, 10) and explanations are reproduced with permission from my article in THEORETICAL ASPECTS OF AGING (ed. by M. Rockstein, Academic Press, Inc., 1974).

Figs. 3, 4 and 5 show schematically what crosslinkages between strands can do to DNA (Bjorksten, 1974).

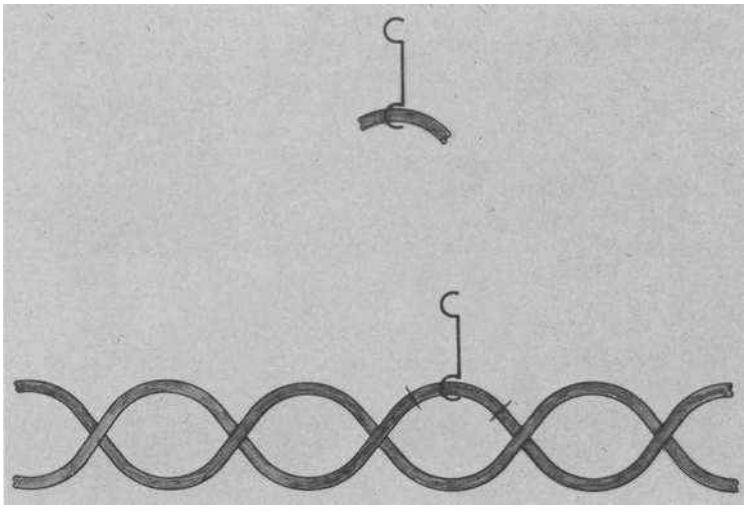


Fig. 3. A crosslinking agent attaches itself at one point of a DNA molecule, involving one strand only. Right, the agent has been excised by defense mechanisms together with a piece of the DNA affected. The damage is then repaired, the unaffected strand being the template.

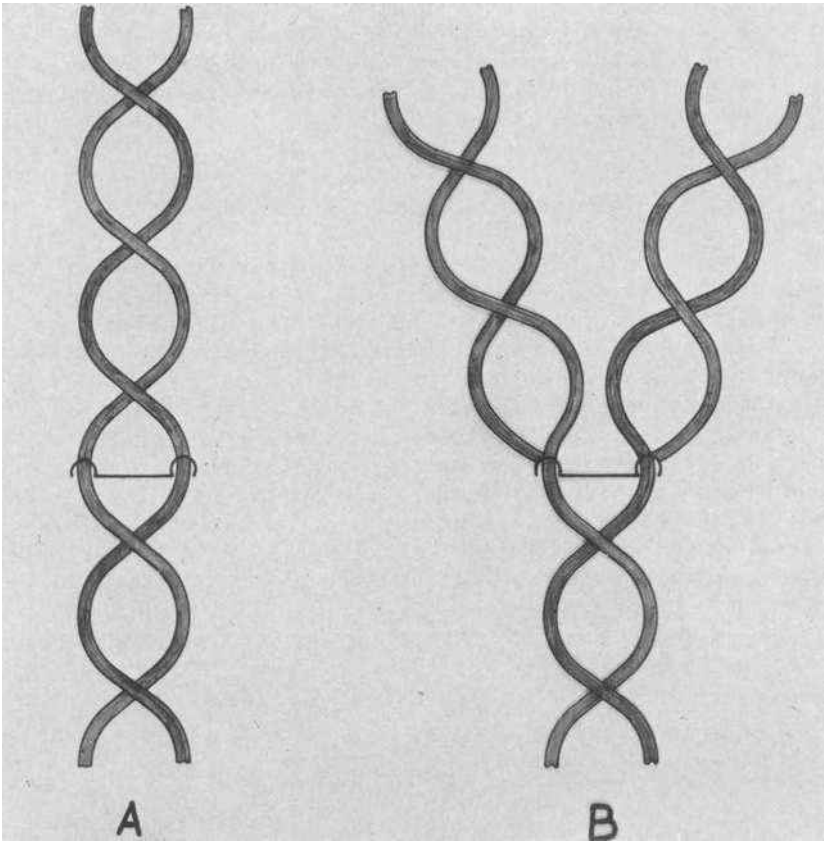


Fig. 4. In A, the crosslinking agent has become attached to the second strand of DNA before the defense mechanism could excise it. When this has happened, the cell is doomed. If the crosslinker is excised, there will be no template for repair as both strands are involved at the same point. If the crosslinker remains as shown in B, it will block the normal parting of strands in mitosis at a stage where the resultant DNA can neither return to normal nor complete the division.

More complex, but nonetheless often fatal damage is done when the crosslinker already attached to one strand of DNA reacts with another different macromolecule, such as a histone (von Hahn, 1964), an RNA, an enzyme, peptide or any other large molecule which has reactive groups (Bjorksten et al., 1971; Acharya et al., 1972).

In spite of the key role which DNA plays in biology, and the proven sensitivity of DNA to crosslinkage, damage to DNA may not be the most important factor in aging. Virtually every large molecule which has more than a purely structural function is susceptible to crosslinkage with any other similarly susceptible molecule. In a lifetime billions of crosslinkages will thus unavoidably be formed. Most of these can be reversed, but some of them cannot. These latter will accumulate over the years. The resultant aggregates are composed of proteins, nucleotides, polymeric fats, polysacharides, any available large molecule at all which can react with any crosslinking agent at all, or which can be directly interlocked, will form parts in the resultant aggregates, as illustrated in Fig. 5.

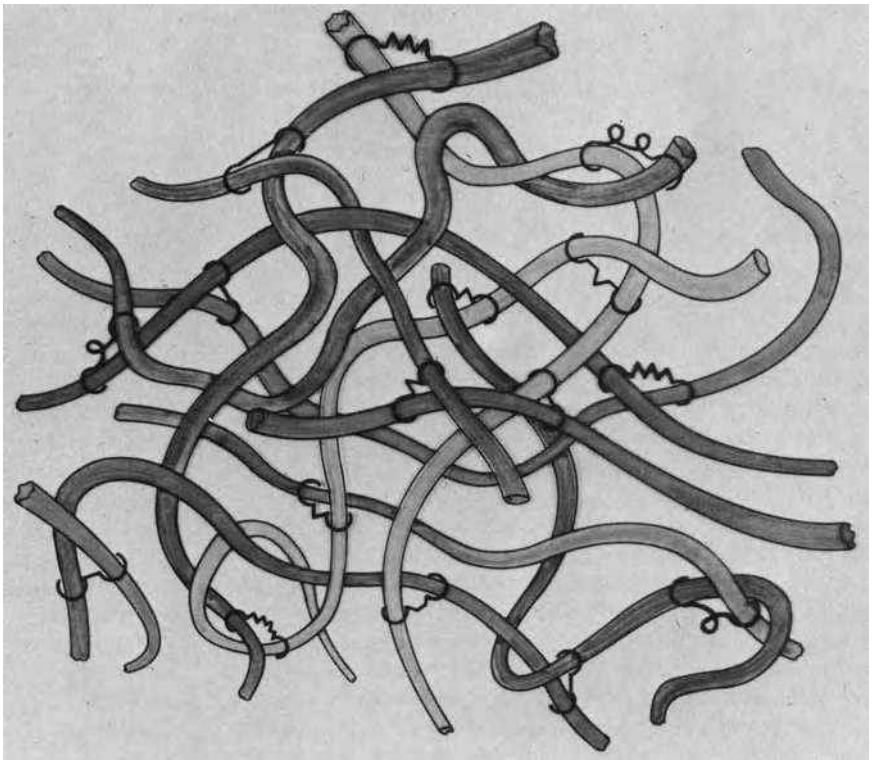


Fig. 5. Random, accidental, mostly nonenzymatic crosslinkages accumulate over a lifetime to form dense aggregates of any available large molecules with any crosslinking agent.

Such essentially nongenetic agglomerates will be whittled away from the surface by proteolytic and other catabolic enzymes, but when the crosslinkages have reached a certain density, the aggregates will become sterically inaccessible to the enzymes available to human metabolism. Because of their much greater quantity, these aggregates may play as large a part in the aging syndrome as does crosslinking of DNA. Tanzer (1973) suggests that crosslinking may prove the "Rosetta Stone of Aging." I could not agree more, having expressed the same conclusion 18 years earlier (Bjorksten, 1955).

The principal group of enzymes which is preferentially increased with age, is that of enzymes which have preponderantly catabolic function, in other words, those most adapted to break down these aggregates (Bjorksten, 1966). It would be very interesting to know if the increase of the activity of these enzymes with age correlates with the life-span in various species in the same way as the speed of repair of DNA studied by Hart and Setlow (1974).

CROSSLINKAGES AS A COMMON BASE FOR DEGENERATIVE DISEASE SYNDROMES

A few major instances will be briefly discussed.

Athero-Arteriosclerosis

It has been fully established (Fishman et al., 1975; Freidman et al., 1975; Constantinides and Wiggers, 1973) that the endothelium becomes permeable to blood serum before infiltration or deposits take place. It has also been established that elasticity of the arteries diminishes with progressive years. Loss of elasticity and embrittlement are well known primary effects of crosslinkage (Bjorksten, 1951, pp. 343, 349).

When loss of elasticity has progressed so far that the endothelium no longer can follow the pulsations, it may rupture at the point of highest hydrostatic pressure, usually at a bend or branching point of the artery, or the media hardens to the point that the intima no longer can stand the squeeze repeated every second between noncompressible liquid and hardening media, or the continuity of cellular adhesion in the endothelium is damaged and gives rise to leaks. Whichever takes place first, the effect is the same: penetration of the endothelium and subintimal deposition of suspended substance. If this is easily resorbable, the lesion may heal, but if it consists of difficult-to-remove particles, for example, of cholesterol or triglycerides, it cannot be resorbed. Such a sequence is shown in Figs. 6, 7, 8, which are copied with permission from a recent article (Bjorksten, 1976).

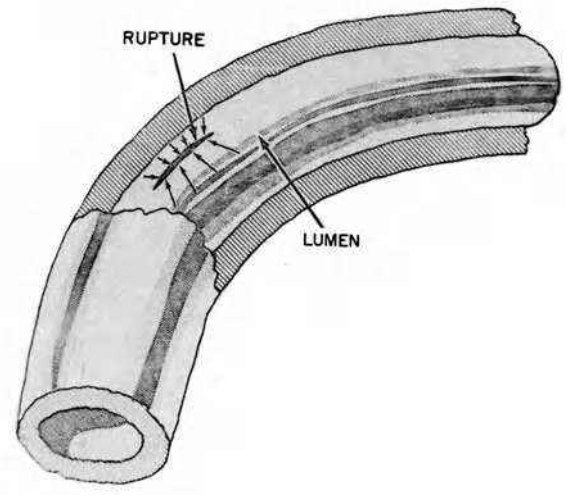


Fig. 6. A rupture has formed in the intima; blood serum filters through and deposits particulate matter in the subintimal region.

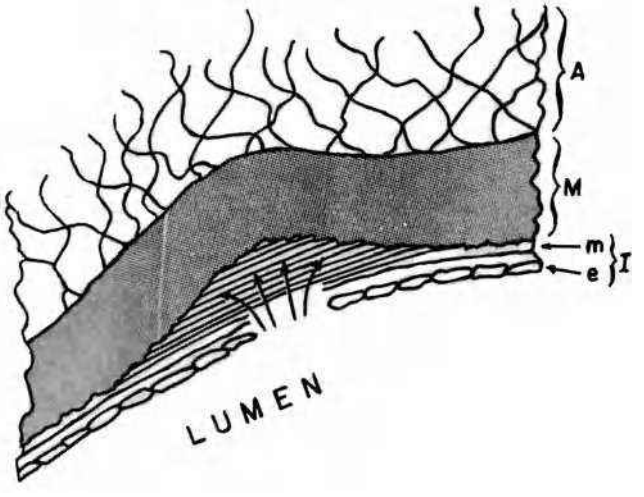


Fig. 7. Diagram of the rupture in section. A = adventitia, M = media, m = membrane, e = endothelium, i = intima

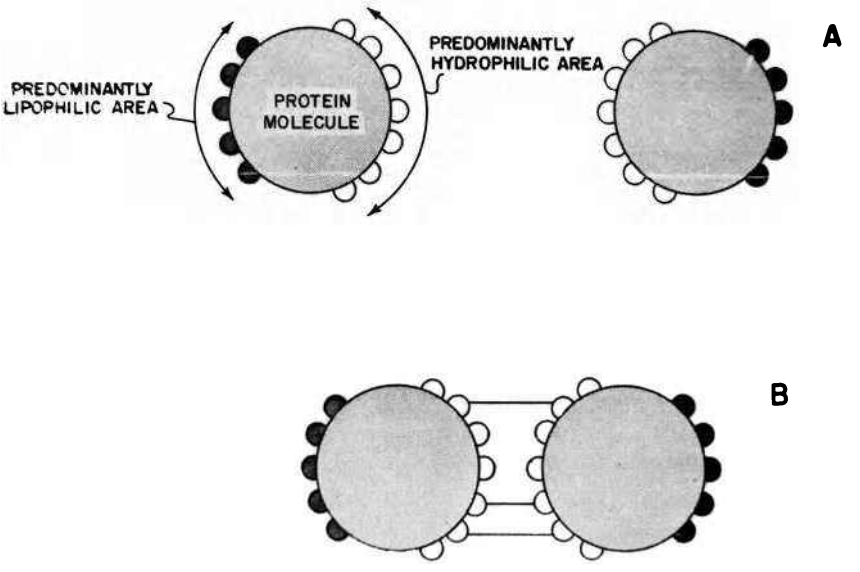


Fig. 8. A. Two protein molecules having both lipophilic (dark) and hydrophilic (white) sites. B. The same molecules cross-linked preferentially at the hydrophilic sites, so as to turn more of the lipophilic sites outward on the average, thus becoming lipophilic.

The affinity to tissue proteins for lipids increases with progressive crosslinkage, for reasons shown in Fig. 8.

On the average, the hydrophilic areas of a macromolecule are more susceptible to crosslinkage than the lipophilic areas, because more crosslinking agents are water soluble, and also because many of the reactions occur more easily in the aqueous phase. Since a preponderance of crosslinkages in both participating macromolecules will be apt to involve their more hydrophilic areas, these will tend to become oriented towards each other, leaving the preponderantly lipophilic areas preferentially exposed. The result is the increasing lipophilicity with progressive crosslinkage phenomena well known and utilized both in the leather manufacture, and in graphic arts to prepare preferentially oil ink receptive printing plates.

This increased lipophilicity of the proteins generally will favor fat deposit on the soft tissues with progressing age.

It may also affect the immunological recognition of the molecules, and could thus well be a factor in autoimmunity (Raff, 1976).

These last mentioned effects of crosslinking may explain the frequent occurrence above age 70 of extensive arterio-atherosclerosis where lipidemia is conspicuously absent. Blumenthal (1975) states in a penetrating article: "There are many curves representing functional declines with age, or changes in the incidence of disease states, which show a middle age peak followed by a decline, and for many of these there is no evident causal link with lipid metabolism; some cancers represent a case in point. All may have a common fundamental underlying cause, not yet elucidated. In any event, these observations raise doubt as to how essential hyperlipidemia is in the genesis of arterio-atherosclerosis, when so many individuals with advanced arterio-atherosclerosis after age 70 do not manifest this biochemical abnormality." Goldstein et al. (1973) found in a study of 500 survivors of myocardial infarct that only 25% of the women and none of the men above 70 showed elevated lipid levels.

The increase in lipophilicity on aging, may explain this. It can be demonstrated easily and even simulated in vitro in gelatin treated with crosslinking agents. Cholesterol adsorption has been induced in vivo by uranium salts, also effective crosslinking agents (Campbell and Longebaugh, 1950). Protein rendered lipophilic by blockage of a sufficient part of its hydrophilic sites, as illustrated in Fig. 8, will absorb lipids even when these are present in low concentrations. Cholesterol and triglycerides will then remain fixed more tenaciously than lipids of a lower melting point or having additional reactive sites.

A crosslinking agent, attached with one end to any surface is capable of capturing with the other end a lipoprotein molecule where the lipid is cholesterol, regardless of the nature of the surface to which it is attached. If the crosslinking agent is strongly ionic, it is even capable of capturing cholesterol connected with nonproteinic colloidal carriers (Bjorksten, 1952); Bjorksten and Gottlieb, 1954). Cholesterol and probably also triglycerides so captured will then remain fixed more tenaciously than lipids of a lower melting point or having additional reactive sites.

Other Clinical Manifestations of Crosslinking

1. Senile Cataract.

In a recent paper, Bellows and Bellows (1976) conclude that crosslinking is the common denominator in past theories of

senile cataracts. Due to its transparency, the lens is an ideal organ for the systematic observation of aging phenomena, and the hardening process can be analyzed in convincing detail. This has been done, and the conclusions stated.

2. Diabetic Sclerosis.

Diabetes is characterized by the inability of the organism to process carbohydrates in the normal pathways. Instead, alternate, and less efficient pathways are taken, with the result that incomplete oxidation products are favored. Some of these are known crosslinking agents.

The shifting of pathways for energy generation which takes place in diabetes is schematically shown in Fig. 9.

Basically, glucose is the optimal biological energy source, because it is the only carbohydrate which can be utilized directly by all tissues. The brain cannot utilize fatty acids or fats under any circumstances, but it can as a poor second choice to glucose, utilize ketone bodies such as pyruvic acid or gamma hydroxy butyric acid.

The muscles can utilize fats or fatty acids for energy production. Therefore, when the glucose available is short of abundance, the brain gets first call on the glucose. This is the evolutionary reason why fats cause an inhibition of insulin function and/or formation. It is the means for forcing the muscles into a fat combustion metabolism in order to secure that the brain gets all of the scarce glucose (Miettinen, 1969). At the same time, protein-amino acid catabolism generates pyruvic acid, which in turn goes to fatty acid to increase the supply of this for the muscles, and in the process also steps up the cholesterol production. This is the reason why cholesterol and fatty acids in the blood increase in diabetes.

It is thus apparent that in diabetes not only the formation of very reactive pyruvic acid is stimulated. Other keto-bodies are formed, not all of which have been defined. Fatty acids and cholesterol have a well known positive correlation to atherosclerosis. The oxidation products of the fats comprise both aldehydes and peroxides, of which many are particularly active crosslinkers (Gustavson, 1956; Bjorksten, 1963; Milch, 1964).

The diabetic metabolism is thus particularly unfavorable from the standpoint of the crosslinking theory of aging. It appears desirable to avoid inhibiting insulin function with diets containing substantial and avoidable quantities of any fats. Several investigations have substantiated the practical soundness

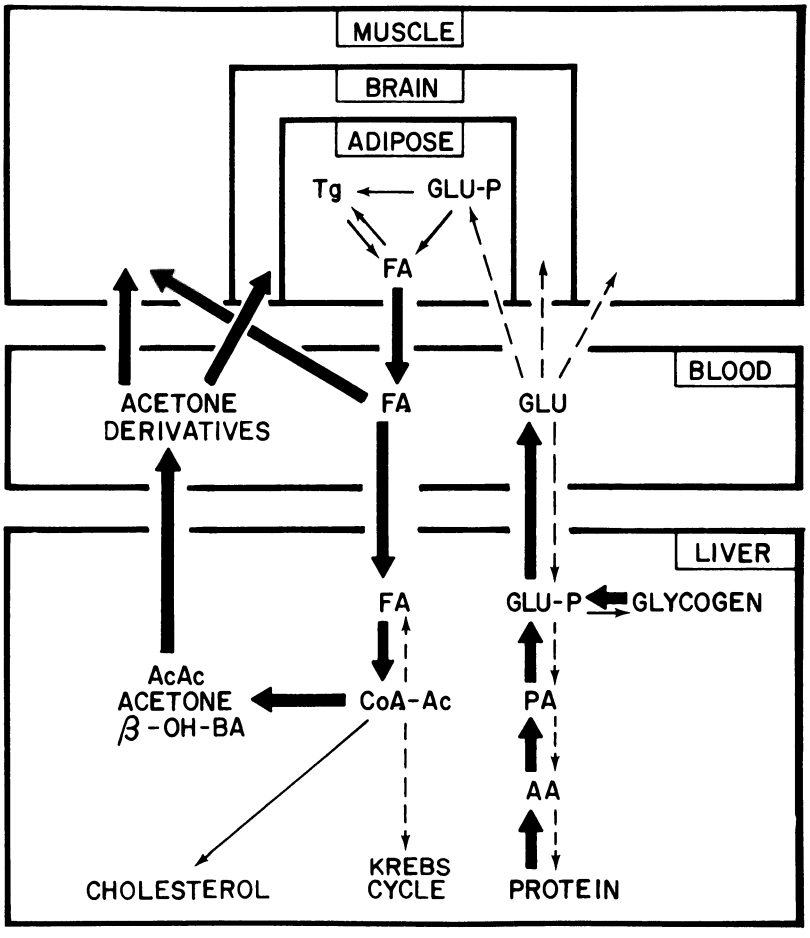


Fig. 9. (After T. Miettinen, Medical Diabetes Symposium, 1969, p. 39.) Diagram showing diabetic metabolism. The metabolic pathways which are restricted in diabetes are shown as dotted arrows; pathways increased in diabetes with heavy arrows. AA = Amino acids; AcAc = Acetoacetic acid; β-OH-BA = β Oxybutyric acid; CoA-Ac = Coenzyme A acetate; FA = Fatty acids; Glu = Glucose; Glu-P = Glucose phosphate; PA = Pyruvic acid; Tg = Triglycerides.

of this general conclusion (Rabinowich, 1935; Buber, 1968; Bierman et al., 1971; Brunzell et al., 1971; Anderson et al., 1973; Weinsier et al., 1974). After a review of the relevant literature, Pritikin (1974) concludes that more than ten % of fat in the diet is disadvantageous. Unquestionably, excessive fat in the diet will force even a normal metabolism part way into diabetic pathways by reducing insulin effects, thus shifting muscle metabolism from carbohydrates to fats as an energy source with consequent increase in the formation of deleterious by-products.

Slow release of glucose is favored by the use in the diet of its polymeric forms, as, in particular, boiled starch, dextrins. Heinonen (1969) states it is well known that persons who eat many small meals show less sclerosis than those who take few large meals. This too avoids release of nutrients at a faster rate than metabolism can handle smoothly, without build-up of reactive intermediate products.

3. Spontaneous Fractures.

Progressive loss of elasticity is a well known effect of extensive crosslinking. Crosslinking on a molecular scale may be likened to the crossbracing which a welder or a carpenter resorts to when he wishes to make a structure very rigid and nonyielding. The result is the same. A bone or a blood vessel or any other tissue is thus rigidized by extensive crosslinkage, any mechanical energy input is dangerously concentrated at the point where it occurs instead of being divided and absorbed over a much larger area as it would be if the resilience of youthful tissue were still present.

The loss of elasticity of the collagenous substance in the bones thus facilitates breakage. The loss of elasticity in arteries has already been discussed. Corresponding phenomena may increase the susceptibility to microfractures in glandular or intestinal organs, where they could become the starting point for continual irritations or ulcerations, and perhaps even malignancies.

4. Cancer

The material at hand is too complex to permit any conclusions at this time. However, a few points may be worth considering:

a. DNA anomalies can be caused by crosslinkages, which could even be their major cause. Such anomalies have been associated with cancer (Failla, 1958; Alexander and Lett, 1960).

b. Almost all of the known carcinogens either are themselves potential crosslinking agents, or have metabolites which are (Alexander et al., 1952, 1959).

c. There is a high correlation between cytotoxicity and crosslinking capacity (Haddow et al., 1948).

d. There is a high correlation between incidence of cancer and exposure to known crosslinking influences, including both ionizing radiation (Upton, 1960) and chemical carcinogens, including "natural" carcinogens such as the aflatoxins. Such relationships are increasingly referred to (for example, Conney, 1973; Nietert et al., 1974; Zeldin et al., 1975).

5. Immunological impairment.

There can be no question at all of the significance of the immunological effects in aging and at least in part in the formation of crosslinked deposits, as in amyloidoses. The primary effect of a considerable part of these changes could very well depend on the effect of crosslinking in changing the antigenic properties.

That changes as profound as those occurring in crosslinking must necessarily result in immunologic recognition, cannot be doubted, although the precise quantitation remains to be worked out.

The illustrations in the paper by Raff (1976) show clearly the significance of crosslinkage in the immune system.

Walford (1964) discussed this in a positive sense, and the questions raised by him (1969) have been answered (Bjorksten, 1974).

The present state of knowledge - and lack thereof in some crucial regards - have been aptly summarized by J. D. Stobo and T. B. Tomasi in an editorial in Journal of Chronic Disease (1975). The crosslinkage concept might well prove rewarding as a guideline in further exploration of the gaps pointed out by these authors.

Prevalence of crosslinked aggregates will certainly greatly disturb intracellular transportation, and might, for example, in this manner cause defects in the interaction between helper T and B cells stressed by Price and Makinodan (1973) as well as interfere with regulatory functions, considerably before the syndrome becomes more general.

COUNTERMEASURES

Various steps for minimizing exposure to random crosslinking have been discussed recently (Bjorksten, 1976). These include nutrition planning to avoid sudden overloads or any "bottleneck" in any metabolic path, favoring paths which involve the minimal amount of chemical processing in the body. Sound guidelines, to which I subscribe as far as they go, have been presented by R. Williams (1976).

What can be done along these lines is necessarily limited, and can be expected to effect life extensions only in the approximate range 5 - 15 years. This, however, can be done now and would gain for some of us the time needed to master the more difficult but also potentially far more rewarding problem of reversing damage already done, beyond what can be done by proper dieting and exercising (Pritikin, 1975; Haeger, 1973, 1974; Williams, 1976).

SOLUBILIZING CROSSLINKED AGGREGATES

These considerations apply both to DNA or RNA molecules repaired within amitotic cells such as neurons, and to breakdown and removal of complex crosslinked aggregates which have been formed during a lifetime and have withstood all enzymic attacks the host organism has been able to mount. Yet, there must be in existence enzymes which can cope with these, for otherwise large fossil deposits of such crosslinked proteins would have been found.

Starting on this premise, suggested by Bjorksten (1955), Bjorksten, Weyer and Ashman (1971) isolated insoluble aggregates from old human brains, suspended them in agar and inoculated them with various microbe rich infusions. Figure 10 shows how a few cultures were capable of dissolving the grey suspended matter surrounding them, forming "halos" in the Petri dishes.

Subcultures were made, grown in 14000 ml fermenters. In 4 years of work an enzyme was separated and purified chromatographically, which was considerably superior to any enzyme previously known to us in solubilizing highly crosslinked protein aggregates, whether natural or artificially prepared by treating gelatin with powerful crosslinking agents.

The enzyme in question was derived from a mutant strain of Bacillus cereus. At least some traces of similar enzymes were found in every spore forming organism examined. Their content of such enzymes appears connected with sporulation. Evolution might have developed this class of very low molecular weight enzymes

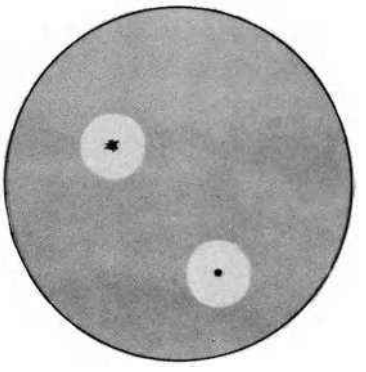


Fig. 10. The dense structures of Fig. 5 were isolated from old human brain, pulverized and dispersed in agar, rendering the agar grey. A suspension of mixed soil organisms was used as inoculum. Two colonies have developed clear halos, showing that these colonies excrete enzymes capable of dissolving the dispersed "gerogenic aggregates.

in order to make possible the dissolution, and perhaps also the synthesis, of the extremely dense and resistant spore shells where access of larger enzyme molecules are sterically hindered. So far only one of these enzymes has been fairly thoroughly studied (Schenk and Bjorksten, 1973). It has only been tested toxicologically, as quantities so far available to us have been insufficient for extensive animal tests. However, enterically coated granules fed to rodents did pass the acidity of the stomach. Some evidence indicates that it has had effects in the bloodstream. It would be almost a miracle if the first member of a family of enzymes should be found to provide the solution to all problems. We have encountered at least 7 promising members of this family of enzymes present in other microorganisms. One of these is now available to researchers from Worthington Biochemical Corporation, Freehold, New Jersey, under the designation "Microprotease, Bac. cereus" (Worthington, 1976).

If these low molecular enzymes are indeed capable of penetrating to organs beyond the circulatory system, they may well provide the key to the long overdue breakaway in the curve of life expectancy at 60, and thus give all of us many additional happy years in good health. Since they appear to destroy most other blood enzymes, their application should probably be intermittent and/or the dosage carefully controlled. The blood enzymes can be

replaced rapidly, the crosslinked aggregates have a vastly slower cycle.

The work along this line has been slowed by the governmental pressures on the pharmaceutical industry, which was supporting our work 1966-1970.

This long term work was one of the first casualties of the reallocation of funds, forced upon the pharmaceutical industry by congressional investigations. Funds allocated for our long range research on aging were among those preempted for defensive research to disprove allegations made. This situation is general and we all have merely begun to pay for it.

In 1961, for example, 39 new single chemical entities appeared--most of these generated by American pharmaceutical industry. In 1974 the number of these was only 18, largely originated abroad, and I see no sign of a reversal.

While hamstringing the most effective mechanism for pharmaceutical advance, the Government has so far been unable to furnish any effective substitute to support innovation in this field.

On September 24, 1883, Oscar Wilde, the famous author, told a distinguished British audience about his extensive travels in America the preceding year. He commented:

"A remarkable characteristic of the American is the manner in which they have applied science to modern life.

"This is apparent in the most cursory stroll through New York. In England an inventor is regarded almost as a crazy man and in too many instances invention ends in disappointment and poverty. In America an inventor is honored, help is forthcoming, and the exercise of ingenuity, the application of science to the work of man, is there the shortest road to wealth."

This happy situation, which made our country great, has been reversed. That work on aging, which if successful, could bring a major breakthrough, has had insufficient support in the USA. Research on the control of aging must be funded and pursued vigorously if those who live now are to benefit.

REFERENCES

Acharya, P.V.N., Ashman, S.M., and Bjorksten, J. (1972). Isolation and Partial Characterization of Age-correlated Oligo Deoxyribo-ribo-nucleo-peptides. Finska Kemists. Medd. 81 No. 3, 70-87.

Alexander, P. (1961). Comparison of the Action of Ionizing Radiations and Biological Ionizing Agents (Nitrogen Mustards) at the Cellular and Sub-cellular Level. Sci. Reports 1st Super Sanita 1, 541-544.

Alexander, P.; Fox, M.; Stacey, K. A.; and Smith, L. F. (1952). The Reactivity of Radiomimetic Compounds. I. Crosslinking of Proteins. Biochem. J. 52, 177-184

Alexander, P., and Lett, J. T. (1960). The Biological Significance of the Changes Produced in the Deoxyribonucleic Acid of Cells Treated with Radiomimetic Alkylating Agents. Biochemical Pharmacology 4, 34-36.

Alexander, P.; Swarcbort, A.: and Stacey, K. A. (1959). The Reactivity of Radiomimetic Compounds. III. Crosslinking of Nucleoprotein. Biochem. & Pharmacol. 2, 133-145.

Anderson, J. W. et al. (1973). Effect of High Glucose and High Sucrose Diets on Glucose Tolerance of Normal Men. Amer. J. Clin. Nutrition 26, 600-7.

Bellows, J. G., and Bellows, E. T. (1976). Crosslinkage Theory of Senile Cataracts. Ann. Ophthalmol. 8, 129-135.

Bierman, E. L.;, Albrink, M. J.; Arky, R. A. et al. (1971). Principles of Nutrition and Dietary Recommendations for Patients with Diabetes Mellitus. Diabetes 20, 633-634.

Bjorksten, J. (1941). Recent Developments in Protein Chemistry. Chem. Ind. 48, 746-751.

Bjorksten, J. (1942). Chemistry of Duplication. Chem. Ind. 50, 68-72.

Bjorksten, J. (1943). Time Saving in Research. Chem. & Eng. News 21, 1324

Bjorksten, J. (1951). Crosslinkages in Protein Chemistry in Ad vances in Protein Chemistry, ed. by Anson, Edsall and Bailey, Vol. 6, pp. 343-381. Academic Press, New York.

Bjorksten, J. (1952). A Mechanism of Cholesterol Deposition on Arterial Walls. Proc. Soc. Exp. Biol. and Med. 81, 350-353.

Bjorksten, J. (1955). Crosslinking, Key to Aging? Chem. & Eng. News 33, 1957.

Bjorksten, J. (1962). Aging: Present Status of our Chemical Knowledge. J. Am. Geriatrics Soc. 10, 125-139.

Bjorksten, J. (1963). Aging: Primary Mechanism. Gerontologia 8, 179-192.

Bjorksten, J. (1966). Enzymes and Aging in Enzymes in Mental Health, ed. by G. J. Martin and B. Kisch, Chap. 6, pp. 84-94. Lippincott Co., Philadelphia.

Bjorksten , J. (1968). The Crosslinkage Theory of Aging. J. Amer. Geriatrics Soc. 16, No. 4, 408-427.

Bjorksten, J. (1969). Theories in Aging Life Processes ed. by Bakerman Chap. 6, pp. 147-149. Charles C. Thomas, Publisher, Springfield, Ill.

Bjorksten, J. (1971a). The Crosslinkage Theory of Aging. Finska Kemists. Medd. 80, No. 2, 23-38.

Bjorksten, J. (1971b). Approaches and Prospects for the Control of Age-Dependent Deterioration. Annals of the N. Y. Acad. of Sci. 184, 95-102.

Bjorksten, J. (1974). Crosslinkage and the Aging Process in Theoretical Aspects of Aging, ed. by M. Rockstein, pp. 43-59. Academic Press, New York.

Bjorksten, J. (1976). The Cross Linkage Theory of Aging: Clinical Implications. Comprehensive Therapy 2, No. 2, 65-74.

Bjorksten, J.; Acharya, P.V.N.; Ashman, S.; and Wetlaufer, D. B. (1971). Gerogenic Fractions in the Tritiated Rat. J. Am. Geriatrics Soc. 19, No. 7, 561-574.

Bjorksten, J., and Andrews, F. (1964). Chemical Mechanisms Underlying the Biological Mechanisms of the Aging Process. J. Am. Geriatrics Soc. 12, No. 7, 627-631.

Bjorksten, J., and Ashman, S. (1970). Nitrogenous Compounds Immobilized in an Aged Rat. J. Am. Geriatrics Soc. 18, No. 2, 116.

Bjorksten,, J. and Champion, W. J. (1942). Mechanical Influence upon Tanning. J. Am. Chem. Soc. 64, 868-869.

Bjorksten, J. and Gottlieb, H. (1954). In Vitro Cholesterol Deposition on Hog Aorta Tissue. Finska Kemists. Medd. 63, No. 3-4, 74-77.

Bjorksten, J.: Weyer, E. R.; Ashman, S. M. (1971). Study of Low Molecular Weight Proteolytic Enzymes. Finska Kemists Medd. 80, 26-46.

Bensusan, H. B. (1972). Investigation of Crosslinks in Collagen Following Enzymic Hydrolysis. Biochem.Biophys.Acta 285,447-452.

Blumenthal, H. T. (1975). Athero-Arteriosclerosis as an Aging Phenomenon in The Physiology and Pathology of Human Aging, p. 142, ed. by R. Goldman and M. Rockstein. Academic Press, New York.

Brunzell, J. D.; Lerner, R. L.; Hazzard, W. R.; Porte, D., Jr.; Bierman, E. L. (1971). Improved Glucose Tolerance with High Carbohydrate Feeding in Mild Diabetes. N. Eng. J. Med. 284, 521-524.

Buber, V. (1968). Improvement of Oral Glucose Tolerance by Acute Drug Induced Lowering of Plasma Free Fatty Acids. Schweiz. Med. W. Schr. 98, 711-2.

Moses, C. and Longabaugh, G. M. (1950). Rapid Production of Atherosclerosis by Administration of Uranium in Presence of Hypercholesterolemia (17819). Proc.Soc.Exp.Biol. & Med. 74, 92-93.

Carpenter, Donald G. (1965). Diffusion Theory of Aging. J. Geront. 20, No. 2, 192-195.

Cater, C. W. (1963). Evaluation of Aldehydes and Other Difunctional

Compounds as Cross-linking Agents for Collagens. J. Soc. Leather Trades Chem. 47, 259.
Charlesby, A. (1953). Effects of High Energy Radiation on Some Long Chain Polymers. Plastics, London, 18, 70, 142-145.
Conney, A. H. (1973). Carcinogen Metabolism and Human Cancer. New Eng. J. Med. 289, 971-973.
Constantinides, P. and Wiggers, K. D. (1973). Direct Visual Evidence of Impermeability of Arterial Endothelium to Lipoprotein Molecules but Permeable Following Insults. Autoradiographic Methodology. Am. J. Pathol. 70, 81a.
Cutler, R. G. (1976). Cross-linkage Hypothesis of Aging: DNA Adducts in Chromatin as a Primary Aging Process, in Aging, Carcinogenesis and Radiation Biology, Kendric C. Smith, ed., Plenum Press, New York, pp. 443-492.
Failla, G. (1958). The Ageing Process and Carcinogenesis. Ann. N. Y. Acad. Sc. 71, 1124-1140.
Fishman, J. A.; Graeme, B. R.; Karnovsky, M. J. (1975). Endothelial Denudation in the Pathogenesis of Myointimal Thickening. Lab. Invest. 32, 339.
Freidman, F. J.; Moore, S.; Singal, D. P. (1975). Repeated Endothelial Injury and the Induction of Atherosclerosis in Normolipemic Rabbits by Human Serum. Lab. Invest. 30, 404.
Goldacre, R. J.; Loveless, A.; and Ross, W. J. C. (1949). Mode of Production of Chromosome Abnormalities by the Nitrogen Mustards. Nature 163, 667.
Goldstein, J.; Hazzard, W. R.; et al. (1973). Hyperlipidemia in Coronary Heart Disease. I. Lipid Levels in 500 Survivors of Myocardial Infarction. J. Clin. Invest. 52, 1533-1543.
Gross, G. (1952). In Metabolic Interrelations, Proc. 4th Josiah Macy Jr. Foundation Conference, E. C. Reifenstein, editor. Progress Associates, Inc., Caldwell, N. J., p. 40.
Gustavson, K. H. (1942). Behaviour of Collagen in Combination with Tanning Agents Towards Trypsin. Svensk. Kem. Tidskr. 54, 251.
Gustavson, K. H. (1956). The Chemistry of Tanning Processes. New York, Academic Press Inc.
Haddow, A.; Kon, G.A.R.; and Ross, W.C.J. (1948). Effects Upon Tumors of Various Haloalkylarylamines. Nature, London, 162, 824.
Hahn, H. P. von (1964). Age Related Alterations in the Structures of DNA. II. The Role of Histones. Gerontologia 10, 174-182.
Haeger, K. (1974). Vitamin E in Intermittent Claudication. Lancet 1, 1352; and (1973) VASA 2, 280-287.
Harman, D. (1956). Aging: A Theory Based on Free Radical and Radiation Chemistry. J. Geront. 11, 293-300.
Hart, R. W. (1976). Role of DNA Repair in Aging in Aging, Carcinogenesis, and Radiation Biology, ed. by Kendric C. Smith, pp. 537-557. Plenum Publishing Co., New York.

Hart, R. W., and Setlow, R. B. (1974). Correlation Between Deoxyribonucleic Acid Excision Repair and Lifespan in a Number of Mammalian Species. Proc. Nat. Acad. Sci. 71, pp. 2169-2173.

Hart, R. W., and Trosko, J. E. (1976)). DNA Repair Processes in Mammals in Interdisc. Topics in Geront. (87 references) 9, 134-167.

Heinonen (1969). Comments (pp. 4 and 7) made in the panel discussion of paper "Diabetic Metabolism" by Tatu Miettinen in Medica Diabetes Symposium, Weilin & Goos Ab, publishers, Tapiola, Finland, pp. 37-41.

Howard-Flanders, P., and Boyce, R. P. (1966). DNA Repair and Genetic Recombination Studies on Mutants of E Coli Defective in These Processes. Radiation Res. Suppl. 6, 156-184.

Johansson, B. V., and Sievers, J. (1966). Spontaneous Course of Intermittent Claudication, Scandinavian J. Clin. Lab. Invest. Suppl. 93, 156.

Kohn, R. R. (1963). Human Aging and Disease. J. Chronic Disease 16, 5-21.

Kohn, R. R., and Rollerson, E. (1960). Aging of Human Collagen in Relation to Susceptibility to the Action of Collagenase. J. Geront. 15, 10-15.

LaBella, F. S., and Paul, G. (1965). Structure of Collagen from Human Tendon as Influenced by Age and Sex. J. Geront. 20, 54.

Lipsitz, P.; Kremen, S. S., and Lollar, R. M. (1949). Studies of Vegetable Tanning. VII. J. Am. Leather Chem. A. 44, 371-387.

Medvedeva, N. B. (1939). The Variations in Nitrogen Compounds and Proteins in the Tissues of the Aged. J. Med. Ukr. 9, 325.

Miettinen, T. (1969). Diabetic Metabolism in Medica Diabetes Symposium, Weilin & Göös Ab, Publishers, Tapiola, Finland, pp. 37-41.

Milch, R. A. (1964). Reaction of Certain Aliphatic Aldehydes with Gelatin. Gerontologia 10, 117-136.

Nietert, W. C.; Kellicutt, L. M.; and Kubinski, H. (1974). DNA-Protein Complexes Produced by a Carcinogen, Beta-Propiolactone. Cancer Research 34, 859-864.

Novak, L. P. (1972). Aging, Total Body K, Fat Free Mass and Cell Mass in Males and Females Between Ages 18 and 85 Years. J. Gerontol. 27, 438.

Orgel, L. E. (1963). The Maintenance of the Accuracy of Protein Synthesis and its Relevance to Aging. Proc. Nat. Acad. Sci. 49, 517.

Piez, K. A. (1968). Cross-Linking of Collagen and Elastin. Ann. Review Biochem. 37, 547.

Powell, J. L. (1958). The Collapse of Time. The Chemist 35, 351-359.

Price, G. B., and Makinodan, T. (1973). Aging: Alteration of DNA Protein Information. Gerontologia 19, 58-70.

Pritikin, N. (1974). Personal Communications.

Pritikin, N. (1975). Physician's Manual: Background Studies and Rationale, LFC Program, Santa Barbara, Cal.

Pritikin, N. (1975). Diet and Exercise as a Total Therapeutic Regimen for the Rehabilitation of Patients with Severe Peripheral Vascular Disease. Paper presented at the 52nd Annual Session, American Congress of Rehabilitation Medicine and the 37th Annual Assembly of the American Academy of Physical Medicine and Rehabilitation, Atlanta, Ga., Nov. 19, 1975.

Rabinowitch, I. M. (1935). Effects of the High Carbohydrate Low Caloric Diet Upon Carbohydrate Tolerance in Diabetes Mellitus. Canad. Med. Ass'n J. 33, 136-44.

Raff, M. C. (1976). Cell-Surface Immunology. Scientific American, 234, No. 5, 30-39 (particularly Fig. p. 34).

Riddick, Thomas M. (1968). Control of Colloid Stability Through Zeta Potential, p. 266-275. Livingston Publ. Co., Wynnewood, Pa.

Schenk, R. U., and Bjorksten, J. (1973). The Search for Microenzymes: The Enzyme of Bacillus Cereus. Finska Kemists. Medd. 82, 26-46.

Schmitt, F. O.; Gross, J.; and Highberger, J. H. (1953). A New Particle Type in Certain Connective Tissue Extracts. Proc. Nat. Acad. Sci. USA 39, 459.

Sinex, F. M. (1964). Cross-linkage and Ageing in Advances in Gerontological Research 1, ed. by Strehler, p. 165. Academic Press, New York.

Smith, Kendric C., editor (1976). Aging, Carcinogenesis and Radiation Biology. Plenum Press, New York.

Tanzer, M. L. (1969). Intermolecular Crosslinks in Reconstituted Collagen Fibrils. J. Biol. Chem. 243, 4045-4054.

Tanzer, M. L. (1973). Cross-Linking of Collagen. Science 180, 561-566.

Tanzer, M. L.; Church, R. L.; Yaeger, J. A.; and Park, E. D. (1975). The Multi-Step Pathway of Collagen Biosynthesis: Pro-Collagen Intermediates in Extracellular Matrix Influences on Gene Expression, ed. by Slavkin and Greulish. Academic Press, Inc., New York.

Stobo, J. D., and Tomasi, T. B. (1975). Editorial. Aging and the Regulation of Immune Reactivity. J. Chron. Dis. 28, 437.

Thomas, A. W., and Seymour-Jones, F. L. (1924). Action of Trypsin upon Diverse Leathers. Indust. Eng. Chem. 16, 157.

Upton, A. C. (1960). Ionizing Radiation and the Aging Process. J. Gerontol. 12, 306-313.

Veis, A., and Anesey, J. (1965). Modes of Intermolecular Crosslinking in Mature Insoluble Collagen. J. Biol. Chem. 240, 3899.

Verzár, F. (1955). Das Altern des Kollagens. Helv. Physiol. Acta 13, 64-67. (1956) 14, 45, 207-221.

Vobra, P., and Kratzer, F. H. (1964). Influence of Various Chelating Agents on the Availability of Zinc. J. Nutrition 82, 249.

Walford, R. L. (1964). Further Considerations Toward an Immunologic Theory of Aging. Exper. Gerontology 1, 73.

Walford, R. (1969). The Immunologic Theory of Aging. Williams and Wilkins, Baltimore.

Weinsier, R. L.; Seeman, A.; Herrara, M. B.; Assal, J. P.; Soeldner, J. S., and Gleason, R. E. (1974). High- and Low-Carbohydrate Diets in Diabetes Mellitus, Study of Effect on Diabetic Control, Insulin, Secretion and Blood Lipids. Ann. Intern. Med. 80, 332-341.

Wilde, Oscar (1906). Impressions of America, ed. by Stuart Mason. Keystone Press, Sunderland.

Williams, Roger J. (1976). On Your Startling Biochemical Individuality. Executive Health XII, No. 8, 1-8.

Worthington Biochemical Corp., Freehold, New Jersey. Catalog 1976, p. 32.

Zeldin, Paul E.; Bhattacharya, P. K.; Kubinski, H.; and Nietert, W. C. (June 1975). Macromolecular Complexes Produced by 1,3-Propane Sulfone. Cancer Research 35, 1445-1452.

Zinsser, H. H.; Butt, E. M.; Leonard, J. (1957). Metal Content Correlation in Aging Aorta. J. Amer. Geriatrics Soc. 5, No. 1, 20.

34

CHEMISTRY OF COLLAGEN CROSSLINKING: RELATIONSHIP TO AGING AND NUTRITION

Philip E. McClain

U. S. Department of Agriculture
Agricultural Research Service, Nutrition Institute
Beltsville, Maryland 20705

Collagen is a major constituent of skin, bone, tendon, and cartilage and is a vital component of the cardiovascular system. Not only is collagen an important protein because of its relative abundance, estimates indicate that it constitutes as much as 40% of the total body protein, but also it is the only protein in the body that has been clearly shown to undergo molecular changes during the aging process in man and animals, i. e., it "ages." One of the outward manifestations of aging in collagen is the wrinkling of the skin which is inevitable in the elderly. Another, which is not so obvious but is of infinitely greater importance to man is the age-associated loss of elasticity in the cardiovascular system.

Aging Changes in Collagen

It has long been recognized that the physical and chemical properties of collagen undergo characteristic changes during the maturation and aging processes. As early as 1847, Wertheim (1) noted that the load-deformation curve of collagen became steeper with advancing animal age. More recently, Rollhäuser (2) reported that collagen becomes "stiffer" with increasing age, and several groups have reported increases in tensile strength with advancing animal age (3,4).

Verzar (5) was the first to show that the degree of thermal shrinkage is dependent on age, and that the tension developed in isometric thermal contraction increases with age. There is also an apparent relationship between animal age and the temperature at which thermal shrinkage occurs (6). The temperature of thermal

Table 1

Age Related Changes in Thermal Shrinkage of Porcine Epimysium

	7 weeks		5 months	
	Onset[1]	Recovery[1]	Onset[1]	Recovery[1]
Mean	46.1	78.8	49.8	81.5
Range	45.0-48.1	77.7-80.3	48.8-52.5	80.3-82.3
Std. Dev.	1.38	1.12	1.84	0.88

[1]Temperature °C differential thermal analysis. McClain et al. (6)

shrinkage, for example, is significantly higher in the epimysial connective tissue from 5-month-old pigs than in that from 7-week-old animals (Table 1). Brown and Consden reported similar results in fibers teased from skin (7).

On collapse of the triple helix during heating, a portion of the collagen in a tissue is released into the surrounding media as gelatin. During maturation the amount of collagen released decreases and during senescence continues to decrease. When the method of thermal dissolution was applied to rat skeletal muscle collagen, Schaub (8) found that there was a decrease from 40% released in a 1-2 month old animal to 10% at 10 months and 3-5% at 30-40 months. From Figure 1 it can be seen that there is a marked increase in the percent of heat labile collagen in bovine skin during the first 4 months of life (9). This increase is thought to reflect the rapid growth and rate of collagen biosynthesis occurring during this period. From 4 months to 1 year there is a rapid decrease in the quantity of heat labile collagen, followed by a gradual decrease which continues throughout the animal's lifespan.

The solubility characteristics of collagen in dilute acetic acid closely parallel the changes observed in thermal labile collagen (Fig.2). From birth to about 2 months of age there is a rapid increase in the amount of acid soluble collagen in porcine L. dorsi muscle collagen. From 2 months to about 6 months of age there is a rapid decrease. After 6 months the solubility continues to decrease slowly. Interestingly, the collagen from the two muscles, L. dorsi and semimembranosus, appear to differ markedly in their age associated solubility characteristics.

All of the workers cited above have attempted to explain their observations on the basis that collagen was forming greater numbers and more stable covalent crosslinks with advancing animal age. From the stress-strain behavior of a thermally contracted sample,

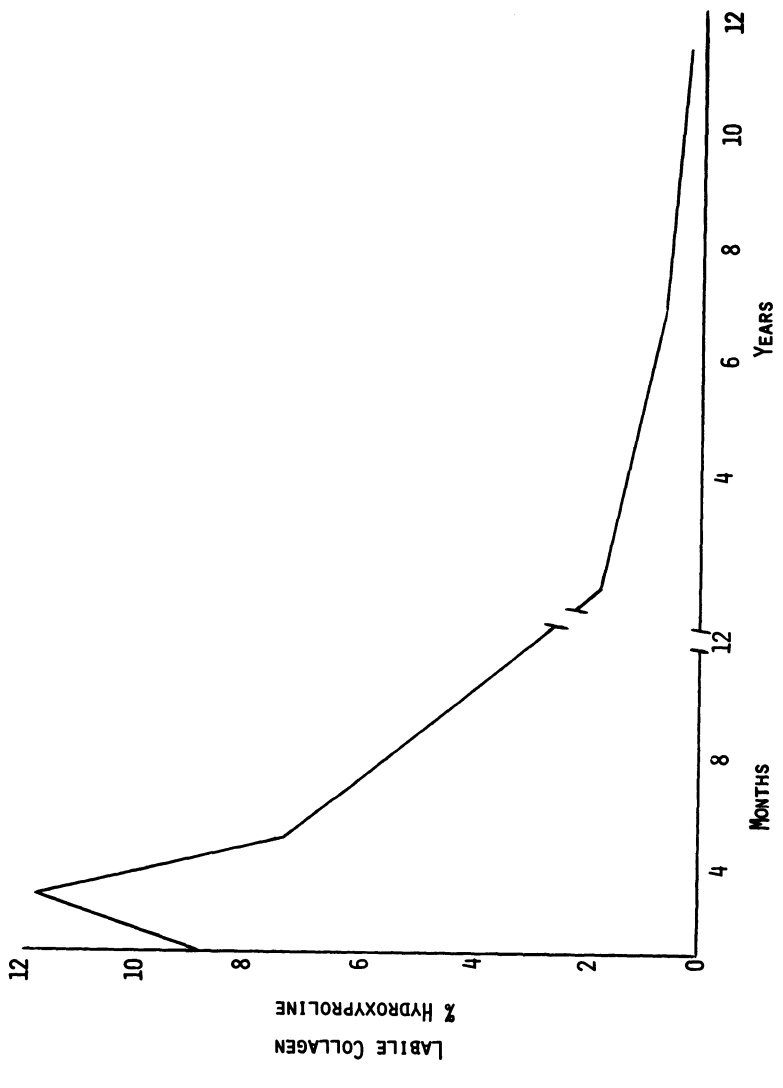


Fig. 1. Changes in heat labile collagen in bovine skin with animal age. Adapted from Verzar (9).

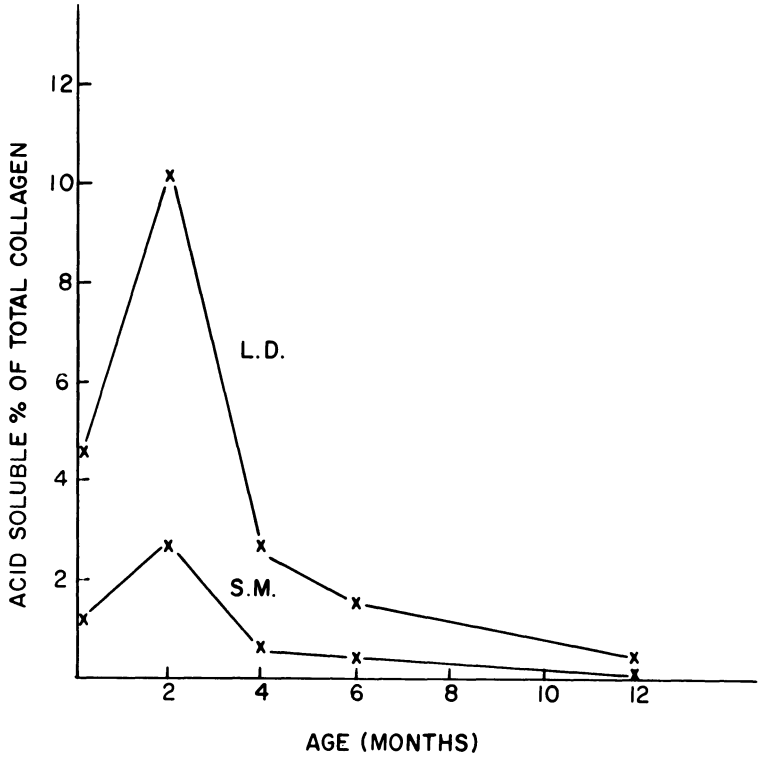


Fig. 2. Changes in solubility of porcine collagen from the L. dorsi (L.D.) and semimembranosus (S.M.) muscles with animal age. McClain (10).

a molecular weight between points of crosslinking can be determined. Utilizing this value, it is possible to calculate the number of crosslinks per unit volume of protein (11). From the data shown in Table 2 it can be seen that the molecular weight between crosslinks decreases from a value of 8.0 in the epimysium of the one week old pig to a value of 4.67 in the 5 month old animal. Conversely the crosslinks per molecule increase from a value of 4.09 in the former to 7.73 in the latter.

In the last few years, major advances have been made in the isolation and characterization of the collagen crosslinks (see reviews in this volume), and convincing evidence is now available that the aging process in collagenous tissues is a consequence of the numbers and types of crosslinks present. Table 3 shows that there is a marked increase in the reducible crosslinks up to 1 year of age in bovine muscle collagen (12), again reflecting the rapid rate of growth occurring during this period. From 1 year to

Table 2
Effect of Biological Aging on the Molecular Weight Between Crosslinks in Porcine Epimysial Connective Tissues[1]

Age	Number of Animals	Molecular Weight x 10^{-4}	Crosslinks per Molecule
1 Week	2	8.00	4.09
7 Week	3	6.58	4.81
5 Month	6	4.67	7.73

[1]McClain et al (11).

Table 3
Variation in Reducible Crosslinks[1] with Age in Bovine Muscle Collagen

	Crosslink Type[2]				
Age	HL	HN	DHLNL	HLNL	HHM
Fetal	2	2	10	9	3
1 Year	7	5	30	27	15
3 Years	8	7	12	5	5
6 Years	9	8	5	4	5
10 Years	10	10	4	3	3
15 Years	20	16	3	2	2

[1]HL = Hexosyl-lysine; HH = Hexosyl-hydroxylysine; DHLNL = Dihydroxylysinonorleucine; HLNL = Hydroxylysinonorleucine; HHM = Histidinohydroxymerodesmosine.
[2]CPM x 10^{-2}, adapted from Shimokamaki et al. (12).

3 years of age there is a sharp drop in the number of reducible crosslinks (with the exception of the hexosyl compounds), followed by a continued gradual decrease throughout the lifespan. These changes closely parallel the decreases in solubility and thermal labile collagen and increases in isometric thermal contraction and tensile strength. To account for these observations it has been proposed that the reducible crosslinks serve only as intermediates and are converted during the maturation and aging process into a nonreducible, more stable form (13).

Crosslinking and Nutrition

The aging of collagen is affected not only by physical forces such as increase of temperature or irradiation, but also by substances which can produce crosslinks or prevent crosslink formation. Such substances may be intrinsic factors from normal or abnormal metabolic processes in the body, or extrinsic factors such as poisons or nutrients ingested in the diet.

Ascorbic Acid and Iron. Several nutritional factors are known to play a major role in collagen crosslinking. For example, the classical symptoms of scurvy have long been recognized to result from a deficiency of Vitamin C. Current evidence indicates that the enzymes responsible for the hydroxylation of prolyl and lysyl residues during collagen synthesis require as cofactors molecular oxygen, ferrous iron, α-ketoglutarate and ascorbic acid. Molecular oxygen is known to be the source of oxygen for the hydroxyl group (14). The roles of the other cofactors are much less clear. It is known, however, that α-ketoglutarate undergoes stoichiometric decarboxylation in relation to the amount of hydroxyl group formed (15). It is also known that the hydroxylase enzymes are activated by binding ferrous iron, which in turn may serve as the binding site for molecular oxygen (16,17). Ascorbic acid has been considered to serve as a reducing agent in these reactions (18). However, evidence has recently been presented indicating that ascorbic acid may also stimulate an activating system for the conversion of an inactive precursor form of the peptidyl hydroxylases to active enzyme (19). The under-hydroxylation of proline is known to markedly alter the stability characteristics of collagen and result in intracellular retention of the newly synthesized collagen molecules (20, 21). Although the role of under-hydroxylated lysine residues is not known with certainty, it may be speculated that in the absence of hydroxylysine in certain regions of the collagen molecule, the relatively stable hydroxylysine containing crosslinks cannot be formed and that the observed clinical phenomena in scurvy may relate at least in part to this defect in crosslinking.

Vitamin D. A similar situation may also exist in Vitamin D deficiency. Toole et al (22) and Barnes et al (23) have reported

that both rachitic and normal bones contain $(\alpha 1)_2\alpha 2$ type collagen. However, the vitamin D deficient collagen contains as much as 50% more hydroxylysine residues than that from normal bone. Mechanic and coworkers (24) reported an increase in the amount of the reduced crosslink dihydroxylysinonorleucine relative to the content of hydroxylysinonorleucine in collagen from rachitic chick bone. It would thus appear that Vitamin D is in some manner involved in the regulation of lysylhydroxylase and, hence, the type of reducible crosslink formed. The role of this regulation in the calcification process remains unclear. However, it has been suggested that the carbohydrate moieties normally associated with hydroxylysine may interfere with the deposition of calcium apatite crystals in the hole regions of the collagen fibers (22).

Copper and Lathyrogens. Two other nutritionally derived components play a more direct role in collagen crosslinking. The earlier literature describing copper deficiency referred to skeletal pathology such as spontaneous fractures and osteoporosis (25). Renewed interest in the role of copper in connective tissue metabolism began with the observation of aortic rupture in copper deficient pigs and chicks (26,27). The total collagen content of chick aorta is apparently not affected by copper deficiency, but there does appear to be a reduction in intermolecular crosslinking as indicated by a higher portion of cold salt and acetic acid soluble collagen (28). Not only is intermolecular crosslinking depressed but intramolecular bonding is similarly affected as indicated by the low proportion of β components in salt soluble collagen from copper deficient chick tendon (29). From these experiments it is clear that dietary copper has a marked effect on the activity of lysyl oxidase, the enzyme responsible for the oxidative deamination of lysine residues to the δ-semialdehyde of α-amino adipic acid (13). The fact that copper remains with the protein during isolation and that it can be reversibly removed has led to the conclusion that lysyl oxidase is in fact, a copper metalloprotein (29).

Lathyrogens, such as β-aminopropionitrile produce an arterial pathology analogous to that of copper deficiency. Changes in the chemical characteristics of aortas due to lathyrism and copper deficiency are hardly distinguishable (30). These observations have led to the suggestion that the pathology of copper deficiency and lathyrism results from the same biochemical lesion, i.e., inhibition of lysyl oxidase and the failure to form crosslinks.

Zinc. The marked abnormalities in tissue growth and repair processes produced by zinc deficiency in man and animals have long been recognized (31). Recent evidence suggests that zinc may be essential for wound healing (32). Preliminary work in our laboratory also indicates that zinc may function in the collagen crosslinking process (33). From Table 4 it can be seen that the solubility of

Table 4

Characteristics of Collagen from Zinc Deficient Animals

	Acid[1] Soluble	Aldehyde[2] Content	Hydroxyproline[3]	Hydroxylysine[3]
Zinc +	49	1.7	94	3.5
Zinc -	58	0.8	94	3.5

[1]Mg collagen/g skin

[2]μM/100 mg collagen

[3]Residues per 1000 residues. McClain et al. (33).

skin collagen from zinc deficient rats was almost 20% higher than that from pair fed controls, suggesting an inhibition of the cross-linking mechanism or an increase in the number of labile aldimine type crosslinks. The more than 45% reduction in the aldehyde content of zinc deficient skin collagen (Table 4) further suggests a possible inhibition of lysyl oxidase. The hydroxyproline and hydroxylysine content of the α chains from zinc deficient skin collagen do not appear to be altered (Table 4), indicating that the hydroxylase enzymes are functioning normally. Although the exact role of zinc remains to be elucidated, the apparent alterations in collagen crosslinking observed in zinc deficient skin could explain the increase in tensile strength and enhanced wound healing attributed to zinc administration (34).

Dietary Restriction. The classical experiments of McKay (35) have clearly shown that dietary level and plane of nutrition can greatly increase longevity in laboratory animals. Chvapil and Hruza (36) reported that the aging of connective tissue in underfed rats was retarded as measured by chemical contraction-relaxation. Deyl and Rosmus (37) observed a significant decrease in β components in acid soluble collagen from rats maintained on a diet restricted to 50% of the normal caloric intake. We have made similar observations on animals maintained on restricted diets (10). As can be seen from Table 5, the quantity of β components was about 20% less in the skin collagen from the diet-restricted rats. The thermal shrinkage temperature was also 2° lower in the animals on the restricted diet, suggesting that the collagen from these animals is indeed a more immature type.

More recently Prasad and Bose (38) reported a reduction in β components in salt soluble collagen and a significantly decreased aldehyde content in protein deficient rats, suggesting an impair-

Table 5
Characteristics of Collagen from Animals on a Restricted Food Intake

Treatment	%α	%β	Ts °C
Control	41	59	58
Food Restricted	53	47	56

McClain (10).

ment of collagen crosslinking. Recent work in our laboratory tends to confirm that conclusion (39). Table 6 shows that the quantity of salt soluble collagen from skins of rats maintained on an 8% protein diet was reduced over 35% in comparison to control animals, suggesting a decrease in collagen biosynthesis. This was confirmed by incorporation studies (Table 6) which revealed that the uptake of both [2-^{14}C] glycine and L-[U-^{14}C] proline was reduced 35 to 46% in the low protein animals. Figure 3 shows the component distribution of the soluble collagen fraction from animals on the two diets. These data reveal that the β components were reduced almost 50% in the animals on the 8% protein diet. Conversely, the aldehyde content of the α1 chains was almost twice as high in animals on the low protein diet (Table 6).

As can be seen in Table 7, the radioactivity of tritiated sodium borohydride reduced skin collagen was almost twice as high in the rats maintained 21 days on the low protein diet than in the control animals. Similar results are evident for new born pups from mothers maintained on the 8% protein diet. It can also be seen that the incorporation of tritium activity into the reducible crosslink, hydroxylysinonorleucine, was markedly higher in the day old pups from mothers on the 8% diet. The hydroxyproline-hydroxylysine content did not differ between the two experimental groups (Table 6).

The exact role of dietary protein in collagen crosslinking can not be determined from these preliminary studies. However, it does appear that the maturation of the reducible crosslinks to nonreducible - stable crosslinks may be impaired in animals maintained on a low level of dietary protein.

Level and Type of Carbohydrate. The results of subsequent studies in our laboratory have revealed that food restriction is not the only nutritional means of altering collagen metabolism and crosslinking. As shown by the reduced yield of salt soluble collagen (Fig. 4), protein free regimes (90% G + 90% F) have effected a greater than 60% reduction in salt-soluble collagen as

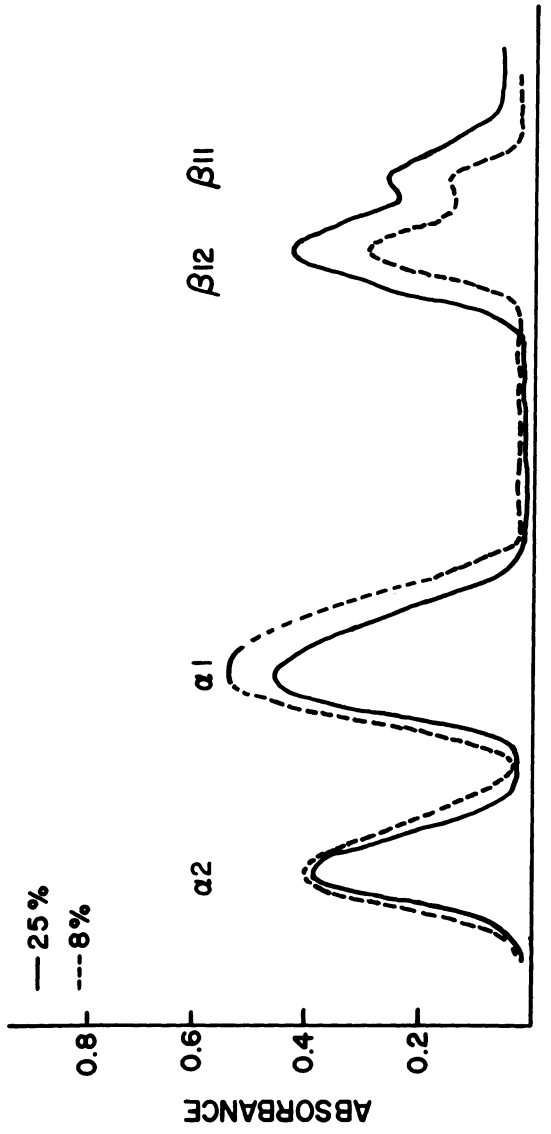


Fig. 3. Densitometer tracing of disc gel patterns obtained from acid soluble collagen. McClain et al (39).

Table 6
Characteristics of Collagen from Animals on a Low Protein Diet

Parameters	Protein Level 25%	8%
Soluble Collagen[1]	9.7	6.3
[2-^{14}C] Glycine[2]	100	54
L-[U-^{14}C] Proline[2]	100	65
Hydroxyproline[3]	92	92
Hydroxylysine[3]	7.4	6.3
Aldehyde[4]	10.4	20.1

[1]Mg collagen/g skin.
[2]Incorporation, DPM per mg skin expressed as a percent of 25% control
[3]Residues per 1000, α1 chains.
[4]nM/mg collagen. McClain et. al. (39).

Table 7
Radioactivity[1] of Skin Collagen After NaB^3H_4 Reduction

Age	Protein Level 25% Skin	25% HLNL	8% Skin	8% HLNL
42 day	141	-	232	-
Birth	265	770	505	1130

[1]CPM/mg collagen.
[2]HLNL = hydroxylysinonorleucine.

compared to animals on a 25% casein diet (65% G + 65% F); suggesting that the primary effect of protein free diets is on reduced collagen biosynthesis. The surprising result of this study was the markedly higher content of both salt and acid soluble fractions from rats on the fructose diets regardless of protein content of the diet (65% and 90% G vs. 65% and 90% F). These results suggest that carbohydrate source has a marked effect not only on collagen synthesis but also on the extent and type of intermolecular crosslink formed. The collagen from animals on the all protein diet (90% C) had solubility characteristics very similar to that from the 65% fructose regimen.

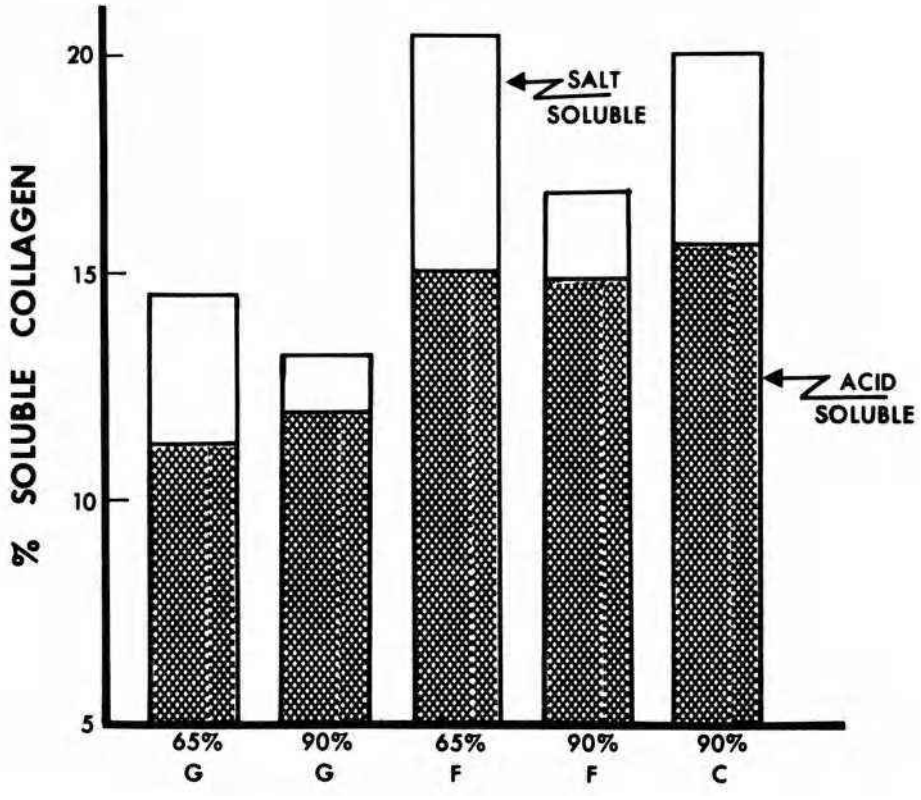


Fig. 4. Influence of dietary carbohydrate on collagen crosslinking. G = glucose, F = fructose, C = casein.

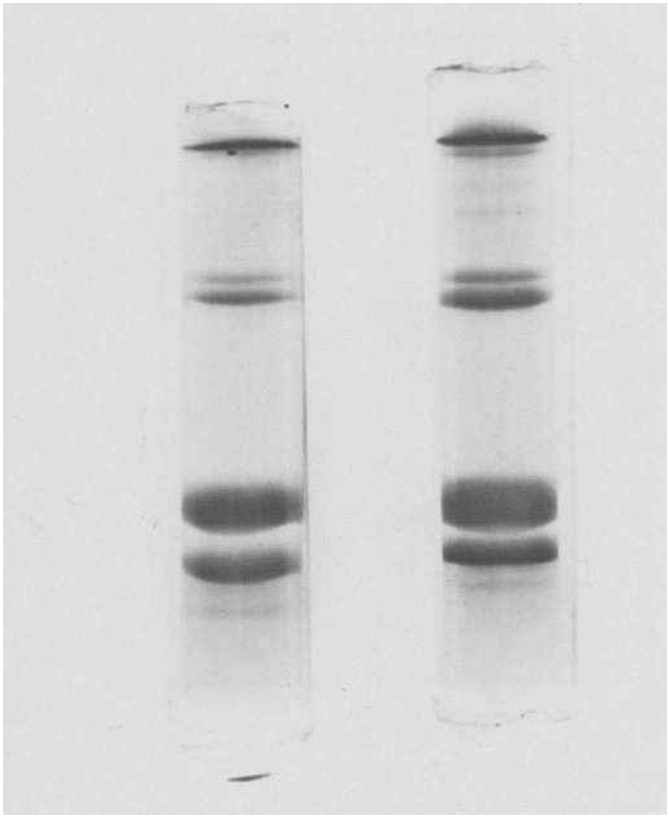


Fig. 5. Disc gel patterns of acid soluble collagen from animals on 25% casein (right) and protein free (left) diets. The bands from bottom to top are identified as $\alpha 2$, $\alpha 1$, $\beta 12$, $\beta 11$ and a trace of γ.

Figure 5 shows the disc gel electrophoresis patterns obtained from rats maintained on diets of 25% casein and protein free diets with glucose as the carbohydrate source (65% G vs. 90% G). The decreased extent of intramolecular crosslinking in the protein free diet is evidenced by the reduction in β components. These data are illustrated in graphic form in Fig. 6. Again the reduction in collagen crosslinking induced by protein free diets (90% G and 90% F) is evident. The influence of carbohydrate source is apparent in the reduced content in β components in animals on fructose diets (65% and 90% G vs. 65% and 90% F). The all protein diet (90% C) resulted in the least amount of crosslinking.

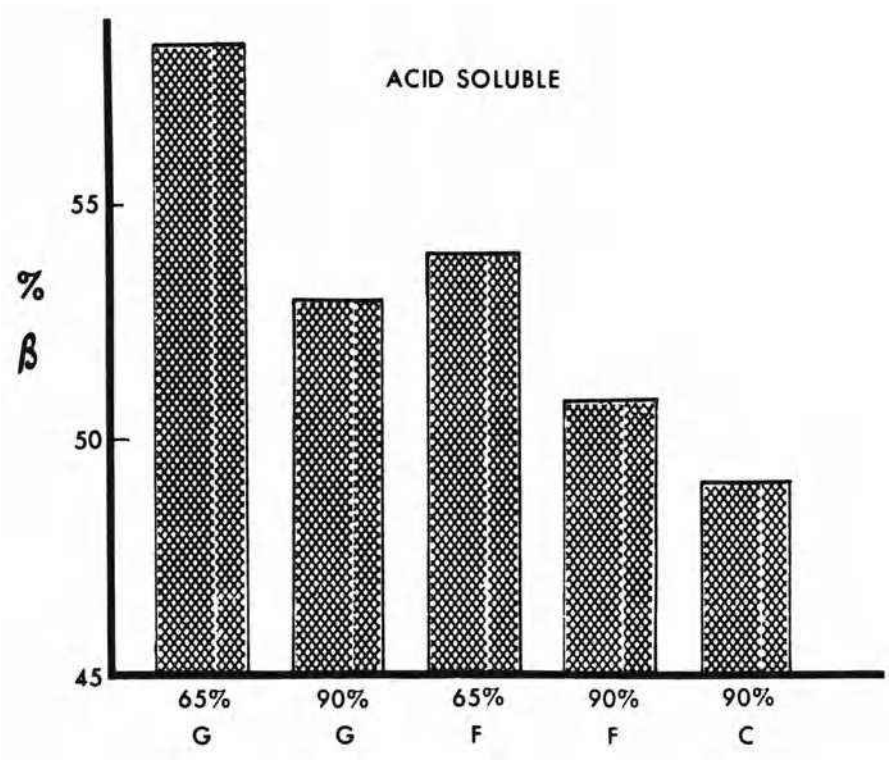


Fig. 6. Influence of dietary carbohydrate on collagen crosslinking. G = glucose, F = fructose, C = casein. McClain (10).

The mechanism whereby these dietary factors are affecting collagen crosslinking remains to be resolved. It is known however, that one manifestation of diabetes, a disease with abnormal carbohydrate metabolism, is an increase in the content of covalently bound carbohydrate in the collagen from the glomerular basement membrane (41). Perhaps dietary factors can induce similar post-synthetic modifications of collagen and thus alter the type and extent of crosslinking. In any case the results of these studies make it abundantly clear that various dietary regimens can elicit rapid and distinct changes in collagen metabolism and crosslinking.

Collagen Crosslinking and Longevity

It is now the general consensus of thought that the intrinsic capability of collagen to form crosslinks is probably not the primary cause of aging. Collagen does, however, play a major secondary adaptive role in the aging process. Its formation,

aggregation and crosslinking compensates for the death of parenchymal cells, injury to blood vessel endothelium, and the weakening of smooth and striated muscles, events which are known to be associated with aging (42). While normal maturation of collagen may not be harmful, any acceleration of the process could be.

Collagen is obviously harmful when it immobilizes a joint, interferes with the function of a glomerulus or when it accumulates in a damaged liver. The age-associated crosslinking of collagen, and the loss of elasticity in the arterial walls of the major vessels would certainly predispose the cardiovascular system to pathological deterioration (42). It is, therefore, logical to conclude that nutritional factors through their influence on the collagen aging or crosslinking process could detract from or enhance the physical well being and lifespan of man.

References

1. Wertheim, M. G., Ann. Chim. et Phys. 21, 385 (1847).
2. Rollhäuser, H., Gegenbaurs Morph. Jb. 90, 157 (1950).
3. Mendoza, S. A. and Milch, R. A., Gerontologia 10, 42 (1964).
4. Nimni, M. E., DeGuia, E. and Baretta, L. A., Nature 207, 865 (1965).
5. Verzar, F., Experientia 11, 230 (1955).
6. McClain, P. E., Pearson, A. M., Miller, E. R. and Dugan, L. R., Biochem. Biophys. Acta 168, 143 (1968).
7. Brown, P. C. and Consden, R., Nature 181, 349 (1958).
8. Schaub, M. C., Gerontologia 8, 114 (1963).
9. Verzar, F., Gerontologia 4, 105 (1960).
10. McClain, P. E., Proc. 29th Ann. Rec. Meat Conf. In Press (1976).
11. McClain, P. E., Kuntz, E. and Pearson, A. M., Agr. and Food Chem. 17, 629 (1969).
12. Shimokamaki, M., Elsden, D. F. and Bailey, A. J., J. Food Sci. 37, 892 (1972).
13. Bailey, A. J., Robins, S. P. and Balian, G., Nature, 251, 105 (1974).
14. Prockop, D., Kaplan, A. and Undenfriend, S., Arch. Biochem. Biophys. 101, 499 (1963).
15. Rhoads, R. E. and Undenfriend, S., Proc. Natl. Acad. Sci. U. S. 60, 1473 (1968).
16. Bhatnagar, R. S., Rapaka, S. R., Liu, R. Z. and Wolfe, S. M., Biochim. Biophys. Acta 271, 125 (1972).
17. Hayaishi, O., In: Biological Oxidations, Ed. T. P. Singer, New York, Interscience P. 581 (1968).
18. Hutton, J J., Jr., Tappel, A. L. and Undenfriend, S., Arch. Biochem. Biophys. 118, 231 (1967).
19. Stassen, F. L. H., Gardinale, G. J. and Unden Friend, S., Proc. Natl. Acad. Sci. U. S., 70, 1090 (1973).

20. Berg, R. A. and Prockop, D. J., Biochem. Biophys. Res. Commun., 52, 115 (1973).
21. Jimenez, S., Harsch, M. and Rosenbloom, J., Biochem. Biophys. Res. Commun., 52, 106 (1973).
22. Toole, B. P., Kang, A. H., Trelstad, R. L. and Gross, J., Biochem. J. 127, 715 (1972).
23. Barnes, M. J., Constable, B. J., Morton, L. F. and Kodicek, E., Biochem. J. 132, 113 (1973).
24. Mechanic, G. L., Toverud, S. V. and Ramp, W. K. Biochem. Biophys. Res. Commun. 47, 760 (1972).
25. Asling, C. W. and Hurley, L. S., Clin. Orthoped. 23, 213 (1963).
26. Shields, G. S., Coulson, W. F., Kimball, D. A., Carnes, W. H., Cartwright, G. E. and Wintrobe, M. M., Am. J. Pathol. 41, 603 (1962).
27. O'Dell, B. L., Hardwick, B. C., Reynold, G. and Savage, J. E., Proc. Soc. Exptl. Biol. Med., 108, 402 (1961).
28. Chou, W. S., Savage, J. E. and O'Dell, B. L., Proc. Soc. Exp. Biol. Med., 128, 948 (1968).
29. Harris, E. D. and O'Dell, B. L., Adv. in Exp. Med. and Biol. 48, 267 (1974).
30. Savage, J. E., Bird, D. W., Reynolds, G. and O'Dell, B. L. J. Nutr. 88, 15 (1966).
31. Todd, W. R., Elvejhem, C. A. and Hart, H. B., Am. J. Physiol. 107, 146 (1934).
32. Pories, W. J., Henzel, J. H., Rob, C. G. and Strain, W. H., Lancet 1, 121 (1967).
33. McClain, P. E., Wiley, E. R., Beecher, G. R., Anthony, W. L. and Hsu, J. M., Biochem. Biophys. Acta 304, 457 (1973).
34. Pories, W. J., Henzel, J. H., Rob, C. G. and Strain, W. H., Ann. Surg. 165, 432 (1967).
35. McKay, C. M., Amer. J. Publ. Hlth. 37, 521 (1947).
36. Chvapil, M. and Hruza, Z., Gerontologia 3, 241 (1959).
37. Deyl, Z. and Rosmus, J., Exp. Geront. 3, 177 (1968).
38. Prasad, C. U. and Bose, S. M., Nutr. Metabol. 16, 172 (1974).
39. McClain, P. E., Wiley, E. R. and Beecher, G. R., Nutr. Rep. Int. 12, 317 (1975).
40. McClain, P. E. and Wiley, E. R. Fed. Proc. 30, 402A (1971).
41. Beisswenger, P. J. and Spiro, R. G. Science, 168, 596 (1970).
42. Sinex, F. M., In: Treatise on Collagen Ed. B. S. Gould. New York: Academic Press p. 409 (1968).

35

NUTRITIONAL COPPER DEFICIENCY AND PENICILLAMINE ADMINISTRATION: SOME EFFECTS ON BONE COLLAGEN AND ARTERIAL ELASTIN CROSSLINKING

R.B. Rucker*, JoAnn Murray*, and R.S. Riggins**

Departments of Nutrition* and Orthopaedic Surgery**

College of Agriculture and Environmental Science* and

School of Medicine**, University of California, Davis,

California, 95616

ABSTRACT

Nutritional copper deficiency effects marked changes in the crosslinking of collagen and elastin, presumably in relationship to copper's role as a cofactor for lysyl oxidase. Lysyl oxidase controls one of the initial steps in the crosslinking of elastin and collagen, i.e., the conversion of peptidyl lysine or hydroxylysine residues to peptidyl α-aminoadipic-δ-semialdehyde derivatives. Once lysine-derived aldehydic functions in collagen and elastin are formed, crosslinks occur via aldol and Schiff-base type condensations. A decrease in the degree of crosslinking results in changes in the biomechanical properties of both collagen- and elastin-rich tissues. Some of these changes are described with respect to chick bone and aorta. Likewise, penicillamine blocks crosslinking reactions. In this case, however, it is probably because of the formation of thiazolidine complexes between penicillamine aldehydic functions. The administration of penicillamine at different levels to young growing chicks allows the isolation of fibrous insoluble elastin varying in aldehyde content.

INTRODUCTION

Copper is a cofactor for a variety of oxidases. One such oxidase, lysyl oxidase, is critical in the initial steps involving

synthesis of amino acid crosslinks in collagen and elastin. The enzyme catalyzes the conversion of specific residues of lysine and hydroxylysine in collagen and lysine in elastin to peptidyl α-amino adipic-δ-semialdehyde or its hydroxylated derivative (Gallop et al., 1972). Once aldehydes are formed they in turn condense with other modified or non-modified lysyl residues to form a variety of Schiff-base and aldol condensation products. The nature of some of these condensation products in elastin and collagen will be described in considerable detail in the papers which follow from the laboratories of Drs. Anwar, Tanzer and Mechanic. Key to the relationships described in our report is the dependency of lysyl oxidase on nutritional copper status (Harris et al., 1974). With copper-deficiency the levels of lysyl oxidase are reduced and concommitantly this appears to result in an overall decrease in the formation of collagen or elastin crosslinks (Sandberg, 1976; Barrow et al., 1974).

When elastin or collagen crosslinking is reduced in animals either experimentally or for other reasons, defects in the integrity of connective tissue components are observed (Carnes, 1971; Hill et al., 1967). Since the comments reported here will be confined to only our experiences using nutritional copper-deficiency and penicillamine to study collagen and elastin, other reviews should also be consulted which deal with the effects of lathyrogens, such as β-aminopropionitrile (BAPN) and related compounds on elastin and collagen crosslinking (Gallop and Paz, 1975; Sandberg, 1976; Franzblau, 1971; Rucker and Tinker, in press; Barrow et al., 1974).

MATERIALS AND METHODS

Studies on the Biomechanical Properties of Bone

Experimental Diets. White leghorn cockerols were fed copper deficient (<1 ppm) and supplemented (25 ppm) diets from the day of hatching. The diet was based on skim milk. Its composition has been described by Starcher et al., 1964. The birds were housed in conventional brooders with stainless steel feeders and supplied distilled water. In addition, for one of the experiments related to the bone studies, groups of day old birds were also fed a calcium deficient (0.2% Ca) and supplemented (1% Ca) diet containing 0.6% phosphorus (Chan et al., 1976).

Studies related to bone strength measurements. Three experiments were performed. In experiment I, two hundred day old chicks were randomly assigned to four experimental groups and fed either the copper deficient or supplemented diets or the calcium deficient or supplemented diets for 20 days.

The birds were then killed, and the femurs, tibias and aortas were removed. In experiment II, two groups of day old chicks were fed the copper deficient or supplemented diet. In this experiment food intake was restricted daily in the control group to that of the deficient group to minimize differences in weight gain.

The criteria used to assess the degree of copper deficiency were the estimation of insoluble elastin (Starcher et al., 1964), increased mortality resulting from dissecting aneurysms, and growth. Calcium deficiency was assessed primarily from the measurements of growth and the content of bone ash (Chan et al., 1976). In these experiments, the most critical measurements were the estimate of bone collagen solubility (Rucker et al., 1969), total collagen (Woessner, 1961), percentage ash, cortical thickness, bone diameter, and tortional breaking strength (Riggins et al., 1974).

In experiment III, femurs obtained from copper deficient or supplemented birds were also used. However, the tortional breaking strength measurements were obtained using femurs treated with solutions of formaldehyde and sodium borohydride in order to introduce chemically derived crosslinks in the organic matrix (Balian et al., 1969). First, the right and left femurs were removed from individual chicks (16-day old). One of the femurs was incubated in a solution containing 0.9% NaCl buffered at pH 7.6 with 0.05 M sodium phosphate as buffer (24 hours at 4°C). The corresponding femur was incubated in the same solution but in addition formaldehyde was added at 0.1 M. After 12 hours at 4°C the femurs in the formaldehyde solution were washed with saline and incubated an additional 12 hours at 4° in buffered 0.9% NaCl containing 5 mg/ml $NaBH_4$. It was assumed that the formaldehyde treatment followed by $NaBH_4$ reduction would result in both the formation of reduced aldimine- and aldol-derived crosslinks in the bone collagen matrix and also introduce methylene crosslinks derived from formaldehyde. Breaking strength measurements were performed immediately following these incubations.

Collagen and ash determinations. The total collagen and ash determinations were performed using tibia taken from individual chicks. The tibia was first cleansed of adhering tissue, lyophilized and the dry bone was powdered by means of a Wiley Mill. An aliquot of the powder was then either ashed for the determination of percentage mineral or hydrolyzed in 6 HCl (24 hours, 110°) to estimate hydroxypropline (Woessner, 1961). It was assumed that one mg of hydroxyproline was equivalent to 7.7 mg of bone collagen (Rucker et al., 1969).

In the collagen solubility studies, powdered tibia samples were also used. In this case fresh bone was cleansed, weighed and homogenized in 0.02 M TRIS buffered (pH 7.0) containing 5 M guanidine-HCl (cf. Rucker et al., 1969 and references cited). Three 24 hour serial extractions were performed at 4°. The soluble components were separated by centrifugation (25000 x g, 60 min.) and the extracts were pooled. An aliquot was then hydrolyzed for the estimation of hydroxyproline (Woessner, 1961).

Measurement of bone breaking strength. For the determination of bone breaking strength, an apparatus designed to measure tortional strength was used. Detailed descriptions of the apparatus and procedures have been published (Riggins et al., 1974; Rucker et al., 1975). The measures obtained were torque (rotary force applied to the bone) and deformation angle (the degree of rotation with respect to the applied torque). In one of the experiments, the amount of strain was also measured. Strain is defined as the radians traversed during fracture divided by bone length. This value is directly related to the angle of deformation occurring during a tortional fracture. Cortical thickness and the diameter of the femurs were also measured from the bone fragments by means of a micrometer.

Other measures. Samples of bone collagen were also characterized as described previously by Miller et al. (1967). Two hundred cylinders of diaphyseal bone (femur and tibia) from normal and copper-deficient chicks were used. In addition an estimation of lysyl oxidase was performed using methods adapted from Siegel et al. (1970), Pinnell and Martin (1968), and Harris et al. (1974). Bone cartilage was used as the source of the enzyme. For these studies the birds were fed the copper-deficient and supplemented diets for 20 days immediately following hatching.

Studies on the Action of Penicillamine

Penicillamine administration. Day-old chicks fed a conventional starter ration were used. Penicillamine in saline was administered intraparentineally and daily in amounts of 0, 25, 50, 100, or 200 mg/kg body weight from the day of hatching for 20 days. Higher doses were not used, because in preliminary studies it was observed that amounts of penicillamine greater than 200 mg/kg body weight were extremely toxic. When the birds were killed, samples of thoracic aorta and Achilles tendon were removed for the isolation of aortic insoluble elastin and to estimate collagen solubility.

Elastin and collagen studies. Tendon collagen was extracted as described by Nimni et al. (1969). The extractability of collagen was used primarily as an index to estimate the degree to

which the chicks responded to penicillamine. Penicillamine presumably blocks crosslinking because of the formation of thiazolidine complexes with prefromed aldehydes in connective tissue proteins (see Discussion). This in part was the rationale for the administration of penicillamine to chicks. We wished to obtain elastins which varied in aldehyde content. In this regard, insoluble elastin was isolated using a non-degradative procedure similar to that described by Miller and Fullmer (1966). One to two grams of aorta were used for each isolation. The aldehyde content of the final insoluble elastin products were determined as described by Miller and Fullmer (1966) after elastase digestion using a method adapted after the procedure of Paz et al. (1965).

Arterial Elastin Studies

Isolation of chick arterial soluble elastin. Routinely 200 to 500 aortas from chicks fed the copper-deficient diet are used as starting material. Procedures for the isolation have been published previously. The criteria for purity are based on electrophoretic mobility, amino acid composition, N- and C-terminal amino acid sequences, and molecular size (Rucker et al., 1975).

Interaction of soluble elastin with carbonyl functions. Two types of experiments were performed. In first series of experiments pyridoxal-5'-phosphate (PLP) was used as a model carbonyl agent. In the other series of experiments, insoluble elastin preparations were used obtained from the birds given penicillamine.

For the PLP-elastin binding studies, chick soluble elastin and PLP were dissolved in 0.05 M phosphate buffer (pH 7.5) containing 0.5 M NaCl. Concentrations of PLP in the equilibrium mixtures were varied from 0 to 20 mM. Soluble elastin was added at 1 mg and adjusted with the buffer solution to give a final volume of 1 ml. The reactions were carried out for 60 minutes at 25°. After completion of the reaction, the mixtures were reduced by the addition of $NaBH_4$ (10 mg/ml in water). Three separate additions of the $NaBH_4$ solution (0.15 ml) were made at 15 minute intervals. Following the last addition of $NaBH_4$, the solutions were adjusted 2 ml with 0.1 M acetic acid and then exhaustively dialyzed at 6° to remove excess reduced PLP. The solutions were passed through a millipore filter and the amount of ε-N-PLP-elastin was measured at 323 nm against a reduced soluble elastin blank. The molar absorbance of peptidyl ε-N-pyridoxyl lysine was taken as 5800 and the molecular weight of soluble elastin as approximately 70,000 (Rucker et al., 1973).

In the second experiment, insoluble elastin preparations with aldehyde contents of 5.2 μmoles/100 mg or 11.8 μmoles/100 mg were used (see Results). For the ligand arterial soluble elastin from arterial extracts was used in which soluble elastin appeared to be the primary product labeled with ^{3}H-lysine. The labeled soluble elastin was prepared from normal chick aortas after incubation in a medium containing ^{3}H-lysine. The details for the incubation have been described previously (Lee et al., in press). Briefly, after incubation the aortas were extracted with 0.5 M acetic acid. Carrier soluble elastin (0.5 mg/ml) was then added to the extract and sodium chloride was added to 1.0 M. These steps were performed at 4°. After adjustment of the pH to 7.6 with disodium phosphate, the mixture was heated to 31° and held for 20 minutes.

A property of soluble elastin is its ability to coacervate and precipitate under these conditions. This provided a way of partially separating elastin from other proteins (cf. Smith et al., 1968 and 1972). After centrifugation the coacervate was redissolved and applied to a column of G-150 Sephadex for further purification (Rucker et al., 1975). Two protein fractions were isolated. The major peak containing radioactivity represented a 70,000 dalton fraction and appeared to be mostly soluble elastin with respect to labeled material. The second fraction represented mostly protein eluting as 20-40,000 dalton subunits (see Results). To estimate size, the distribution of radioactive label in the two fractions was assessed using a polyacrylamide gel electrophoresis technique employing sodium dodecyl sulfate (SDS) in the buffers (Rucker et al., 1975). The amino acid composition of the two fraction was also determined.

With the two soluble elastin fractions and the two preparations of insoluble elastin containing differing amounts of aldehydic functions (see Results), several experiments were then performed to determine the interaction between radioactively labeled soluble arterial protein and insoluble elastin. The radioactively labeled protein fractions were added in amounts ranging from 0 to 500 μg/mg of insoluble elastin in two milliliters of HEPES buffer (pH 7.0). After 12 hours of equilibration, the reaction mixtures were reduced with $NaBH_4$ as described above in the studies on the interaction between PLP and elastin. The reduced insoluble residue was then centrifuged and washed repeatedly with 0.5 M acetic acid. This step was followed by a wash with solution containing H_2O:n-propanal-n-butanol (2:3:5). The rationale for the wash with the aqueous alcohol mixture was taken from the observations of Sandberg et al. (1975).

The insoluble residues were then hydrolyzed (48 h, 110°, 6N HCl). The amount of soluble elastin bound was determined by the estimation of the radioactivity in the hydrolyzates. Portions of

the hydrolyzates were also electrophoresced using a Savant high voltage electrophoresis apparatus according to the method of Moczar et al. (1974). This method easily separates most the crosslinking amino acids from lysine. It was assumed that radioactivity migrating as crosslinks was derived from lysine.

Metabolic studies. Lastly, attempts have been made to estimate the effect that nutritional copper deficiency has on the metabolism and turnover arterial elastin. Some of this work has been summarized elsewhere (Rucker and Riemann, 1972; Rucker et al., 1973; Rucker et al., 1975; Lee et al., in press). For the studies reported here two groups of day old chicks were fed the copper-deficient or supplemented diet for 20 days. Selected birds from each group were then injected with 250 μCi ^{3}H-valine/kg of body weight. At 3, 6, 12, 36, or 72 hours post injection, 6 birds from each of the groups were killed. The aortas were removed and homogenized individually using a glass homogenizer in a solution containing 0.5 M NaCl and 0.05 M phosphate buffer (pH 7.6). Attempts were then made to isolate crude fractions of soluble elastin. The details of these steps have been described (Rucker et al., 1975). Briefly, the radioactivity and protein isolated by coacervation (Smith et al., 1968) were estimated. In addition, the protein and radioactivity in the fractions were characterized by means of polyacrylamide gel electrophoresis using a cationic system in which 4M urea was incorporated into the separating gel (Rucker et al., 1975).

RESULTS

Studies on the Biomechanical Properties of Bone

Figure 1 shows a typical stress strain curve for bones from chicks fed diets in which the copper or calcium content had been altered (experiment I). The curves from bones from chicks fed adequate diets indicate a much higher stress per unit strain than bones from deficient birds. The major portions of the strain are elastic, but just prior to fracture plastic deformation occurs, i.e., a low stress (torque) per unit strain. In contrast the bone from copper deficient chicks exhibit deformation under less stress and no plastic deformation prior to fracture. The bone from calcium deficient chicks has the least rigidity, but considerable plastic deformation prior to fracture. These observations relate primarily to the effects of the different diets on diaphyseal bone. The articular ends of the bone were firmly potted in molds containing a dental acrylic. Upon hardening the ends which were molded in acrylic were then fastened to a freely rotating grip. The amount or length of bone shaft which was exposed to torsion was constant. In most instances the bones fractured at the mid section of the shaft.

TABLE 1

Dietary copper and calcium restrictions and their relationships to the amount of torque and deformation required to fracture chick femur

Diet	Torque	Joules
	Newton-meters	10^3
A. 1% Ca, 0.6% P	0.23 ± 0.02	8.7 ± 1.3
B. Skim milk, 25 ppm Cu	0.22 ± 0.06	8.6 ± 1.2
C. Skim milk, <1 ppm Cu	0.10 ± 0.01	2.4 ± 0.6
D. 0.2% Ca, 0.6% P	0.03 ± 0.01	1.3 ± 0.3

Each value is the average of 8-10 determinations ± S.E.M. The total energy associated with fracture is expressed as Joules and is proportional to the area under the curves obtained from plots of torque vs. deformation angle.

The values given in Table 1 summarize the average values for torque obtained from this experiment. The total energy (Joules) required for fracture is also given. In Table 2 are values which indicate differences observed in the mineral and collagen content of bone after the various treatments. Also, values for lysyl oxidase are given (note: Footnote to Table 2). It was observed that a reduction in lysyl oxidase occurred. Although there was little difference in the total amount of collagen in bones for copper-deficient and supplemented chicks, the collagen in bone from the copper-deficient chicks was more easily solubilized than that from normal chicks. Likewise, the data given in Figure 2 also represents a measure of decreased bone collagen crosslinking. For this experiment, chick bone was extracted and purified as described by Miller et al. (1965) using diaphyseal bone from copper-deficient and sufficient chicks. The decrease in the amount of crosslinking is reflected qualitatively by the greater percentage of total α-chains of collagen to β-collagen (dimerized collagen α-chains) in the extracts from copper-deficient birds. The ratio for the amount of α_1- to α_2-chains in bone collagen was approximately two to one, respectively. The increase in the amount of β_{12}-collagen reflects an increase in the amount of α_1-collagen crosslinked to α_2-collagen. Similar results were also obtained using polyacrylamide gel electrophoresis techniques to assess the nature and amounts of monomer to dimer collagen in the bone extracts.

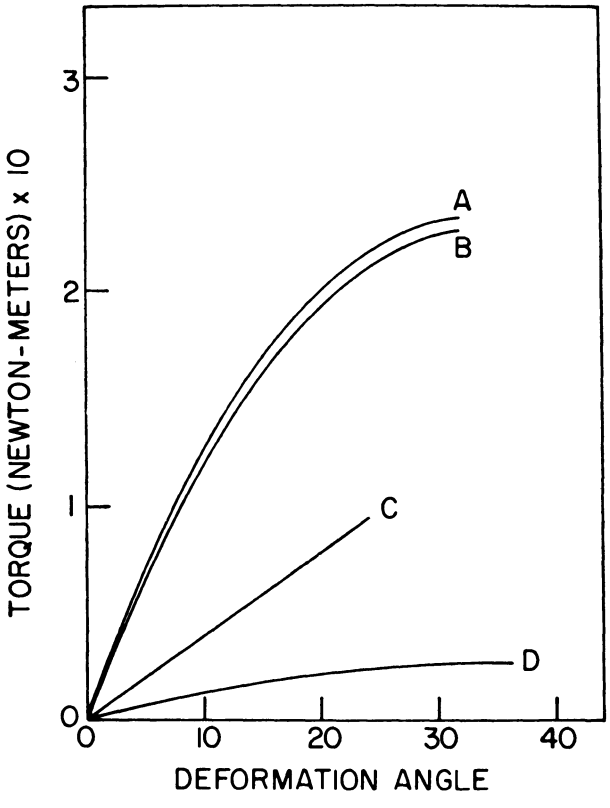


Fig. 1. Breaking strength curves. The curves were obtained using fresh femur from 20-day-old chicks fed a semi-purified diet containing 1% Ca and 0.6% P (A); a skim milk based diet with 25 ppm copper (B); a skim milk diet with <1 ppm copper (C); or a semi-purified diet containing 0.2% Ca and 0.6% P (D). The curves represent typical tracings from a device designed to measure torque and deformation angle during bone fracture.

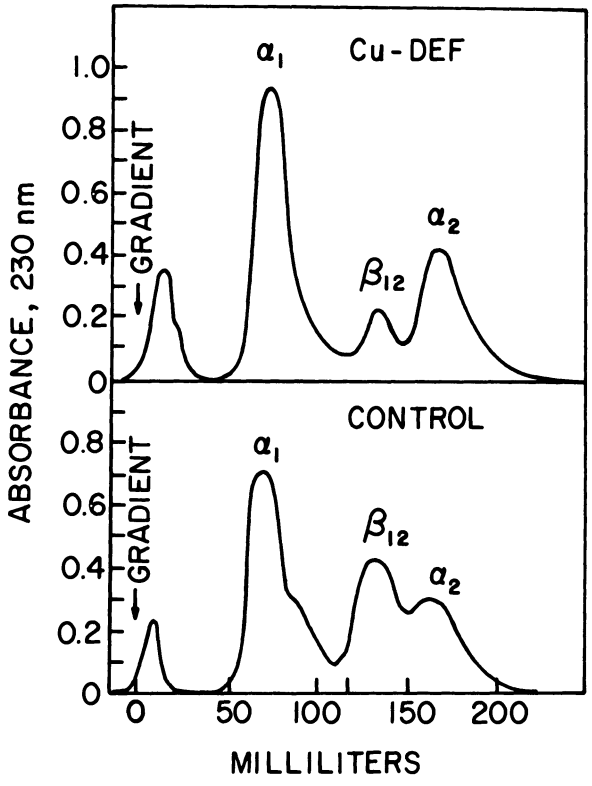


Fig. 2. Carboxymethyl cellulose chromatography of bone collagen extracted with 0.5 M acetic acid. Conditions were identical to those reported by Miller et al. (1965). The β-collagens migrate between the two α-chains.

Because variations in body weight markedly effect bone size and subsequently bone strain (Figure 3), another experiment was also performed in order to rule out factors related to differences in chick growth (Experiment 2). In this case, only copper deficient and supplemented birds were used and food was restricted in the control group so that weight gain did not differ from that for the deficient group. As in the first experiment the measurements for torque and deformation were reduced when the values obtained from copper deficient birds were compared with those from the control (Table 3). Growth restriction in the control group resulted in femurs of approximately the same cortical thickness and diameter as those in the copper deficient group. The characteristics of the stress strain in the bone from copper deficient were identical to those shown in Figure 1.

TABLE 2

Dietary copper and calcium restriction and their effects on chick weight, bone corticle thickness, bone ash, bone collagen and cartilage lysyl oxidase

	Dietary Treatments			
	1% Ca (A)	Skim milk +Cu (B)	Skim milk -Cu (C)	0.2% Ca (D)
Body Wt. (g)	222 ± 21	214 ± 15	132 ± 18	127 ± 12
Cortical Thickness (mm)	0.53 ± 0.08	0.44 ± 0.03	0.27 ± 0.02	0.21 ± 0.03
Bone Ash (%)	40 ± 2	39 ± 2	36 ± 3	21 ± 3
Total Collagen (%)	47 ± 2	48 ± 2	48 ± 2	68 ± 3
Collagen Solubility (mg/g)	3.6 ± 1.0	4.1 ± 1.2	15.2 ± 3.1	5.8 ± 2.0
Lysyl Oxidase (cpm/g)	---	11068 ± 1028	---	168 ± 52

The birds were 20-days old at the time of measurements. The percentage bone ash and collagen are expressed in relationship to fat-free bone. Collagen solubility represents the mg collagen extracted per g of fresh bone. Lysyl oxidase represents the cpm of ^{3}H released when 1 ml of a urea extract from 1 gram of bone cartilage was used. The gram of cartilage was extracted 2x with 10 ml of 6M urea (pH 7.6), dialyzed and then an aliquot was incubated with substrate (300000 cpm ^{3}H) for 5 hours at 40°C (cf. Pinnell and Martin, 1968).

Since the effects of copper deficiency on bone strength appeared to be independent of alterations in growth, mineral content, cortical thickness, and bone diameter, it was felt crosslinking was probably the major feature related to increased fragility. However, to confirm this Experiment 3 was performed in which an attempt was made to introduce artificial crosslinks into bone. It was felt there should be an increase in the degree

TABLE 3

Properties of bone from copper-deficient and supplemented chicks of the same body weight

Diet	Body Weight	Ash	Cortical Thickness	Torque	Deformation
	g	%		Newton-meters	radians/cm
Skim Milk + Cu	156 ± 23	39	0.28±0.04	0.15±0.03	0.16±0.01
Skim Milk - Cu	148 ± 15	37	0.27±0.04	0.08±0.04	0.12±0.02

Average of 8-10 determinations. Deformation in this case is expressed as the angle traversed during fracture converted to radians ÷ the length of bone undergoing fracture.

of deformation and the amount of torque required to fracture bone from copper deficient chicks after introduction of crosslinks. As shown in Figure 4, stress strain curves for copper-deficient bone after formaldehyde treatment and $NaBH_4$ reduction were similar to those obtained of bone from control birds. The curves given in Figure 4 represent composites for 6 independent determinations. At the moment of fracture the amount of deformation (strain) observed for control and treated copper deficient bone was significantly greater than that observed for nontreated bones from copper deficient chicks.

Studies on the Action of Penicillamine

The effects of penicillamine on collagen and elastin crosslinking in the chick were less dramatic than those from severe copper deficiency. Figure 5 gives results related to the extractability of collagen and elastin from the chick tendon and aorta, respectively. Also, values for the aldehyde content of insoluble elastin are given. Extraction of chick tendon collagen with 0.15 M NaCl, 0.5 M NaCl and citrate buffer indicated that the collagen from penicillamine treated birds at the highest level was 3 to 4 times more soluble than that from controls. Although elastin solubility was not affected, the aldehyde content of insoluble elastin from the aortas of penicillamine-treated chicks did increase in relationship to the administered dose. Not shown is the

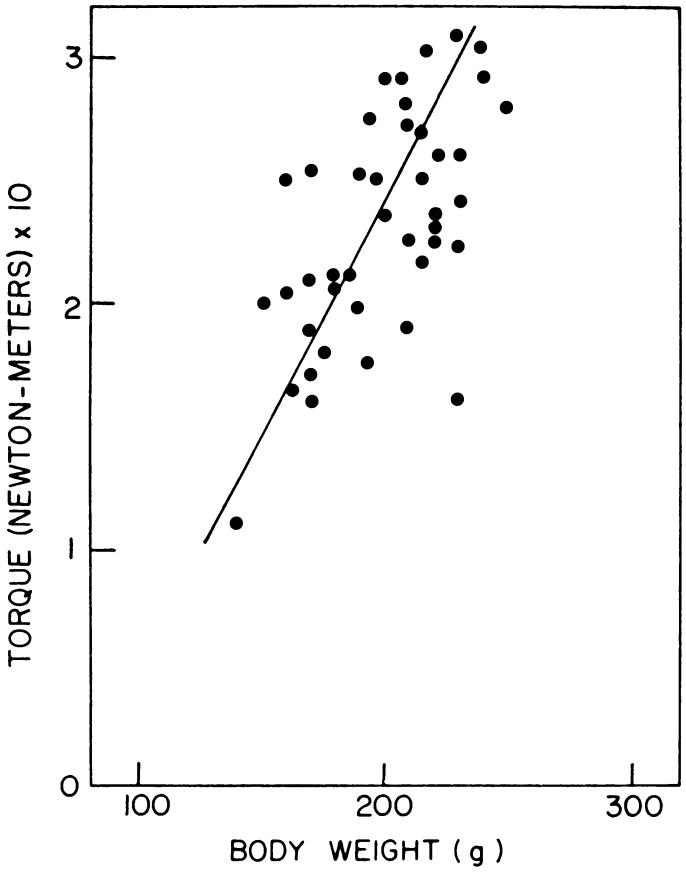


Fig. 3. Relationship between bone strength and chick body weight (20-day-old chicks). For some of the birds food intake was restricted to provide variation in body weight. They were all fed a normal diet.

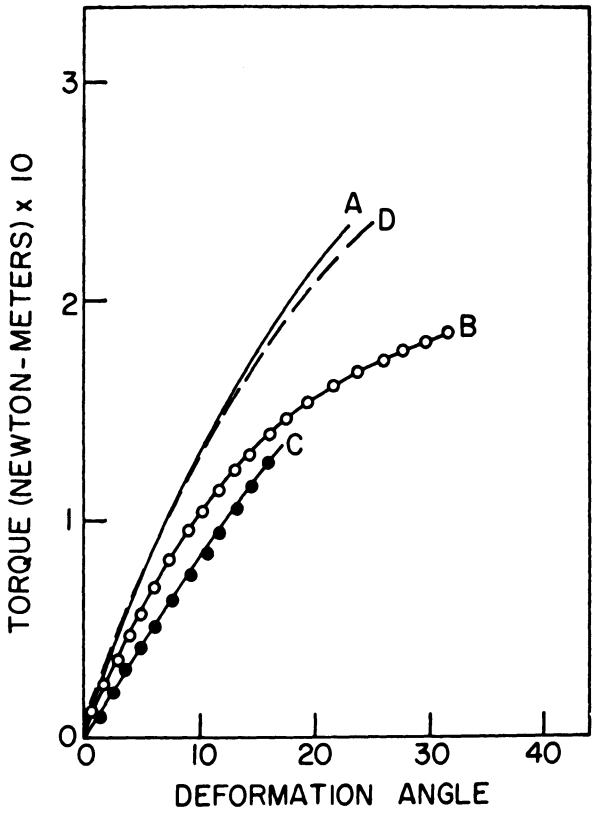


Fig. 4. Effect of formaldehyde and sodium borohydride treatment on bone strength. Curves A and C represent measurements from control (——) and copper-deficient chikc (– – – –) bone pretreated (24 hours) with only a buffered solution of 0.9% NaCl before fracture, respectively. Curves B and D represent control (●——●) and copper-deficient chick (●●●) bones pretreated with buffered 0.1 M formaldehyde (12 hours) followed by sodium borohydride reduction (5 mg/ml, 12 hours), respectively.

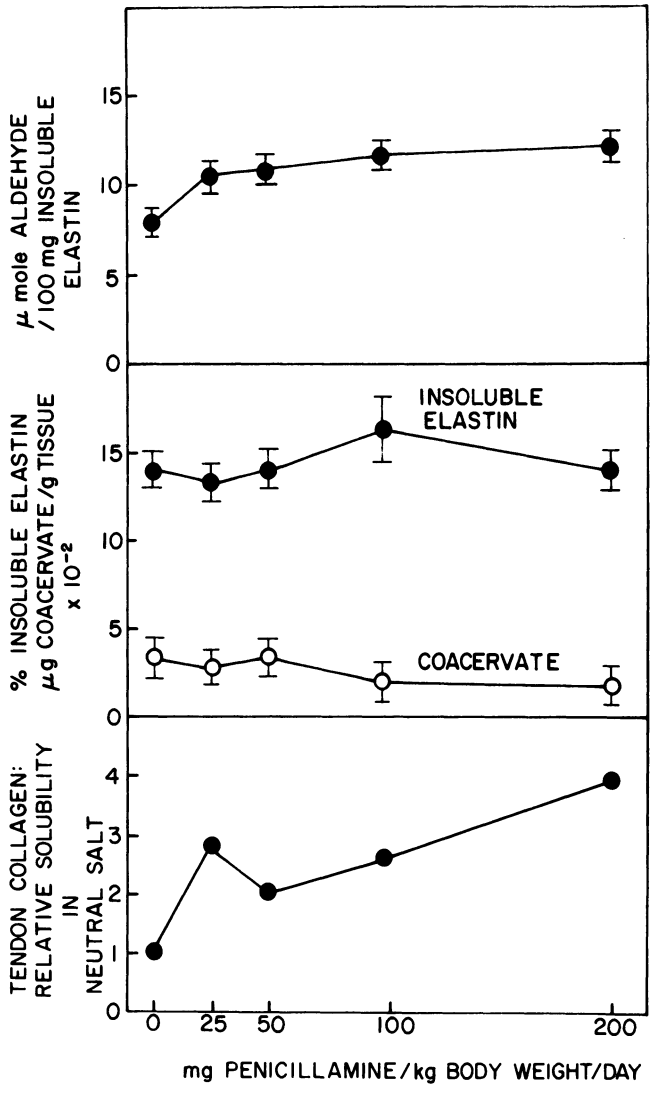


Fig. 5. Effects of penicillamine on the extractability of chick tendon collagen and the aldehyde content of aorta elastin. For collagen, the relative values were obtained by dividing the total amount of collagen at each penicillamine dose by the amount extracted from the control which was injected only with saline. The term coacervate is probably misused in the figure. Before the values for insoluble elastin were obtained (see text), the aortas were extracted with 0.05 M phosphate buffer (7.6) containing 0.5 M NaCl. After centrifugation, the extract was heated to 40°C for one hour and recentrifuged. The values represent the total amount of protein which coacervated or precipitated/g fresh aorta.

value for the aldehyde content of copper-deficient insoluble elastin. Approximately 5.2 mmoles aldehyde/100 mg insoluble elastin was present in this elastin. This value (60% of the control) would be expected with a decrease in lysyl oxidase.

Studies on Arterial Elastin

The amino acid composition of soluble elastin and its comparison to insoluble elastin from normal and copper deficient chicks is shown in Table 4. It should be noted that there is an increase in the amount of lysine in the insoluble arterial elastin fraction from copper-deficient compared to that from control birds. This is presumably due to a decrease in lysyl oxidase and concommitantly the decrease in the oxidation of lysine to α-amino adipic-δ-semialdehyde. Other details on the role of soluble elastin as a precursor to insoluble elastin and its isolation and properties are covered in the reviews mentioned previously.

With respect to the investigation related to the interaction of soluble elastins with carbonyl functions, the data in Figure 6 indicated the binding of PLP to soluble elastin after equilibration and reaction. PLP binds to soluble elastin to form a Schiff-base product with peptidyl lysines in the protein (Rucker et al., 1973). The nature of the binding curve suggests little if any cooperative binding effects. A Hill plot of the values obtained at different pyridoxal concentrations gave essentially a straight line (cf. Rucker et al., 1973). The stability constant for the reaction was 5,100.

The maximum number of available lysyl residues in soluble elastin that reacted with PLP was determined from the Schatchard plot shown in Figure 7. The values for $\bar{v}$ were calculated from the initial binding data given in Figure 6. This value is equivalent to the moles of lysine per mole of soluble elastin reacting with PLP assuming a molecular weight of approximately 70,000. Twenty-five residues of lysine represents about 80% of the total lysine residues in soluble elastin, i.e., 32-33 residues/mole. Furthermore, in order to determine the stoichiometry of binding, samples or reduced PLP-soluble elastin were also hydrolyzed and the total amount of lysine in selected samples were determined by ion-exchange chromatography using an amino acid analyzer. The lysine residues missing after reaction of PLP with soluble elastin were in good agreement with the ε-N-pyridoxyl lysine content determined spectrophotometrically (Table 5). It should also be noted that temperature had little effect on the amount of pyridoxal-phosphate bound to soluble elastin. Soluble elastin usually coacervates between 20 and 25 degrees in the presence of 0.5 M NaCl. Reaction mixtures above 20° were turbid in appearance, i.e., coacervated, but apparently this had little effect on the binding of lysyl groups by PLP (cf. Figure 7).

TABLE 4

Amino acid composition of chick tropoelastin expressed as residues per 1000 residues

Amino Acid	Soluble Elastin	Insoluble Elastin	
		Deficient	Supplemented
Lys	44	21	6
Arg	5	7	6
Hyp	9	12	10
Asp*	8	6	6
Thr	9	10	15
Ser	15	17	15
Glu*	18	17	17
Pro	128	131	136
Gly	328	332	336
Ala	173	173	173
Val	172	172	173
Ile	16	20	22
Leu	48	46	47
Tyr	10	11	12
Phe	17	21	20
Des**	-	1	2
LNL**	-	<1	1
Other***	-	2	5

* Approximately 80 amidated (Kagan and Leach, 1976).

** Total desmosines,

*** Equivalent to leucine, assumed to be crosslinks.

TABLE 5

Cromatographic analysis of lysine in soluble elastin after equilibration and reduction in the presence of pyridoxyl-5'-phosphate.

Sample	Lysine content residues/1000	ε-N-pyridoxyl lysine content residues/1000	Total lysine residues/1000
1. No additions	42	0	42
2. Pyridoxyl added and reduced	8	32	40

Some of the data for studies in which aldehyde-rich or poor insoluble elastin and radioactively labeled soluble elastin were used are shown in Figures 8 and 9. Figure 8 contains data related to the characterization of the soluble radioactively labeled protein from aortas. Figure 9 shows the association curves following the incubation and reduction of the insoluble elastin fractions with radioactively labeled soluble arterial protein.

As shown in Figure 8 most of the radioactively labeled lysine eluted at the void volume. Fractions I and II were collected and lyophilized. The column buffer was 0.2M pyridine acetate (pH 5.6) and was easily removed upon lyophilization (cf. Rucker et al., 1975). Aorta from young chicks (2 to 4 wks) incorporate a large percentage of radioactivity labeled amino acids into elastin (Lee et al., in press). The birds used to prepare the labeled ligands were 3 weeks old. It is necessary to note, however, that based on amino acid composition 20% of the protein in fraction I was collagen. Fraction II contained about 40% elastin-like peptides and 60% of the protein appeared to have the composition of microfibrillar protein (Ross, 1974). Although carrier was added and a partial isolation of elastin was attempted, clearly not all of the labeled soluble components were elastin. Subsequently, the data in Figure 9 is viewed as preliminary and reference to the ^{3}H-lysine soluble protein fractions as elastin is tentative and based primarily on the behavior of the ^{3}H-lysine labeled protein when electrophoresed in polyacrylamide gel containing SDS. The distribution of radioactivity on SDS-polyacrylamide did behave as soluble elastin and migrated with a soluble elastin standard. Previous experiences with the in vitro labeling of soluble elastin peptides indicated that 70-80% of the radioactivity in Fraction I was derived from soluble elastin (cf. Lee et al., in press). That most of the radioactivity in Fraction II was as soluble elastin is not certain.

When these fractions were incubated with insoluble elastin (Figure 9) significant binding did occur. Radioactivity was observed after reduction and hydrolysis in compounds migrating as crosslinking amino acids. The figure is drawn so that each μgram of the soluble components allowed to equilibrate with insoluble elastin is equal to 730 DPMs/μgram. It was observed that at given concentrations of the soluble components 20-40 percent of the total radioactivity appeared to bind with the insoluble elastin

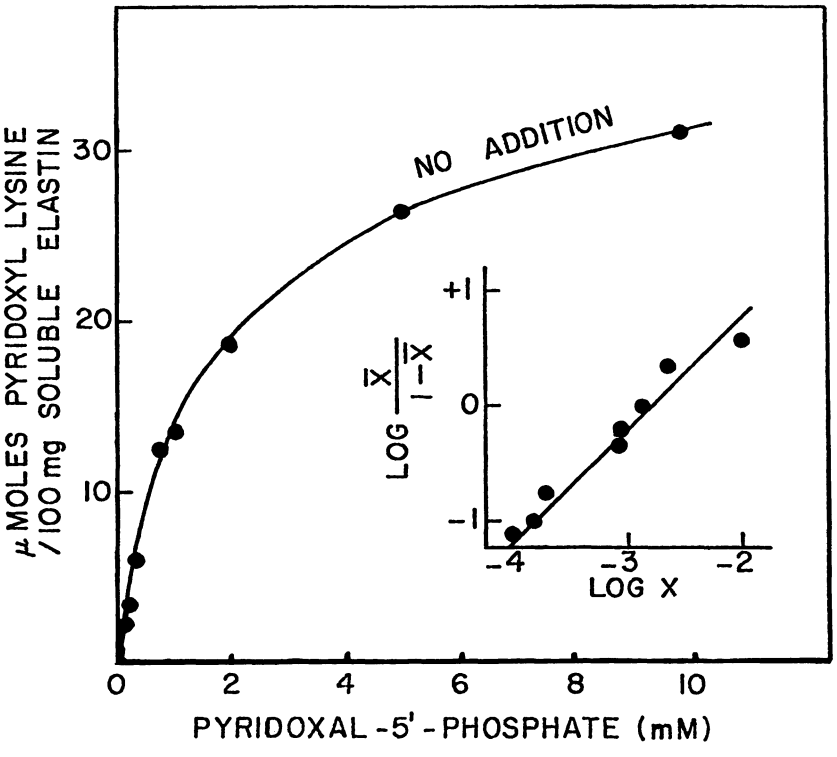


Fig. 6. Binding of pyridoxyl-5'-phosphate by soluble elastin. Elastin (1 mg/ml) was incubated in the presence of the pyridoxal-5'-phosphate at the concentrations indicated. A Hill plot of data was obtained (small figure). X is the fractional saturations of elastin with pyridoxal-5'-phosphate.

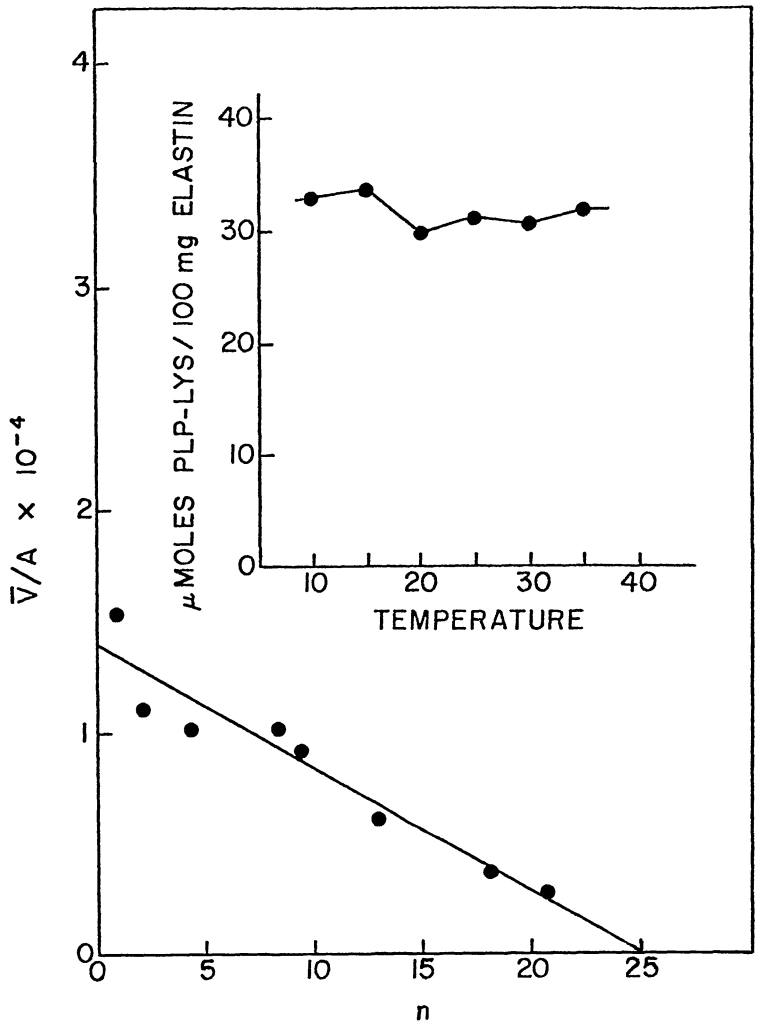


Fig. 7. Binding of pyridoxyl-5'-phosphate by soluble elastin. The data in figure 6 are given in the form of a Scatchard plot. The value ($\bar{v}$) represents the moles of pyridoxyl-5'-phosphate bound per mole of soluble elastin. The value (A) is equal to the molar concentration of pyridoxyl-5'-phosphate in solution. The value (N) is the extrapolated number of lysyl residues which equibrated with pyridoxyl-5'-phosphate. The small figure indicates the effects of temperature on the formation of ε-N-pyridoxyl soluble elastin. Pyridoxyl-5'-phosphate was added at 10 mM.

preparations. Most important, the association of radioactivity with insoluble elastin was greater when the aldehyde content of the insoluble elastin was high (11.8 μmoles/100 mg compared to 5.2 μmoles/100 mg). Also, after reduction and hydrolysis, no radioactivity corresponding to crosslink amino acids was observed

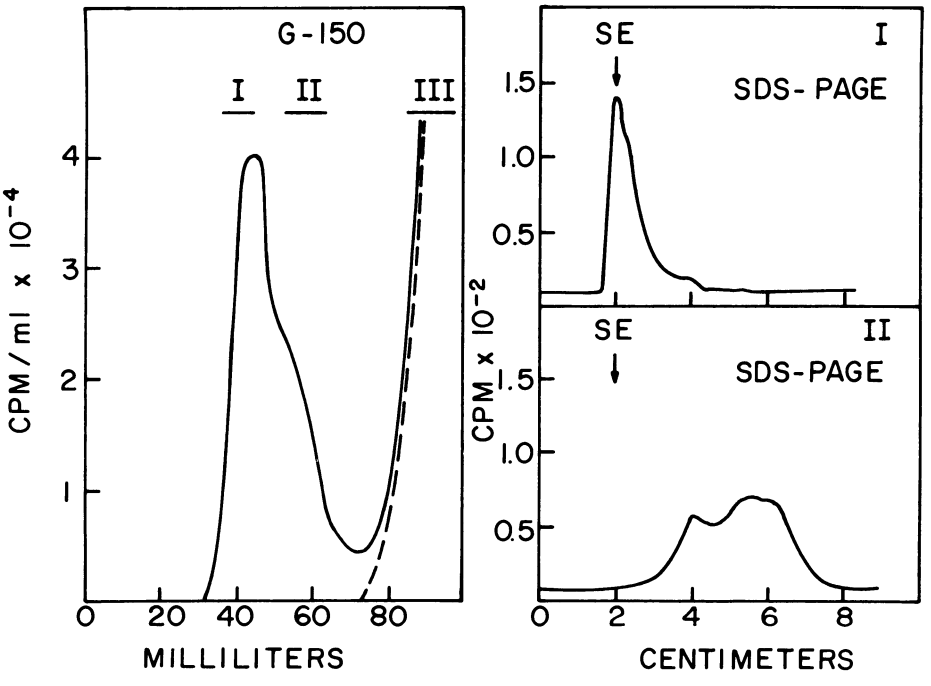


Fig. 8. Partial characterization of ^{3}H-lysine labeled protein after extraction and coacervation of soluble protein from aortas incubated in the presence of ^{3}H-L-lysine. The elution of radioactivity from G-150 column using 0.2M pyridine acetate (pH 5.6) is shown. The protein from labeled fractions I and II were also applied to 10% polyacrylamide gels containing sodium dodecyl sulfate. The designation SE indicates the migration of highly purified soluble elastin. The molecular size of soluble elastin is approximately 70000 daltons. Most of the radioactivity in Fraction II migrated as 20-40000 dalton components. Fraction III was over 85 percent ^{3}H-lysine from the incubation medium and was discarded. The dashed line corresponds to ^{14}C-lysine which was applied to the column as a marker.

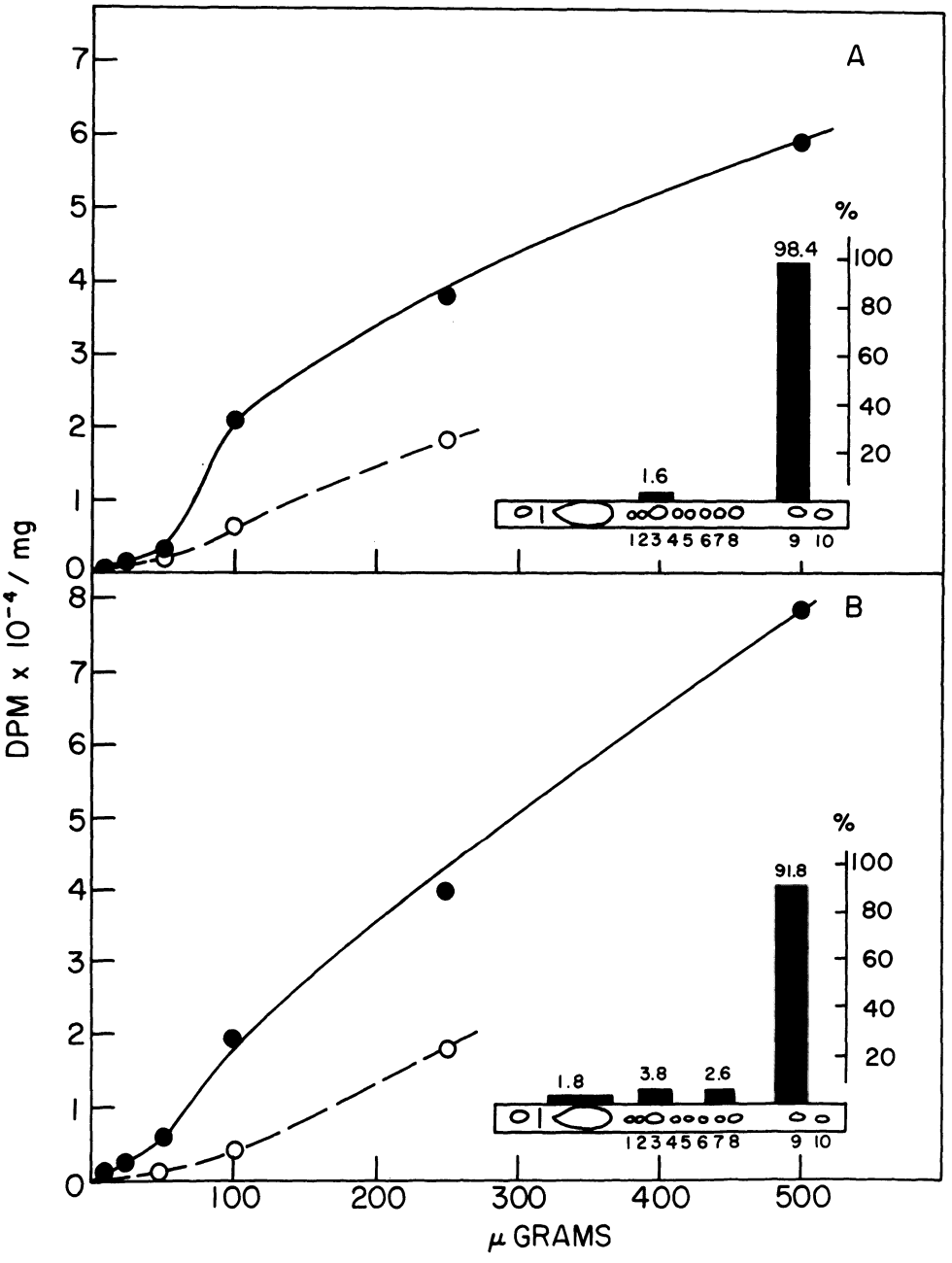


Fig. 9. Binding of arterial soluble protein labeled with ^{3}H-lysine to insoluble elastin from copper-deficient (O--O) and penicillamine treated chicks. Penicillamine was administered daily at 200 mg/kg body weight. Part A indicates the binding characteristics of a protein fraction 20-40000 daltons in size. Part B indicates

Fig. 9 (continued)

the binding characteristics of the protein fraction which was 70000 daltons in size (presumably soluble elastin). The small figure inserts show the distribution of ^{3}H-lysine found after the incubation of 500 µg of soluble protein (730 DPM/µgram) with soluble elastin. No radioactivity was associated with amino acids other than lysine when insoluble elastin from copper-deficient chicks were used using the insoluble elastin from penicillamine-treated birds the distributions which are shown were observed. From left to right, known amino acids standards which cochromatograph are as follows: left of origin, aspartic acid; large area to the right of the origin, neutral and acidic amino acids; (1) unknown; (2), unknown; (3), desmosine and isodesmosine; (4), unknown; (5), unknown; (6), unknown; (7) lysinonorleucine; (8), lysinoalanine; (9), lysine, and (10), arginine. The buffer was pyridine/acetic acid/H_2O (1:10:89 v/v/v), pH 3.8 and a voltage of 2000 v was applied for 2 hours. The samples were applied to thin layer microfine cellulose sheets. For the estimation of radioactivity 1 cm segments were used.

---

after the association of soluble fractions with insoluble elastin of low aldehyde content, i.e., that from copper-deficient chicks. Radioactivity as crosslinking amino acids was only observed when fraction I (presumably the 70000 dalton soluble elastin) was used in the incubations (Part B, Figure 9).

Finally, some data related to metabolic studies involving copper deficiency and elastin metabolism are included and given in Figures 10 and 11. The data for ^{3}H-valine incorporation arterial soluble elastin in vivo indicate that copper deficiency appears to retard the "turnover" extractible soluble elastin. This presumably occurs because the incorporation of soluble elastin into the mature cross-link product is partially blocked. Although the procedure used in the isolation of the soluble elastin resulted again in the precipitation of some of soluble collagen in the extracts, gel electrophoresis of the isolated protein indicated only elastin was significantly labeled (Figure 11). The gel shown in Figure 11 was sliced so that the radioactivity associated with the migrating protein could be determined. The gel represents soluble elastin-rich fraction from a control chick aorta. Soluble collagen, the major contaminant, appeared to represent 33 to 40% of the total protein in this fraction. Given in Table 6 are data related to the soluble protein extracted from aorta. The amount of coacervate was used to calculate the specific activities indicated in Figure 10.

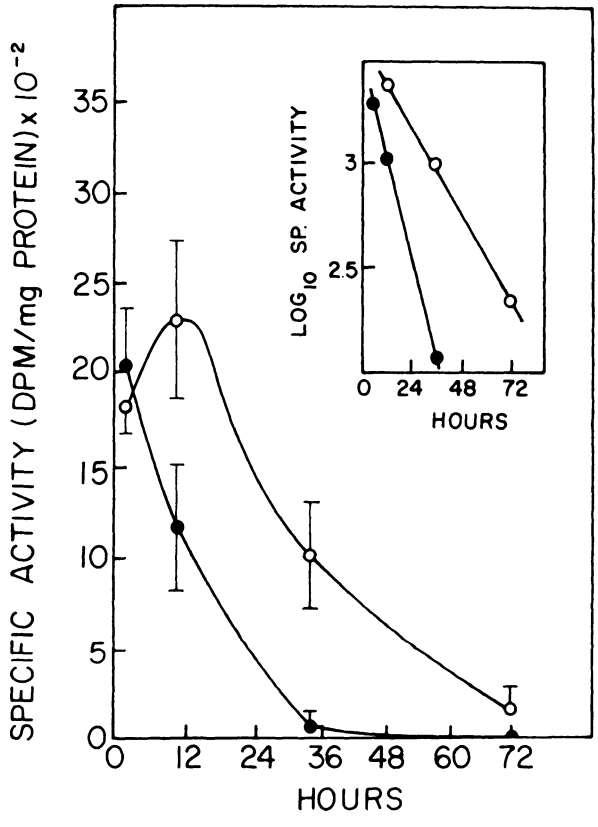


Fig. 10. The specific activities of coacervated protein obtained from copper-deficient (○—○) and -supplemented (●—●) chicks after a single injection of [^{3}H]valine. The small insert represents a semilog plot of the specific activity versus time.

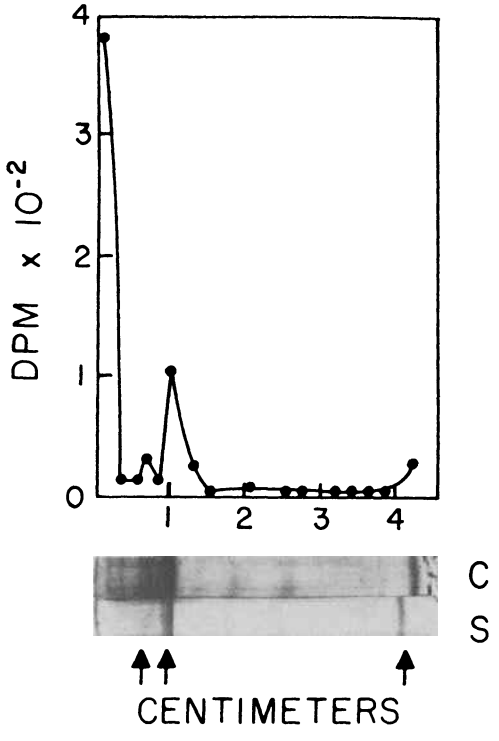


Fig. 11. Radioactivity associated with coacervated protein obtained from chick aortas after an injection of L-[G-^{3}H]valine. Approximately 200 µg of protein was applied to polyacrylamide gels containing 4 M urea buffered at pH 4.3 in the separating gel. After electrophoresis, the gel was cut into contiguous sections and radioactivity in the sections determined. Gel C represents protein from an arterial extract from copper-supplemented birds. Gel S is a sample of purified soluble elastin. From left to right, the first arrow represents the area in which α-collagens normally migrate. Bands of α_1 and α_2 collagen are clearly seen as contaminants in the crude coacervated sample. The next arrow represents elastin and the last arrow shows the migration of the tracking dye. Only elastin and higher molecular weight aggregates appear to be labeled.

TABLE 6

Effects of copper deficiency on the amounts of soluble protein in the chick aorta

Diets	Total protein extracted	Coacervate	Insoluble elastin
	mg/100 mg	mg/100 mg	%
<1 ppm Cu	1.6 ± 0.3	0.82 ± 0.21	6.2 ± 1.3
25 ppm Cu	0.9 ± 0.2	0.14 ± 0.11	10.4 ± 1.5

Values for soluble protein represent the protein soluble in 0.5 M NaCl (pH 7.6) at 4°. It should be noted some of this protein is plasma protein. The coacervate is the mg of soluble protein which coacervated or precipitated upon elevation of the temperature of the extracts to 35°. Insoluble elastin is the percentage insoluble residue after extraction of fresh aortas at 95° for 45 minutes in 0.1 N NaOH.

The time required to maximally label the elastin-rich protein fraction in vivo appeared to be at least three times longer in the copper deficient chicks than in the controls. Furthermore, the disappearance of radioactive protein from the control elastin-rich fraction was two times greater than that from copper deficient chick aortas. These differences probably reflect the differences in the pool size of elastin and differences in the rate of elastin cross-link formation. An important point is that the rate of disappearance of radioactivity associated with the soluble elastin fraction appears to be similar to that previously reported for soluble α -chains of collagens in skin and other soft tissues. The $t_{1/2}$ values obtained from negative slopes given in Figure 10 indicate that approximately 10 and 36 hours were required before 1/2 of the labelled protein disappeared from control and copper deficient chicks arterial extracts, respectively (cf. Rucker et al., 1975).

DISCUSSION

The results point out some of the physiological consequences of impaired crosslinking in collagen and elastin. Bone fragility is often seen in animals that are copper deficient (Underwood, 1971), even in cattle and sheep grazing on copper-deficient pastures. Much of this response appears to be due to defective crosslinking. With a torsional load, the effects of copper

deficiency on bone appear to be both a reduction in stress and a tolerance to strain. That bone collagen crosslinking is a component of these features is clear, particularly in view of the more normal biomechanical properties after artificial crosslinks were introduced into bone. Treatment with formaldehyde increased bone strength both by increasing bone rigidity and its tolerance to strain. Also, some degree of plastic deformation prior to fracture was restored.

Our interest in penicillamine stemmed from early studies by Marcel Nimni and his coworkers (Nimni et al., 1969). When penicillamine is given there are two pronounced effects in animals. One is the chelation of copper, zinc and similar metals. The other is the apparent formation of thiazolidine complexes with aldehydes. Even though tissue copper was presumably complexed in the chick at the doses described in this report, the increase in the aldehyde content of insoluble elstin suggested that copper associated with lysyl oxidase was not markedly affected, but the aldehyde residues which were formed were blocked at the steps preceeding condensation to crosslinks. Pinnell et al. (1969) have also demonstrated in tissue culture, penicillamine causes marked increase in the formation of allysine in elastin and a reduction in the desmosine content. This is in contrast to the effects of BAPN or copper-deficiency which cause a reduction in both allysine and desmosine. Although it did not appear penicillamine had a dramatic effect on elastin crosslinking from the standpoint of increased solubility of elastin, this is probably due to the high number of crosslinks per mole of elastin subunit. Changes in tendon collagen solubility are easily observed in that tendon contains only one or two crosslinks per mole of α-chain.

With respect to the studies on the interaction of soluble elastin with carbonyl functions, it appeared that approximately 80 percent of the lysines in soluble elastin form Shiff-base products with PLP. This is about the same number that appear modified in the formation of crosslinks. Furthermore, it is of interest that in the preliminary studies using aldehyde-rich insoluble elastin and soluble protein fractions from aorta containing ^{3}H-lysine labeled soluble elastin that crosslinks appear to be formed. Recently, Narayanan et al. (1975) have demonstrated that the interaction between lysyl oxidase and soluble elastin under appropriate conditions results in desmosine formation. Narayanan et al. (1975) have suggested no other enzymes are involved in crosslink formation after allysine is formed. Our results also imply crosslinking may occur spontaneously. Further comments regarding the potential mechanisms for crosslink formation in elastin are described in the paper by Dr. Anwar.

Lastly, some data on soluble elastin metabolism was included to point out that protein appears to be rapidly synthesized in vivo in growing animals. The protein disappears from its soluble pool at rates similar to those for collagen α-chains in most tissues. Work currently in progress in which antibody specific for soluble elastin has been used to separate soluble elastin from other protein suggests the same half-life (10-12 hours for normal 3-wk-old birds, unpublished data). Also, we have reported on critical growth periods in the chick when arterial elastin synthesis and metabolism seem to be markedly stimulated (Lee et al., in press). Two to five weeks after hatching there is significant increase in the elastin content of aortas from young growing chicks. The effects of decreased elastin crosslinking during this period are very dramatic. It is during this period that dissecting anurysms and internal hemmorrhage occur resulting in the death of the animals.

Certainly, the process of intra and inter molecular cross-linking in collagen and elastin is a complex one involving numerous facets. Current information would suggest that the process is in no way random and is part of the information programmed into the structure of fibrous proteins. What we have highlighted represents only a few of the physiological consequences resulting from the decreased crosslinking of fibrous tissue proteins.

ACKNOWLEDGEMENTS

This work was supported in part by HL-15965. Joann Murray currently holds a predoctoral fellowship sponsored by the National Institute of Dental Research (HD05219).

REFERENCES

Balian, G. A., Bowes, J. H., and Cater, C. W. (1969) Stabilization of crosslinks in collagen by borohydride reduction. Biochim. Biophys. Acta 81, 331-333.

Barrow, M. V., Simpson, C. F., and Miller, E. J. (1974) Lathyrism: A review. Quarterly Rev. Biol., 49, 101-128.

Carnes, W. H. (1971) Role of copper in connective tissue metabolism. Federation Proceedings 30, 995-1000.

Chan, M. M., Rucker, R. B., and Riggins, R. S. (1976) Effects of fluoride and ethane-1-hydroxy-1,1-diphosphonate on bone metabolism in the growing chick. J. Nutrition 106, 802-811.

Franzblau, C. (1971) Elastin. Comprehensive Biochemistry, 26C, 659-712.

Gallop, P. M., Blumenfeld, O. O., and Seifter, S. (1972) Structure and metabolism of connective tissue protein. Annual Review of Biochemistry, 41, 617-665.

Gallop, P. M. and Paz, M. A. (1975) Posttranslational protein modifications, with special attention to collagen and elastin. Physiological Reviews, 55, 418-487.

Harris, E. D., Gonnerman, W. A., Savage, J. E., and O'Dell, B. L. (1974) Connective tissue amino oxidase. II. Purification and partial characterization of lysyl oxidase from chick aorta. Biochim. Biophys. Acta, 341, 332-344.

Hill, C. H., Starcher, B., and Kim, C. (1967) Role of copper in the formation of elastin. Federation Proceedings, 26, 129-133.

Lee, I., Yau, M. C., and Rucker, R. B. (1976) Arterial elastin synthesis in the young chick. Biochim. Biophys. Acta, in press.

Miller, E. J., Martin, G. R., Piez, K. A. and Powers, M. J. (1967) Characterization of chick bone collagen and compositional changes associated with maturation. J. Biol. Chem. 242, 5481-5489.

Moczar, E., Robert, B., and Robert, L. (1972) Separation and determination of cross-linking amino acids by high-voltage paper electrophoresis. Analytical Biochemistry 45, 422-427.

Narayanan, A. S. and Page, R. C. (1976) Demonstration of a precursor-product relationship between soluble and crosslinked elastin and the biosynthesis of the desmosines in vitro. J. Biol. Chem., 251, 1125-1130.

Nimni, M. E., Deshmukh, K., Gerth, N., and Bavetta, L. A. (1969) Changes in collagen metabolism associated with the administration of penicillamine and various amino and thiol compound. Biochemical Pharmacology 18, 707-714.

Pinnell, S. R. and Martin, G. R. (1968) The crosslinking of collagen and elastin: Enzymatic convertion of lysine to allysine by an extract from bone. Proc. Nat. Acad. Sci. 61, 708-717.

Paz, M. A., Blumenfeld, O. O., Rojkind, M., Henson, E., Furfine, C., and Gallop, P. (1965) Determination of carbonyl compounds with N-methyl benzothiozolone hydrazone. Arch. Biochem. Biophys. 109, 548-557.

Riggins, R. S., Zeman, F., and Moon, D. (1974) The affects of fluoride on bone breaking strength. Calif. Tiss. Res. 14, 283-289.

Ross, R. (1973) The elastic fiber. A reivew. J. Histochem. Cytochem., 21, 199-208.

Rucker, R. B., Parker, H. E. and Rogler, J. C. (1969) Effect of copper deficiency on chick bone bollagen and selected bone enzymes. J. Nutr. 98, 57-63.

Rucker, R. B., Parker, H. E. and Rogler, J. C. (1969) The effects of copper on collagen cross-linking. Biochem. Biophys. Res. Commun. 34, 28-32.

Rucker, R. B. and Riemann, W. (1972) Isolation and properties of soluble elastin from copper-deficient chicks. J. Nutrition 102, 563-570.

Rucker, R. B., Riemann, W., and Tom, K. (1973) Properties of chick tropoelastin. Biochim. Biophys. Acta 317, 193-201.

Rucker, R. B., Riggins, R. S., Laughlin, R., Chan, M. M., Chen, M. and Tom, K. (1975) Effects of nutritional copper deficiency on the biomechanical properties of bone and arterial elastin metabolism in the chick. J. Nutr., 105, 1062-1070.

Rucker, R. B., Tom, K., Tanaka, M., Haniu, M., and Yasunobu, K. T. (1975) Chick tropoelastin. Isolation and partial chemical characterization. Biochem. Biophys. Res. Commun., 66 287-292.

Rucker, R. B., Goetlich-Rieman, W., Tom, K., Chen, M., Poaster, J. and Koerner, S. (1975) Effects of high dietary levels of cholesterol on the metabolism of tropoelastin and proteolytic enzymes in the chick aorta. J. Nutr., 105, 46-56.

Rucker, R. B., and Tinker, D. (1976) Arterial elastin structure and metabolism, International Review of Experimental Pathology. Academic Press, New York, in press.

Sandberg, L. B. (1976) Elastin structure in health and disease. International Reviews of Connective Tissue Research 7, 159-199.

Sandberg, L. B., Bruenger, E. and Cleary, E. G. (1975) Tropoelastin purification: Improvements using enzyme inhibitors. Analytical Biochemistry, 64, 2490254.

CHEMICAL BASIS FOR PHARMACOLOGICAL AND THERAPEUTIC ACTIONS OF PENICILLAMINE

Mendel Friedman

Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Berkeley, California 94710

ABSTRACT

The pharmacological and therapeutic action of penicillamine are very largely explained by its ability to chelate metal ions and take part in oxidation-reduction reactions, sulfhydryl-disulfide interchange, and nucleophilic addition. Effects of penicillamine on particular enzymes are explained by its chemical properties. Possible interactions with amino acids, tissue proteins, food constituents, and intermediates in the metabolism and biosynthesis of sulfur containing amino acids are discussed.

INTRODUCTION

Penicillamine is a trifunctional amino acid in which a carboxyl group and an amino group are attached to one carbon atom and a sulfhydryl and two methyl groups to a second. It differs from valine in having a sulfhydryl group. It differs from cysteine in that two hydrogen atoms attached to the second carbon atom of cysteine are replaced in penicillamine by methyl groups. The SH group in penicillamine is therefore much more sterically hindered than the corresponding group in cysteine. D-penicillamine is usually or always more active pharmacologically, while the L-isomer occurs "naturally." The three functional groups in penicillamine undergo characteristic chemical reactions. These include acid-base equilibria, nucleophilic addition and displacement, combination with various metals, oxidation, and free radical transformations induced by ultraviolet and gamma radiation and by free radicals such as hydrogen and hydroxyl radicals. The three functional groups in penicillamine differ enormously in their ability to participate in these

reactions. Factors that influence such differences include the pH of the reaction medium, ionization (as specified by pKa values), and the respective nucleophilic and electrophilic reactivities, which are influenced in turn by steric and electronic factors as well as by hydrogen-bonding and hydrophobic interactions. The influence of these factors on the reactivity of penicillamine *in vitro* and *in vivo* will be discussed primarily in terms of organic reaction mechanisms. An attempt will be made to explain the behaviour and role of penicillamine in terms of its known chemical properties in normal and diseased states including Wilson's disease, metal poisoning, cystinosis, cystinuria, and rheumatoid arthritis and related connective tissue abnormalities (Perings & Junge, 1975). I also outline possible interactions of penicillamine with diet components including carbohydrates and proteins.

SYNTHESES AND PHYSICOCHEMICAL PROPERTIES

The various ways of synthesizing D-penicillamine are reviewed by Weigert *et al.* (1975). The extensively studied physical-chemical properties of D- and L-penicillamine and their metal complexes are detailed in the following references, which provide excellent access to the literature: Budzinski and Box (1971); Cockerill *et al.* (1974); Cothern *et al.* (1976); Copeland (1970); Doornbos and Feitsma (1967); Freeman *et al.* (1976); Friedman (1973); Lal *et al.* (1975); Letter and Jordaan (1975); Purdie *et al.* (1973); Rao *et al.* (1973); Rabenstein and Fairhurst (1975); Rosenfield & Pathasarathy (1975); Snow *et al.* (1975, 1976); Thich *et al.* (1974); Vasiliev *et al.* (1973, 1974); Wu and Kuntz (1975); Kojima *et al.* (1976).

METAL BINDING

Penicillamine is used therapeutically to increase excretion of copper by patients who suffer from Wilson's disease (hepatolenticular degeneration) (Friedman, 1973; Friedman, 1974; Henkin, 1974; Henkin and Bradley, 1969; Jones and Pratt, 1976). This disease is caused by inherited error in copper metabolism and is characterized by an accumulation of copper in the body. Schizophrenic patients are also reported to suffer from hypercupremia. Penicillamine is also used as an antidote in mercury and lead poisoning (MacGregor and Clarkson, 1974; Lilis and Fishbein, 1976). The chemical basis for these therapeutic uses of penicillamine is that it forms stable, soluble chelates with these metals over a wide pH range that includes physiological pH's. The bound metal ions are then removed from the circulatory system by urinary excretion.

From a detailed study of the interaction of D-penicillamine and triethylenetetramine(trien) with copper ions *in vitro* and *in vivo*, Sarkar *et al.* (1977) concluded that (a) Trien competes more effectively than penicillamine, for copper bound to albumin

and to whole human serum; (b) Penicillamine, but not trien, can enter the liver and mobilize copper there; (c) Both compounds mobilize copper in the kidney, so both help to remove copper by urinary excretion. These authors state that their results support conclusions of Walshe (1973) that D-penicillamine and trien act on different pools of copper in patients with Wilson's disease, but they do not agree that trien removes copper from tissue while penicillamine binds plasma copper, thus rendering it available for filtration from the glomerulus.

In their critical review of mercurial toxicity, MacGregor and Clarkson (1974) note that penicillamine appears to be a better antidote for mercury poisoning than BAL (2,3-dimercaptopropanol). Both D-penicillamine and N-acetyl-DL-penicillamine are orally effective antidotes to mercuric chloride. The acetyl derivative is also effective against mercury vapor intoxication.

Administration of penicillamine increases urinary excretion of lead in hyperactive children with raised blood lead levels (Darrow and Schroeder, 1974).

Ercoli (1968) discovered that penicillamine reduces the toxic but not therapeutic effects of drugs that contain arsenic and antimony that are used in treating parasitic diseases caused by schistosomes leishmania, and trypanosomes. This discovery led to the preparation of several penicillamine chelates, including those of oxyphenarsine (mepharsen) and sodium potassium antimony tartrate (tartar emetic). In each case, chelation enhances the chemotherapeutic-toxicological index.

PENICILLAMINE CONGENERS AND DERIVATIVES

Studies by Sweetman et al. (1971) and by Field et al. (1973) revealed that all three functional groups of (COOH, NH_2, and SH) of D-penicillamine are needed to lower rat skin tensile strength, a measure of anti-arthritic effectiveness. These authors synthesized various congeners of penicillamine and demonstrated that the following three compounds were effective in lowering skin tensile strength: The zinc chelate of penicillamine, 2,2,5,5-tetramethyl-4-thiazolidinecarboxylic acid hydrochloride, and DL-2-(1-mercaptocyclopentyl) glycine hydrochloride. Both skeletal modification and latentiation were used to design penicillamine derivatives that compare favorably with penicillamine in therapeutic activity toward rheumatoid arthritis.

In attempts to develop new nethods to measure the half-cystine content of proteins, to make new internal standards for amino acid analysis, and to elucidate the principles that govern the binding of mercuric and methyl mercuric chloride and other metal salts to keratin proteins, we have synthesized several model amino acid derivatives including derivatives of cysteine and penicillamine derivatives with modified chelating sites. These include S-β-(2-pyridylethyl)-L-cysteine (2-PEC), S-β-(4-pyridylethyl)-L-cysteine (4-PEC), S-β-(2-quinolylethyl)-L-

cysteine (2-QEC), S-β-(2-pyridylethyl)-D-penicillamine (2-PEP), S-β-(4-pyridylethyl)-D-penicillamine (4-PEP), S-β-(p-nitrobenzyl)-DL-penicillamine, and S-β-(p-nitrophenethyl)-DL-penicillamine (Friedman and Krull, 1969; Friedman et al. 1970; Friedman and Noma, 1970; Wu et al. 1971; Cavins and Friedman, 1970; Krull et al. 1971; Friedman et al. 1973; Masri et al. 1972; Friedman and Waiss, 1972; Friedman et al. 1973; Friedman and Masri, 1974; Masri and Friedman, 1974). Studies carried out in collaboration with Dr. A. N. Booth of this laboratory (Tables 1 and 2) show that the cysteine derivatives show a low order of toxicity. Since one of the compounds (2-PEC) was shown to be an excellent chelating agent for mercuric chloride in vitro (Fish and Friedman, 1972) and since S-pyridylethyl wool showed increased binding capacity for mercuric and methylmercuric chlorides and for several other toxic and industrial metal salts, such synthetic cysteine and penicillamine derivatives may be useful as therapeutic agents in metal poisoning.

Table 1

Acute Toxicity of S-β-(2-Pyridylethyl)-L-Cysteine (2-PEC)

Mouse #	Body Wt.	Dose and Route	7-Day Effect	mg/kg Body Wt.
1	20.5	0.5 ml (66 mg) I.P.	Survived	3300
2	18.5	0.5 ml (66 ml) I.P.	Survived	3564
3	19.0	0.5 ml (66 mg) I.P.	Survived	3500
4	24.0	1.0 ml (125 mg) I.P.	Survived	5200
5	20.0	1.0 ml (125 mg) I.P.	Survived	6250
	LD_{50} Intraperitoneal = 6250 mg/kg body wt.			
6	21.0	1.0 ml (125 mg) oral	Died	6000
7	18.0	1.0 ml (125 mg) oral	Survived	6900
8	24.0	1.0 ml (125 mg) oral	Died	5200
9	21.0	0.75 ml (112.5 mg) oral	Survived	5424
10	22.5	0.75 ml (112.5 mg) oral	Survived	4972
	LD_{50} oral = 5,600; 5200 mg/kg body wt.			

Swiss-Webster male mice were dosed orally or intraperitoneally with the compound. All mice given oral dosage in divided dose because of volume. No feed overnight before dosage. The synthesis of 2-PEC is described by Friedman and Noma (1970).

Table 2

Toxicity of Three Cysteine Derivatives: S-β-(4-Pyridylethyl)-L-Cysteine (4-PEC), S-β-(2-Quinolylethyl)-L-Cysteine (2-QEC), S-β-bis-(2-chloroethyl)-phosphonylethyl-L-Cysteine (CPC)

Compound	Route	LD_{50}
4-PEC	Oral	5.9 g/kg body wt.
4-PEC	I. P.	1.2 to 1.5 g/kg
2-QEC	Oral	1.1 to 1.9 g/kg
2-QEC	I. P.	1.2 to 2.1 g/kg
CPC	Oral	2.1 g/kg
CPC	I. P.	2.2 g/kg

Swiss-Webster male mice (young adult) were dosed orally or intraperitoneally with the test compound. Feed was withheld overnight before oral dosage. Results are based on ten-day survival. The sysnthesis of 4-PEC is described by Cavins and Friedman (1970), of 2-QEC by Krull et al. (1971), and of CPC by Friedman and Tillin (1970).

In tests of possible value in mercury poisoning, young adult male Sprague-Dawley rats were given $HgCl_2$ by intraperitoneal injection (3 mg/kg body weight) as described by Aposhian and Asposhian (1959). We were, however, surprised to find that administration of 2-PEC to rats appears to increase the toxicity of mercuric chloride. The reason for this is not clear. One possible explanation is that 2-PEC chelates the mercury in vivo and then, transports it across the blood-brain barrier more rapidly than mercuric chloride passes by itself, and more rapidly than it is excreted. The toxic effect is then manifested when SH-compounds in the brain (for example) competitively remove the mercury salt from 2-PEC. These preliminary results are reported here in the hope that they will stimulate additional studies in this area since we are presently not pursuing this problem. A primary objective of such studies should be to demonstrate the statistical significance of the cited effects.

Although in fact 2-PEC appears actually to increase the toxicity of $HgCl_2$ to rats, tests of the therapeutic use of penicillamine derivatives and analogs such as shown in Tables 2-3 would still be worthwhile.

THIAZOLIDINE FORMATION

Pyridoxal-5-phosphate is a coenzyme of amino-acid decarboxylases and transaminases. Extensive studies with enzyme models have been conducted to discover the catalytic mechanisms mediated by this and related compounds.

Table 3

Mouse LD_{50} Values for Two Organic Phosphate Compounds

No. Mice	Oral Dose (mg/kg body wt.)	I. P. Dose (mg/kg body wt.)	LD_{50}
N-β-bis(β'-chloroethyl)phosphonylethyl-L-phenylalanine (Fig 3)			
3	1240	-	1240
2	2100	-	2100
3	-	1200	1200
3	-	600	600
3	-	1060	1060
N-β-bis(β'-chloroethyl)phosphonylethyl)L-tyrosine (Fig 3)			
3	1370	-	1370
3	2060	-	2060
3	-	1300	1300
3	-	560	560
3	-	960	960

Approximate LD_{50} values have been determined in young adult male Swiss-Webster mice. Feed was withheld overnight from those mice receiving the compounds by oral tube. For the intraperitoneal injections feed was not withheld. The oral LD_{50} is about 2100 mg/kg body weight for both compounds; the intraperitoneal LD_{50} is between 600 and 1000 mg/kg body weight for both compounds. The synthesis of the phenylalanine derivative is described by Friedman and Romersberger (1968) and Friedman and Boyd (1967). The tyrosine derivative was prepared similarly (M. Friedman, unpublished results).

Penicillamine readily participates in reactions with carbonyl compounds including ninhydrin and pyridoxal (Friedman and Sigel, 1966; Abbott and Martell, 1970; Friedman, 1973; Friedman and Williams, 1974).

The equilibrium constant for forming thiazolidine derivatives from pyridoxal phosphate and aminothiols (eq 1) are high, ranging from 10^6 M for cysteine to 10^7 M for penicillamine. Penicillamine displaces many of the common amino acids from Schiff's bases to form stable thiazolidine derivatives. Thus, Schonbeck

$$K = \frac{(\text{thiazolidine})\ (H_2O)}{(\text{pyridoxal})\ (\text{aminothiol})} = 10^6 - 10^7\ \underline{M} \qquad (1)$$

et al. (1975) measured rates of equilibration of cysteine and penicillamine with the Schiff base N^6-(P-pyridoxylidene)-6-aminocaproic acid to form the thiazolidine derivatives N^2S^3-(P-pyridooxylidene)-cysteine and N^2S^3-(P-pyridooxylidene)-penicillamine (eq 2) as models for transaldimination and resolution of pyridoxal-P enzymes by aminothiols. They found that the thiazolidine formed from penicillamine is about 12 times more stable than the thiazolidine formed from cysteine. These results imply that penicillamine can exert some of its biological effects by forming relatively stable thiazolidine derivatives with the pyridoxal moiety of pyridoxal-phosphate-requiring enzymes and thus alter or interfere with their normal catalytic activity. The kinetic analysis also suggests that penicillamine can successfully compete with cysteine in thiazolidine formation in vivo.

$$\text{Schiff base of 6-aminocaproic aicd + penicillamine} \underset{}{\overset{Kc}{\rightleftharpoons}} \text{6-aminocaproic acid + thiazolidine derivative of penicillamine} \qquad (2)$$

The mechanism of inhibition of collagen formation by penicillamine will be considered only briefly here.

The time-course of penicillamine inhibition of collagen formation was examined by Ruiz-Torres (1974) who concludes that D-penicillamine exerts its influence on collagen biosynthesis indirectly by increasing the pool of tropocollagen which depresses the rate of collagen-biosynthesis (Cf. also Ruiz-Torres and Kurten, 1974).

The complex physiological consequences of collagen crosslink inhibition by D-penicillamine are examined in detail by Rucker et al. (1977). Their results support the view that penicillamine-aldehyde interaction to form thiazolidine derivatives is the primary mechanism. Decreased crosslinking causes, in chickens, bone fragility, dissecting aneurysms, internal hemorrhage, and death.

Studies by Otsuka and Mori (1976) suggest that the anti-rheumatoid effect of penicillamine may be due to its inhibition of lysosomal enzyme release in connective tissue membranes. Similarly, Abe et al. (1973) found that rats treated with either DL- or D-penicillamine grew more slowly and showed marked changes in liver phospholipids. The phospholipid effect could be reversed by pyridoxine.

Thiazolidine formation in vivo appears to depend on which isomer is given. Thus, Tomono et al. (1973) found that administration of penicillamine to rats lowers the activities of the pyridoxal-requiring enzymes glutamate decarboxylase in the brain and ornithine amino-transferase in the small intestine, but increases the activities of these enzymes in the liver. DL-penicillamine-induced changes were eliminated when pyridoxine was given simultaneously. This result may mean that pyridoxine (vitamin B_6) successfully competes with pyridoxal for penicillamine in vivo or is changed to pyridoxal and replaces pyridoxal bound by penicillamine. In contrast to DL-penicillamine, D-penicillamine did not affect ornithine aminotransferase activity in any of the organs tested (liver, small intestine, kidney); therefore changes in pyridoxal enzyme activities caused by DL-penicillamine administration appear due to the L-isomer. Sigmund et al. (1968) were unable to confirm the reported (Kuchinskas and du Vigneaud, 1957) inhibition of aspartic trasaminase by L-penicillamine. If we accept that neither D- or L-penicillamine inhibits the enzyme, and since the rate of thiazolidine formation between either penicillamine isomer and pyridoxal phosphate would be expected to be the same, the observed anti-vitamin B_6 activity of L-penicillamine cannot be rationalized in terms of differences in rates of formation of the respective thiazolidines. The authors suggest that the anti-vitamin B_6 action of penicillamines may result not from thiazolidine formation but from interaction with other keto acids that participate in transamination (Hasenbank et al. 1968). In contrast, both enantiomers of penicillamine inhibit alanine transaminase (Sigmund et al. 1968). Studies by Nagasawa et al. (1975) reveal that intraperitoneally injected D-penicillamine combines with ethanol-derived acetaldehyde to form 2, 5, 5-trimethyl-D-thiazolidine-4-carboxylic acid.

These authors suggest that D-penicillamine may be valuable for counteracting ethanol intoxication, which appears mainly due to acetaldehyde, so that penicillamine should be investigated also as a possible protective agent against overdoses of other aldehydes, such as chloral hydrate, paraldehyde, and formaldehyde (possibly derived from methanol), as well as two toxic compounds derived from vinyl chloride, chloracetaldehyde and chlorethylene oxide. Each of these compounds is expected, like acetaldehyde, to react readily with penicillimine to form a possibly less toxic derivative. (Cf. also, Cederbaum and Rubin, 1976).

A related possibility may also merit study. Bjorksten (1977) has suggested that formaldehyde may crosslink DNA molecules and that the resulting impairment may be an important part of aging. Anti-crosslinking agents may therefore perhaps be useful for increasing the lifespan of animals, including human beings. If this conjecture is correct, penicillamine, which can combine with formaldehyde to form thiazolidine, may help to preserve youthful vigor by inhibiting crosslinking by formaldehyde.

INTERACTIONS WITH QUINONES

Okumura et al. (1974) carried out equilibration studies of several sulfhydryl-disulfide-containing compounds including penicillamine. From the results they calculated oxidation-reduction potentials of the sulfhydryl-disulfide couples as follows.

$$E(MPGSH)-E(CSH)=-0.029\ V$$
$$E(MPGSH)-E(GSH)=-0.015\ V$$
$$E(PSH)-E(CSH)=0.032\ V$$
$$E(PSH)-E(GSH)=0.046\ V$$

CSH=cysteine; PSH= penicillamine; MPGSH=mercaptopropionylglycine; GSH = reduced glutathione.

Of the four compounds tested, penicillamine shows the lowest reducing activity:

$$MPGSH > GSH > CSH > PSH$$

In spite of the fact that penicillamine has less reducing power than some of the other biologically important thiols, penicillamine is an effective biochemical reducing agent. Some possibilities of pharmacological interest will be outlined.

Quinones undergo two kinds of reaction with thiols (Friedman 1973; Mason and Peterson, 1965; Powell et al. 1969). Two molecules of a thiol may participate in oxidation-reduction with a quinone to form the corresponding hydroquinone and disulfide. Alternatively, thiol anions may add to the conjugated system of a quinone to form a substituted hydroquinone. Excess quinone in the reaction mixture usually oxidizes the monosubstituted hydroquinone to the corresponding quinone derivative, which may then participate in another nucleophilic addition, and so on. In addition, the amino and SH groups of aminothiols can both condense with a quinone to give cyclized products. These various reactions are important in biochemistry and pharmacology.

To obtain evidence relevant to the controversial use of D-penicillamine in schizophrenia, Mattke & Adler (1971) evaluated the therapeutic effectiveness of D-penicillamine on clinical symptoms. They noted a statistically significant improvement in patients treated with the drug and conclude that their data are

consistent with the view that D-penicillamine exerts an anti-schizophrenic effect at the molecular level by combining with essential copper, thus blocking copper-enzymes needed for catecholamine synthesis. This inhibition also leads to suppression of melanogenesis.

A relevant study by Lovstadt (1976), designed to establish the role of penicillamine in schizophernia and melanin biosynthesis, revealed that penicillamine appears to prevent biosynthesis of the intermediate dopachrome by reducing an active oxidation product of DOPA in an one-electron reduction to an unidentified free radical. Reduced penicillamine was itself oxidized to penicillamine disulfide. Biosynthesis of melanin through the tyrosinase-catalyzed oxidation of DOPA recommenced as soon as all of the added penicillamine was consumed in the reduction.

The described reduction may be one mechanism by which penicillamine affects the skin pigmentation of schizophrenic patients treated with penicillamine and prevents formation of adrenochrome from adrenalin.

Other reactions inhibiting melanin biosynthesis are also possible. For example, cysteine reacts with dopaquinone in feather papillae of embryonic New Hampshire chicks to form 5-S-cysteinyldopa (Misuraca et al. 1969, Rorsman et al. 1973). This compound is further oxidized to phaeomelanin. My guess is that penicillamine may interact similarly with the conjugated system of dopaquinone to give an analogous derivative (5-S-penicillaminyl DOPA) or reduce the dopaquinane to a hydroquinone-type intermediate and thus block melanin biosynthesis at the dopaquinone state (Fig 1).

An investigation of the relative reactivities of several thiols and aminothiols and the nature of products formed in their reactions with adrenochrome revealed that penicillamine reacted only 1/30 as rapidly as cysteine or homocysteine and formed melanin rather than the expected reduction products or adducts.

The interaction of oxidized catechol derivatives, such as oxidized epinephrine, with thiols has been studied extensively because of its possible significance in melanin formation and other biological processes. Much of the chemical information is ambiguous (Mattok 1967).

Finally, the reader is referred to a discussion by Chauffe et al. (1975) of reduction pathways of quinone moieties in melanin and to a description by Blois (1974) of interrelationships of melanin and adrenalin (epinephrine) biosynthesis via dopaquinone.

DISULFIDE CLEAVAGE AND SULFHYDRYL-DISULFIDE INTERCHANGE

Penicillamine depolymerizes high-molecular-weight macroglobulins found in sera of patients with the neoplastic disease, Waldenstrom's macroglobulinemia (Bloch et al. 1960; Schneider, 1967). Depolymerization probably takes place by sulfhydryl-

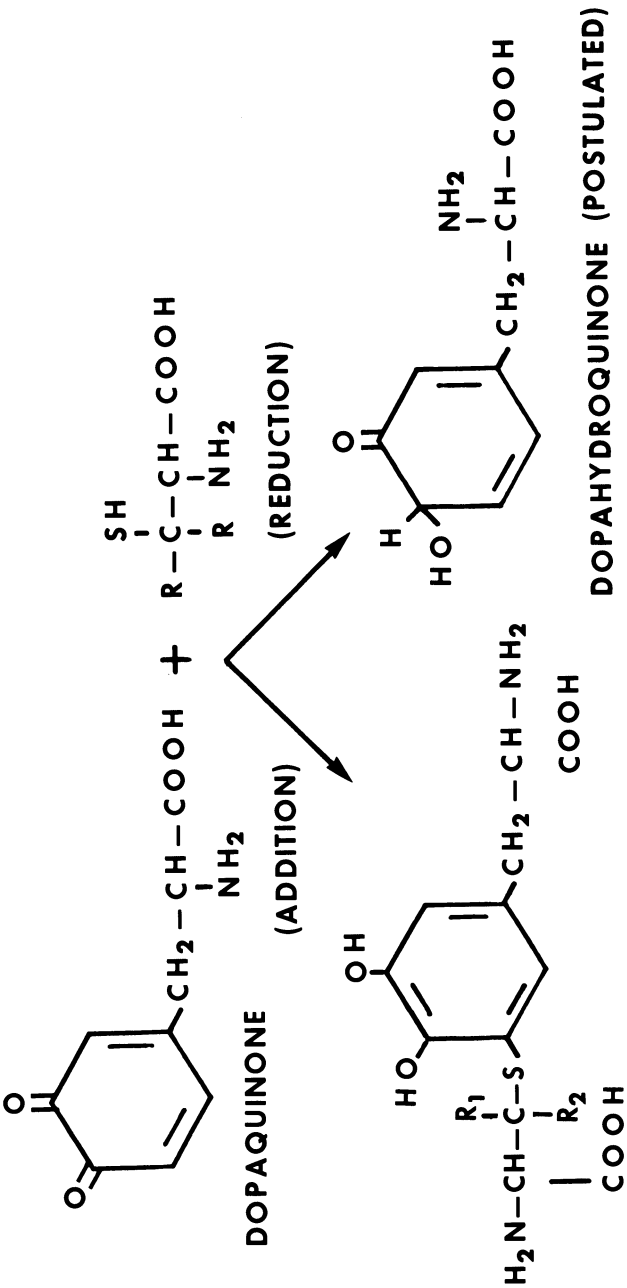


Fig. 1. Possible modes of reaction of penicillamine with dopaquinone.

disulfide interchange, leading to the formation of penicillamine-protein mixed disulfides with lower molecular weight.

In vitro studies by Raab (1974) show that D-penicillamine and N-acetylcysteine increased the solubility of dermal proteins, including collagen. The authors suggest that the solubilizing effect may be due to cleavage of disulfide bonds by both penicillamine and acetylsteine in analogy with the observed depolymerizing effect of such compounds on pathological macroglobulins. Gerber (1972) suggests that penicillamine and gold thiomalate exert their beneficial effects in rheumatoid arthritis by suppressing gamma globulin aggregation. This result, in the case of penicillamine, could arise from cleavage of disulfide bonds in the protein to form mixed penicillamine-gamma globulin disulfides.

The mucolytic and protein-cleaving activity of such compounds as N-acetylcysteine and penicillamine is presumably due to their ability to break -S-S- bonds to form mixed disulfides of lower molecular weight and to their ability to initiate sulfhydryl-disulfide interchanges, possibly converting interchain disulfide bonds of proteins present in mucus to intrachain bonds.

Planas-Bohne (1973) showed that D-penicillamine forms a mixed disulfide with serum proteins. Since cystine, cysteine, and the SH blocking agent N-ethylmaleimide all inhibit protein binding of D-penicillamine, the author suggests that protein SH groups, which are present in 4 to 6 X 10^{-4} molar concentration in serum proteins, participate in disulfide bond formation. The participation of albumin disulfide bonds, however, cannot be excluded. It is also noteworthy that the mixed cysteine-penicillamine disulfide appears to react more slowly with SH groups ($k = 0.001\ min^{-1}$) than the mixed disulfide derived from albumin and penicillamine ($k = 0.035\ min^{-1}$). This comparison suggests that penicillamine will be liberated more rapidly and thus be more readily available for therapeutic activity from the mixed protein-penicillamine disulfide via sulfhydryl-disulfide interchange than from the smaller mixed disulfide.

In a study of the therapeutic mechanisms of penicillamine, Raab and Morth (1974) evaluated its effects on the alkaline phosphatases from calf intestine, human serum, and rat kidney. In each case, a strong inhibitory effect was noted. Cobalt ions partly reduced the inhibition. Zinc ions overcame it completely. Two possible mechanisms are cited to account for this inhibitory effect. Penicillamine may take part in SH-SS interchange with the enzyme or it may chelate zinc ions essential for activity. The disulfide interchange would result in blocking an essential SH group or in changing a necessary configuration. Both mechanisms may operate. These results also imply that patients receiving penicillamine very likely show altered serum enzyme profiles.

In related studies, Raab & Gmeiner (1976) showed that D-penicillamine in high concentrations (above 6.7 mmol/l) inhibited glucose-6-phosphate dehydrogenase (G-6-P) in purified en-

zyme preparations and in human hemolysates. The inhibitory influence may again be due to formation of a penicillamine-enzyme disulfide in which essential enzyme SH groups are blocked.

Low concentrations of D-penicillamine partially off-set the inhibitory action of zinc ions on G-6-P activity. The protection was not shown by higher concentrations of D-penicillamine. Evidently, at low levels, penicillamine preferentially chelates zinc ions, thus preventing their inhibitory influence on enzyme activity. However, at higher levels, D-penicillamine not only chelates zinc and other metal ions but begins to exert an inhibitory effect of its own. Although D-penicillamine concentrations used in these in vitro studies were high, they approach concentration levels sometimes used in therapy (about 6g/day). Thus, the cited effects may operate in vivo. These studies also imply that D-penicillamine may affect keratinization in skin by participating in sulfhydryl-disulfide interchange reactions with the SH and S-S bonds of keratin proteins. Sulfur compounds have a key role in keratinization and are also important structural elements of hair and skin (Maltoltsy, 1976).

Cystinosis is a rare congenital disease of childhood in which deposits of cystine are found throughout the body (Kroll and Lichte, 1973). The associated clinical manifestations of the disease include cystinuria and are referred to as the Fanconi syndrome (Crawhall, 1974). In an attempt to discover the biochemical causes and manifestations of cystinosis, Patrick (1962, 1965) observed that (a) GSH concentration and the reduction of cystine to cysteine by cystine reductase and by glutathione-cysteine transhydrogenase are normal in cystinotic liver; and (b) sulfhydryl-dependent enzymes (glucose-6-phosphate dehydrogenase, hexokinase, succinate dehydrogenase, alcohol dehydrogenase, amino-levulinate dehydrase, and coenzyme A) are specifically inactivated by cystine in cystinotic liver. Patrick suggests that impairment of sulfhydryl-dependent enzyme systems in cystinotic livers and other tissues probably results from reaction between cystine and the SH-enzyme to form an inactive mixed enzyme-cysteine disulfide. It should be pointed out, however, that since the cystine is deposited as a solid in various tissues, it would appear that this interchange would have to occur under heterogenous conditions. Cystine is, of course, very poorly soluble, but I expect that the small amount in solution can react, setting up a dynamic equilibrium based on Le Chatelier's principle, so that more solid dissolves as formation of the soluble disulfide progresses (Cf. Kallistratos and Timmermann, 1966).

From these facts, one would expect that administration of SH compounds such as penicillamine should decrease the cystine deposits of patients with cystinosis and cystinuria by SH displacement. This expectation was, indeed, realized. Crawhall and Thompson (1965) found that oral administration of D-penicillamine lowered plasma cystine levels of patients with cystinuria.

The decrease accompanied formation of cysteine-penicillamine disulfide (Cf. also, Ekberg et al. 1974).

Schulman and Bradley (1970) discovered that (a) cystinosis is a derangement of lysosomal disulfide metabolism or transport and (b) exposure of cultured fibroblasts from cystinotic, but not from normal, individuals to L-cysteine-D-penicillamine disulfide induces vacuolation, so that (c) this vacuolation furnishes a histological criterion that may be useful for diagnosis. This criterion may, of course, not be a primary etiologic mechanism.

RELATIVE REACTIVITIES

Amino and SH groups in aminomercapto acids such as penicillamine may react concurrently with a large number of structurally different compounds. We (Cavins and Friedman, 1968, 1970; Friedman, 1967, 1977a, b; Friedman and Noma, 1970; Friedman and Romersberger, 1968; Friedman and Tillin, 1974; Friedman and Wall, 1964, 1966; Friedman et al., 1965, 1970; Snow et al., 1975) investigated factors that govern nucleophilic reactivities of functional groups in amino-mercapto acids and related compounds with α,β-unsaturated compounds such as acrylonitrile, methyl acrylate, vinylpyridine, and dehydroalanine. The ratio of reaction rates of the SH to the NH_2 groups in compounds in which SH groups are attached to primary carbon atoms, as in cysteine and glutathione, vary from 300 to 1300. The SH groups in penicillamine and β-mercaptoisoleucine (methylethylcysteine) were only about seven times as reactive as their amino groups. Steric factors hindering access of the vinyl-type reagents to the SH groups appear to be responsible for the lower reactivity of the SH group in penicillamine, β-mercaptoisoleucine, and their corresponding N-acetyl derivatives. Sulfhydryl groups in these compounds are attached to tertiary carbon atoms, whereas the corresponding groups in cysteine or glutathione are attached more accessibly to primary carbon atoms.

In related studies, Snow et al. (1975) noted that the SH group of penicillamine reacted only 1/40 as rapidly as the SH group of N-acetyldehydroalanine. Boyd (1968) found that cyanide ions react only one-sixteenth as fast with the mixed disulfide of penicillamine and cysteine (PSSCy) as with cystine (CySSCy). The slower rates are further evidence of steric hindrance by the two methyl groups attached to the carbon atom of penicillamine.

A study of sulfite ion cleavage of unsymmetrical disulfides including penicillamine-cysteamine disulfide by VanRensburg and Swanepoel (1967) revealed that steric factors are mainly responsible for the observed direction of the displacement and that the pH of the medium and electronic effects have only minor influence on the products.

What are some implications of these observations with respect to pharmacological activity? First, the fact that the SH

group of penicillamine is much less reactive than SH groups in other biological thiols such as cysteine and glutathione has a limiting effect on the number of reactions in which penicillamine can take part during its passage through an animal. The most probable reactions are fast reactions such as metal chelation and thiazolidine ring formation in which both the SH and NH_2 groups participate. Slower reactions such as formation of mixed disulfides with proteins can also take place. These appear to be useful in some diseased states in which penicillamine depolymerizes high molecular weight proteins. It appears that penicillamine would be less effective for this purpose if it were highly reactive so that it would be more easily tied up with normal tissue proteins. Second, the fact that penicillamine has the lowest reducing power of aminothiols tested (Okumura et al. 1974) implies that it will be less readily inactivated than other biological thiols in vivo by oxidation to the disulfide or higher oxidation states by oxidizing agents, which may include not only catalase and peroxidase but also methionine sulfoxide present in some diets (Walker et al. 1975; Friedman, 1975), as well as various intermediates in the biosynthesis and metabolism of sulfur amino acids. These intermediates, which could include hypotaurine, cysteine sulfenic acid, cysteine sulfinic acid, and cystine monoxide, would be expected to interact readily with penicillamine, (Allison, 1976; Baker, 1976; Hayes, 1976; Kochakian and Marcaise, 1974), possibly as suggested in Figure 2. Some of the pharmacological and therapeutic properties of penicillamine may very well result from such reactions, which, however, remain to be demonstrated.

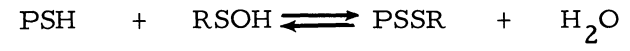
Snow et al. (1975) measured oxidation rates of SH groups of penicillamine, cysteine, and glutathione to the SS form by dimethyl sulfoxide and other sulfoxides including methionine sulfoxide. Penicillamine was found to be 1/4 and 1/10 as reactive as glutathione and cysteine, respectively. As in other cases, two factors may contribute to the lower reactivity of penicillamine; greater steric hindrance and the consequent lower reducing power of its SH group. The amino group of penicillamine may catalyse reduction of the sulfoxide by participating in hydrogen bonding interactions with S=O groups.

Besides possibly reacting with methionine sulfoxide and aldehydic food constituents such as glucose, our kinetic studies imply that penicillamine can also interact with other natural sulfoxides. These include S-propenyl-L-cysteine sulfoxide, S-allyl cysteine sulfoxide, and cycloallin, and allyl propyl disulfide present in onions and garlic (Augusti, 1974; Augusti and Mathew, 1974; Schwimmer and Friedman, 1972; Carson and Boggs, 1966), lenthionine, present in mushrooms (Kyoden et al. 1971), cystathionine sulfoxide, present in animal tissues (Datko et al. 1975), and ergothioneine, present in Neurospora crassa (Ishikawa et al. 1974).

In conclusion, although my objective has been to offer a unified presentation of the molecular reactions of penicillamine

1. Penicillamine Plus Cysteine or Cysteamine Sulfenic Acids:

   a. Nucleophilic Displacement:

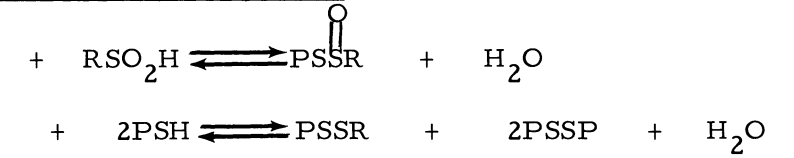


   b. Reduction:

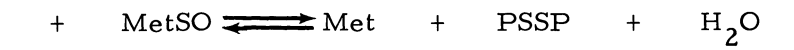
2. Penicillamine Plus Cysteine Sulfinic Acid or Cysteamine Sulfinic Acid (Hypotaurine):

   a. Reduction:

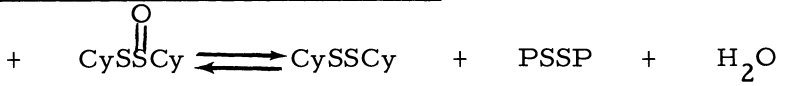
   b. Nucleophilic Displacement:



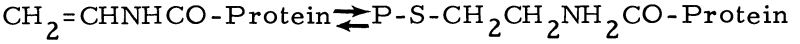
3. Penicillamine Plus Methionine Sulfoxide:



4. Penicillamine Plus Cystine Monoxide:



5. Penicillamine Plus Dehydroalanylprotein:



PSH = penicillamine

PSSP = oxidized penicillamine

PSSR = penicillamine mixed disulfide

RSOH = $NH_2CH_2CH_2SOH$ (cysteamine sulfenic acid) or $NH_2CH(COOH)CH_2SOH$ (cysteine sulfenic acid).

RSO_2H = $NH_2CH_2CH_2SO_2H$ (cysteamine sulfinic acid or hypotaurine); $NH_2CH(COOH)CH_2SO_2H$ (cysteine sulfinic acid).

MetSO =  (methionine sulfoxide).

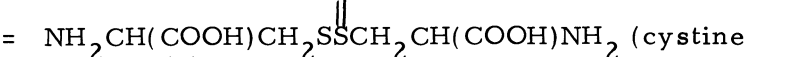
CySSCy =  (cystine monoxide).

Fig. 2. Postulated interactions of penicillamine with thiols, disulfides, and intermediates in the biosynthesis and metabolism of sulfur amino acids and dehydroproteins.

based on its chemical properties, this treatment has been only partly successful, primarily because of the lack of specificity in penicillamine action. The compound participates in too many interactions which are quite general, affecting several enzyme and organ systems, to be easily understood. Nevertheless, I believe that an understanding of its specific biochemical actions offers great hope for more effective use in several diseased states such as metal poisoning, schizophrenia, arthritis, and cystinosis, and may be used to guide the design of improved drugs.

ACKNOWLEDGEMENT

I am grateful to Dr. A. N. Booth for the toxicity data.

A slightly different version of this paper is being published in the Proceedings of the Symposium on "Penicillamine at 21," which will appear in the Proceedings of the Royal Society of Medicine.

REFERENCES

Allison, W. S. (1976). Formation and reactions of sulfenic acids in proteins. Accounts Chem. Res., 9, 293-299.

Abe, M., Mizuno, A., Suda, T., Tomino, I. and Matsuda, M. (1973). Influence of penicillamine on phospholid metabolism. Vitamins (Japan), 47, 43-50.

Abbott, E. H. and Martell, A. E. (1970). Pyridoxine and pyridoxal analogs. A nuclear magnetic resonance study of the condensation of polyfunctional amino acids with pyridoxal. J. Am. Chem. Soc., 92, 1754-1759.

Aposhian, H. V. and Aposhian, M. M. (1959). N-Acetyl-DL-penicillamine, a new oral protective agent against the lethal effects of mercuric chloride. J. Pharmacol. Exptl. Therap., 126, 131-135.

Augusti, K. T. (1974). Lipid lowering effect of allyl propyl disulphide isolated from Allium cepa Linn. on long term feeding to normal rats. Indian J. Biochem. Biophys., 11, 264-265.

Augusti, K. T. and Mathew, P. T. (1974). Lipid lowering effect of allicin (diallyl disulphide-oxide) on long-term feeding to normal rats. Experientia, 30, 468-469.

Baker, D. H. (1976). Nutritional and metabolic interrelationships among sulfur compounds in avian nutrition. Fed. Proc., 35, 1917-1922.

Bjorksten, J. (1977). Some therapeutic implications of the crosslinking theory of aging. This volume.

Bloch, H. S., Prasad, A., Anastasi, A. and Briggs, D. R. (1960). Serum protein changes in Waldenstrom's macroglobulinemia during administration of a low molecular weight thiol (penicillamine). J. Lab. Clin. Med., 56, 212-217.

Blois, M. S. (1974). Melanocytic neoplams. Clin. Pharmacol.

Therapeut., 16, 925-930.
Boyd, T. R. C. (1968). The reaction between cyanide and the mixed disulfide of cysteine and penicillamine. J. Chem. Soc. (C), 2751-2753.
Budzinski, E. E. and Box, H. C. (1971). The oxidation and reduction of organic compounds by ionizing radiation: L-penicillamine hydrochloride. J. Phys. Chem., 75, 2564-2570.
Carson, J. F. and Boggs, L. E. (1966). The synthesis and base-catalyzed cyclization of (+) and (-) cis-S-(1-propenyl)-L-cysteine sulfoxides. J. Org. Chem., 31, 2862-2864.
Cavins, J. F. and Friedman, M. (1970). Preparation and evaluation of S-β-(4-pyridylethyl)-L-cysteine as an internal standard for amino acid analyses. Analyt. Biochem., 35, 489-493.
Cavins, J. F. and Friedman, M. (1968). Specific modification of protein sulfhydryl groups with α,β-unsaturated compounds. J. Biol. Chem., 243, 3357-3360.
Cederbarum, A. I. and Rubin, E. (1976). Mechanism of the protective action of cysteine and penicillamine against acetaldehyde induced mitochondrial injury. Biochem. Pharmacol., 25, 2179-2185.
Chauffe, L., Windle, J. J. and Friedman, M. (1975). Electron spin resonance study of melanin treated with reducing agents. Biophysical J., 15, 565-572.
Cockerill, A. F., Davies, G. L. O., Harrison, R. G. and Rackham, D. M. (1974). NMR determination of the enantiomer composition of penicillamine using an optically active Europium shift reagent. Org. Magn. Res., 6, 669-670.
Copeland, E. S. (1970). Production of free radicals in reduced glutathione and penicillamine by thermal hydrogen atoms and X-radiation. Int. J. Radiat. Biol., 16, 113-120.
Cothern, C. R., Moddeman, W. E., Albridge, R. G., Sanders, W. J., Kelly, P. L., Hanley, W. S. and Field, L. (1976). Some bonding properties of D-penicillamine and related compounds measured by X-ray photoelectron spectroscopy. Anal. Chem., 48, 162-166.
Crawhall, J. C. (1974). The uncommon disorders of sulfur amino acid metabolism. In "Heritable Disorders of Amino Acid Metabolism," W. H. Nyham (Editor), John Wiley & Sons, Inc., p. 467-476.
Crawhall, J. C. and Thompson, C. J. (1965). Cystinuria: effect of D-penicillamine on plasma and urinary cystine concentrations. Science, 147, 1459-1460.
Darrow, D. K. and Schroeder, H. A. (1974). Childhood exposure to environmental lead. In "Protein-Metal Interactions," M. Friedman (Editor), Plenum, New York, pp. 425-445.
Datko, A., Giovanelli, J. and Mudd, H. S. (1975). Preparation of cystathionine sulfoxide and sulfone and some properties relating to their differentiation. Anal. Biochem., 64, 80-84.

Doornbos, D. A. and Feitsma, M. T. (1967). The acid strength of the sulfhydryl and ammonium groups in L-cysteine and D-penicillamine: the determination of the micro acid stability constants. Pharm. Weekblad, 102, 587-598.

Ekberg, M., Jeppsson, J. O. and Denneberg, T. (1974). Penicillamine treatment of cystinuria. Acta Med. Scand., 195, 415-419.

Ercoli, N. (1968). Chemotherapeutic and toxicological properties of antimonyl tartrate-dimethylcysteine chelates. Proc. Soc. Expertl. Biol. Med., 129, 284-290.

Field, L., Hanley, W. S., Kelly, P. L., Sanders, W. J., White, J. E., Jaffe, I. A. and Merryman, P. (1973). Further principles of structure activity relationships for penicillamine analogs and derivatives. J. Med. Chem., 16, 1152-1157.

Fish, R. lH. and Friedman, M. (1972). A novel mercuric chloride complex of S-β-(2-pyridylethyl)-L-cysteine. J. Chem. Soc. Chem. Commun., 812.

Freeman, H., Hug, F. and Stevens, G. N. (1976). Metal binding by D-penicillamine: crystal structure of D-penicillaminatocadmium (II) hydrate. J. Chem. Soc. Chem. Commun., 90-91.

Friedman, M. (1977a). Crosslinking amino acids--stereochemistry and nomenclature. This volume.

Friedman, M. (1977b). Effects of lysine group modification on chemical, physical, nutritive, and functional properties of proteins. In "Food Proteins," J. R. Whitaker and S. R. Tannenbaum (Eds.), Avi, Westport, Connecticut.

Friedman, M. (1975). Protein Nutritional Quality of Foods and Feeds. M. Friedman (Editor). Part 1: Assay Methods--Biological, Biochemical, Chemical. 626 + xx pages. Part 2: Quality Factors -- Plant Breeding, Composition, Processing, Antinutrients. 676 + xx pages. Marcel Dekker, New York.

Friedman, M. (1974). "Protein-Metal Interactions." Advances in Experimental Medicine and Biology, M. Friedman (Editor), Vol. 48, Plenum Press, New York, 692 + x pages.

Friedman, M. (1973). "The Chemistry and Biochemistry of the Sulfhydryl Group in Amino Acids, Peptides, and Proteins." Permagon Press, Oxford England and Elmsford, New York, 485 + viii pages.

Friedman, M. (1967). Solvent effects in reactions of amino groups in amino acids, peptides, and proteins with α,β-unsaturated compounds. J. Am. Chem. Soc., 89, 4709-4713.

Friedman, M. and Boyd, W. A. (1967). A nuclear magnetic double resonance study of N-β-bis(β-chloroethyl) phosphonylethyl-DL-phenylalanine. 154th Amer. Chem. Soc. Meeting, Chicago, Illinois (Abstracts p. C53).

Friedman, M. and Krull, L. H. (1969). A novel spectrophotometric procedure for the determination of half-cystine resi-

dues in proteins. Biochem. Biophys. Res. Commun., 37, 630-633.
Friedman, M. and Masri, M. S. (1974). Interactions of mercury compounds with wool and related biopolymers. In "Protein-Metal Interactions," M. Friedman (Editor), Plenum Press, New York, pp. 505-550.
Friedman, M. and Noma, A. T. (1970). Cystine content of wool. Text. Res. J., 40, 1073-1078.
Friedman, M. and Romersberger, J. A. (1968). Relative influences of electron-withdrawing functional groups on basicities of amino acid derivatives. J. Org. Chem., 33, 154-157.
Friedman, M. and Sigel, C. W. (1966). A kinetic study of the ninhydrin reaction. Biochemistry, 5, 478-484.
Friedman, M. and Tillin, S. (1974). Reactions of amino acid, peptides, and proteins with α,β-unsaturated compounds. XXX. Partly-reduced-alkylated wool. Text. Res. J., 44, 578-580.
Friedman, M. and Tillin, S. (1970). Flame-resistant wool. Text. Res. J., 40, 1045-1047.
Friedman, M. and Waiss, A. J., Jr. (1972). Mercury uptake by selected agricultural products and byproducts. Environmental Science and Technology, 6, 457-458.
Friedman, M. and Wall, J. S. (1966). Additive linear free energy relationships in reaction kinetics of amino groups with α,β-unsaturated compounds. J. Org. Chem., 31, 2888-2894.
Friedman, M. and Wall, J. S. (1964). Application of a Hammett-Taft relation to kinetics of alkylation of amino acid and peptide model compounds with acrylonitrile. J. Am. Chem. Soc., 86, 3735-3741.
Friedman, M. and Williams, L. D. (1974). The ninhydrin reaction. VII. Stochiometry formation of Ruhemann's purple in the ninhydrin reaction. Bioorg. Chem., 3, 267-280.
Friedman, M., Cavins, J. F. and Wall, J. S. (1965). Relative nucleophilic reactivities of amino groups and mercaptide ions in addition reactions with α,β-unsaturated compounds. J. Am. Chem. Soc., 87, 3572-3682.
Friedman, M., Harrison, C. S., Ward, W. H. and Lundgren, H. P. (1973). Sorption behavior of mercuric and methylmercuric salts on wool. J. Appl. Polym. Sci., 17, 377-390.
Friedman, M., Krull, L. H. and Cavins, J. F. (1970). The chromatographic determination of cysteine and half-cysteine residues in proteins as S-β-(4-pyridylethyl)-L-cysteine. J. Biol. Chem., 245, 3868-3871.
Friedman, M., Noma, A. T. and Masri, M. S. (1973). New internal standards for basic amino acid analyses. Analyt. Biochem., 51, 280-287.
Gerber, D. A. (1972). Heat aggregation of human gamma globulin: inhibition by histidine, gold thiomalate, and penicillamine. Clin. Res., XX, 509 (Abstract).
Hasenbank, G., Korber, F. and Siegmund, P. (1968). Uber

die Raktionenen von D- und L-Penicillamin mit Alanintransaminase und mit einigen Aldiminen des Pyridoxalphosphats. Z. Physiol. Chem., 349, 310-316.

Hayes, K. C. (1976). A review of the biological function of taurine. Nutrition Reviews, 34, 161-165.

Henkin, R. I. (1974). Metal-albumin acid interactions: chemical and physiological interrelationships. In "Protein-Metal Interactions," M. Friedman (Editor), Plenum, New York, pp. 299-328.

Henkin, R. I. and Bradley, D. F. (1969). Regulation of taste acuity by thiols and metal ions. Proc. Natl. Acad. Sci. USA, 62, 30-37.

Ishikawa, Y., Israel, S. E. and Melville, D. B. (1974). Participation of an intermediate sulfoxide in the enzymatic thiolation of the imidazole ring of hercyonine to form ergothioneine. J. Biol. Chem., 249, 4420-4427.

Jones, M. M. and Pratt, T. H. (1976). Therapeutic chelating agents. J. Chem. Ed., 53, 342-347.

Kallistratos, G. and Timmermann, A. (1966). Die chemische Auflosung von Cystinsteinen durch Mercapto-Verbindungen. Arzneimittelforschung, 16, 781-782.

Kochakian, C. D. and Marcais, J. (1974). The free amino acids, hypotaurine and other ninhydrin reacting substances of the prostate and seminal vesicles of the guinea pig: regulation by testosterone. Alabama J. Medical Sciences, 11 (3), 240-251.

Kojima, N., Sugiura, Y. and Tanaka, H. (1976). Indium (III) complexes of DL-penicillamine in aqueous solution. Evidence for the formation of protonated and hydrolyzed complexes. Bull. Chem. Soc. Japan, 49, 1294-1300.

Kroll, W. and Lichte, K. H. (1973). Cystinosis: a review of the different forms and recent advances. Humangenetik, 20, 75-87.

Krull, L. H., Gibbs, D. E. and Friedman, M. (1971). 2-Vinylquinoline, a reagent to determine protein sulfhydryl groups spectrophotometrically. Anal. Biochem., 40, 80-85.

Kuchinskas, E. J. and du Vigneaud, V. (1957). An increased vitamin B_6 requirement in the rat on a diet containing L-penicillamine. Arch. Biochem. Biophys., 66, 1-9 and earlier cited references.

Kyoden, Y., Iwami, K. and Mitsuda, H. (1971). A new sulfur-containing peptide from Lentinus edodes acting as a precursor for lenthionine. Agr. Biol. Chem., 35, 2059-2069.

Lal, M., Lin, W. S., Gaucher, G. M. and Armstrong, D. A. (1975). The repair, protection and sensitization of papain with respect to inactivation by H_2O_2 and OH: effects of dithiothreitol, penicillamine, cystine, and penicillamine disulfide. Int. J. Radiat. Biol., 28, 549-564.

Letter, J. E., Jr. and Jordan, R. B. (1975). Complexing of nickel (II) by cysteine, tyrosine, and related ligands and evi-

dence for zwitterion reactivity. J. Am. Chem. Soc., 97, 2381-2390.
Lilis, R. and Fischbein, A. (1976). Chelation therapy in workers exposed to lead. J. Am. Med. Assn., 235, 2823-2824.
Lovstad, R. (1976). Effect of penicillamine on the conversion of DOPA to dopachrome in the presence of tyrosinase or ceruloplasmin. Biochem. Pharmacol., 25, 533-535.
Mason, H. S. and Peterson, E. W. (1965). Melanoproteins. I. Reactions between enzyme-generaged quinones and amino acids. Biochim. Biophys. Acta, 111, 134-146.
Masri, M. S. and Friedman, M. (1974). Interactions of keratins with metal ions: uptake profiles, mode of binding, and effects on properties of wool. In "Protein-Metal Interactions," M. Friedman (Editor), Plenum Press, New York, pp. 551-587.
Masri, M. S., Windle, J. J. and Friedman, M. (1972). p-Nitrostyrene: new alkylating agent for sulfhydryl groups in soluble proteins and keratins. Biochem. Biophys. Res. Commun., 47, 1408-1413.
Matoltsy, A. G. (1976). Keratinization. J. Invest. Dermatol., 67, 20-25.
Mattke, D. J. and Adler, M. (1971). Mode of action of penicillamine in chronic schizophrenia. Dis. Nerv. Syst., 32, 388-391.
Mattok, G. L. (1967). Reactions of adrenochrome with some thiols. Archiv. Biochem. Biophys., 120, 170-174.
Misuraca, G., Nicolaus, R. A., Prota, G. and Ghiara, G. (1969). A cytochemical study of phaeomelanin formation in feather papillae of New Hampshire chick embryos. Experientia, 25, 920-922.
Nagasawa, H. T., Goon, D. J. W., Constantino, N. V. and Alexander, C. S. (1975). Diversion of ethanol metabolism by sulfhydryl amino acids. D-penicillamine-directed excretion of 2,5,5-trimethyl-D-thiazolidine-4-carboxylic acid in the urine of rats after ethanol administration. Life Sci., 17, 707-714.
Okumura, S., Toshioka, N., Asakura, S., Ohya, M. and Nagamori, S. I. (1974). Studies of the oxidation-reduction potentials of 2-mercaptopropionylglycine and penicillamine using thiol-disulfide exchange reactions with cysteine and glutathione. Yakugaku Zasshi, 94 (6), 655-659.
Otsuka, K. and Mori, Y. (1976). Inhibitory effect of D-peniciallmine on degradation of hexosamine-containing substances in the involution of carrageenin granuloma induced by calcium chelate ethylene-diamine tetraacetate. Chem. Pharm. Bull., 24, 215-219.
Patrick, A. D. (1965). Deficiencies of -SH-dependent enzymes in cystinosis. Clin. Sci., 28, 427-443.
Patrick, A. D. (1962). The degradative metabolism of L-cysteine and L-cystine in vitro by liver in cystinosis.

Biochem. J., 83, 248-256.
Perings, E. and Junge, U. (1975). Wirkungen und Nebenwirkungen von D-Penicillamine. Wochenschrift fur Klinik und Praxis, 70, 1265-1274.
Planas-Bohne, F. (1973). Untersuchung zur Bildung gemischter Disulfide zwischen D-Penicillamin und Serumproteinen. Res. Exp. Med., 161, 289-297.
Powell, W., Heacock, R. A., Mattock, G. L. and Wilson, D. L. (1969). Chemistry of the aminochromes. Part X. Some further observations on the reactions of aminochromes with thiols. Canad. J. Chem., 47, 467-476.
Purdie, J. W., Gillis, H. A. and Klassen, N. V. (1973). The pulse radiolysis of penicillamine and penicillamine disulfide in aqueous solution. Canad. J. Chem., 51, 3132-3142.
Raab, W. P. and Gmeiner, B. M. (1976). The influence of D-penicillamine on enzymatic activities: glucose-6-phosphate dehydrogenase. Correlation with serum levels measured in humans. J. Clin. Chem. Clin. Biochem., 14, 173-176.
Raab, W. and Morth, C. (1974). Inhibition of alkaline phosphatase activity by D-penicillamine. Z. Klin. Chem. Biochem., 12, 309-310.
Rabenstein, D. L. and Fairhurst, M. T. (1975). The binding of methyl-mercury by sulfhydryl-containing amino acids and by glutathione. J. Am. Chem. Soc., 97, 2086-2092.
Rao, S. N., Parthasarathy, R. and Cole, F. E. (1973). Crystal and molecular structure of penicillamine hydrochloride monohydrate. Acta. Cryst., B29, 2373-2378.
Rorsman, H., Rosengren, A. M. and Rosengren, E. (1973). Determination of 5-S-cysteinyldopa in melanomas with a fluorimetric method. Yale J. Biol. Med., 46, 516-522.
Rosenfield, R. E., Jr. and Parthasarathy, R. (1975). The crystal structure and absolute configuration of D-penicillamine disulfide dihydrochloride: an unusually short N-H...S contact distance. Acta Cryst., B31, 462-468.
Rucker, R. B., Murray, J. A. and Riggins, R. S. (1977). Nutritional copper deficiency and penicillamine administration: some effects on bone collagen and arterial elastin crosslinking. This volume.
Ruiz-Torres, A. (1974). Zur Frage der Penicillaminwirkung auf die Kollagensynthese. Arzneim.-Forsch (Drug Res.), 24 (1), 43-45.
Ruiz-Torres, A. and Kurten, I. (1974). Zur Pharmakokinetak und zum Stoffwechsel von D- and L-Penicillamin. 3. Resorption, Ausscheidung und Metabolisierung. Arzneimittelforschung, 24 (9), 1258-1261.
Sarkar, B., Sass-Kortsak, A., Clarke, R., Laurie, S. H. and Wei, P. (1977). A comparative study of in vitro and in vivo interaction of D-penicillamne and triethylenetetramine with copper. In press. I thank Dr. Laurie for a preprint of this paper.

Schwimmer, S. and Friedman, M. (1972). Enzymatic and non-enzymathc genesis of volatile sulfur-containing food flavors. Flavour Industry, p. 137-145.

Schneider, W. (1967). Die Wirkung von Penicillamin au pathologische Makromolekule. Munch. med. Wochenschrift, 109, 531-535.

Schonbeck, N. D., Skalski, M. and Shafer, J. A. (1975). Reactions of pyridoxal 5'-phosphate, 6-aminocaproic acid, cysteine, and penicillamine. Models for reactions of Schiff base linkages in pyridoxal 5'-phosphate-requiring enzymes. J. Biol. Chem., 250, 5343-5351.

Schulman, J. D. and Bradley, K. H. (1970). Cystinosis: selective induction of vacuolation in fibroblasts by L-cysteine -D-penicillamine disulfide. Science, 169, 595-596.

Sigmund, P., Hasenbak, G. and Korber, F. (1968). Uber die Wirkung der Penicillamin-Antipoden auf Aspartattransaminase und die Reaktion ihrer Pyridoxalphosphat-thiazolidin mit Apo-Aspartattransaminase. Z. Physiol. Chem., 349, 1062-1070.

Snow, J. T., Finley, J. W. and Friedman, M. (1976). Relative reactivities of sulfhydryl groups with N-acetyl dehydroalanine and N-acetyldehydroalanine methyl ester. Int. J. Peptide Protein Res., 8, 57-64.

Snow, J. T., Finley, J. W. and Friedman, M. (1975). Oxidation of sulfhydryl groups to disulfides by sulfoxides. Biochem. Biophys. Res. Commun., 64, 441-447.

Sweetman, B. J., Vestling, M. M., Ticaric, S. T., Kelly, P. L. and Field, L. (1971). Structure-activity relationships of penicillamine analogs and derivatives. J. Med. Chem., 14, 868-872.

Thich, J. A., Mastropaolo, D., Potenza, J. and Schugar, H. J. (1974). Crystal and molecular structure of bis (copper II) D-penicillamine disulfide nonhydrate. J. Am. Chem. Soc., 96, 726-731.

Tomono, I., Abe, M. and Matsuda, M. (1973). Effect of penicillamine (a vitamin B_6 antagonist) on pyridoxal enzymes. J. Biochem., 74, 587-592.

Van Rensburg, N. J. J. and Swanepoel, O. A. (1967). Reactions of unsymmetrical disulfides. I. Sulfitolysis of derivatives of cysteamine and cysteine. Archiv. Biochem. Biophys., 118, 531-535.

Vasiliev, V. I., Korobkin, V. N., Korchagin, V. B., Fedorets, T. M. and Kaverkina, T. D. (1974). Differential determination of functional groups in penicillamine hydrochloride (Russian). Antibiotiki, XIX, 146-150.

Vasiliev, V. I., Usvyatsov, A. A., Korchagin, V. B., Fedorets, T. M., Korobkin, V. N. and Motmaschik, A. D. (1973). Amperometric titration of D-penicillamine hydrochloride (Russian). Antibiotiki, XVIII, 420-407.

Walker, H. G., Jr., Kohler, G. O., Kuzmicky, D. D. and Witt, S. B. (1975). Problems in analysis of sulfur amino acids in feeds and foods. In "Protein Nutritional Quality of Foods and Feeds," M. Friedman (Editor), Dekker, New York, pp. 549-567.

Walshe, J. M. (1973). Copper chelation in patients with Wilson's disease. Quart. J. Med. New Series, XLII, 441-452.

Weigert, W. M., Offermanns, H. and Scherberich, P. (1975). D-penicillamine-production and properties. Angew. Chem. Internat. Ed., 14, 330-336.

Wu, J. T. and Kuntz, R. R. (1975). The reactions of hydrogen atoms in aqueous solutions: effect of pH with cysteine and penicillamine. Radiation Res., 64, 662-666.

Wu, Y. V., Cluskey, J. E., Krull, L. H. and Friedman, M. (1971). Some optical properties of S-β-(4-pyridylethyl)-L-cysteine and its wheat gluten and serum albumin derivatives. Canad. J. Biochem., 49, 1042-1049.

37

LOCATION OF THE INTERMOLECULAR CROSSLINKING SITES IN COLLAGEN

Norma P. Stimler and Marvin L. Tanzer

Department of Biochemistry

University of Connecticut Health Center

Farmington, Connecticut 06032

ABSTRACT

Collagen is stabilized by intermolecular crosslinks which occur following the enzymatic conversion of the ε-amino groups of selected lysyl and hydroxylysyl residues to the aldehydes, allysine and hydroxyallysine. These aldehydes combine specifically with other allysine or hydroxyallysine residues and/or the side chains of lysine, hydroxylysine or histidine to produce characteristic crosslinks composed of two, three, or four amino acid components. These crosslinks may be converted to more complex compounds by reaction with other amino acids. The newly discovered crosslink, hydroxyaldol-histidine, appears to arise by condensation of one residue each of allysine, hydroxyallysine, and histidine. It was found in a peptide from a tryptic digest of insoluble calf skin collagen which contains three constituent chains, one arising from a region of α1-CB5 and one from α1-CB6. Data obtained in this laboratory, from peptides which were originally isolated from an extensive proteolytic digest of reconstituted calf skin collagen are consistent with a crosslink between the hydroxylysine at residue 927 in α1-CB6 and the allysine at residue 9^{N} in α1-CB(0,1). This is supported by other investigators who have isolated an analogous crosslinked peptide from CNBr digests. The amino acid sequence of a peptide derived from limited alkaline hydrolysis of insoluble calf bone collagen indicates the presence of the crosslink δ,δ'-dihydroxylysinonorleucine at residue 671 in α1-CB7. It is apparent, on both theoretical and experimental bases, that many more crosslinked peptides occur; detailed studies should elucidate the three-dimensional structure of the collagen fibril.

INTRODUCTION

In contrast to most other naturally occurring crosslinked proteins, the most abundant types of mature collagens are devoid of disulfide bridges. Instead, this connective tissue protein is covalently joined by a series of lysine and histidine derived crosslinks to form a network of molecules which is extremely resistant to external forces. Some of the crosslinking components derived from lysine are also found in elastin, but at present it appears that the histidine containing compounds are unique to collagen.

A number of excellent review articles have appeared in the literature over the past several years concerning the topic of crosslinking in connective tissues (Piez, 1972; Gallop and Paz, 1975; Tanzer, 1973, 1976). Since that time sufficient new data has become available concerning the primary structure of collagen to warrant its discussion in relation to crosslinking. Specifically, the entire sequence of the α1 chain and approximately half of the α2 chain of type I collagen are now known (Fietzek and Kuhn, 1976). Progress has been made in determining the nature of the crosslinking components as they exist in vivo, and a number of new crosslinked peptides have been isolated and characterized. Taken in concert with the well established structural parameters derived from electron microscopic and x-ray diffraction studies, these data can be used to construct a sound model of the collagen fibril.

FIBRIL STRUCTURE

The collagen fibril is assembled from a highly ordered array of monomeric units, the collagen molecules. These collagen molecules are composed of three similar or identical polypeptide chains, termed α chains, each of which has a molecular weight of about 100,000 daltons. Each α chain is coiled into a polyproline type II helix (Ramachandran, 1967), three of them being wrapped around each other to form a superhelical rigid rod, 2800 A in length and about 15 A wide (Boedtker and Doty, 1956). The rod-like molecules are packed together in a "quarter stagger" array with all the molecules polarized in the same direction, giving rise to the characteristic banding pattern observed in the electron microscope (Hodge and Schmitt, 1960). These relationships are shown schematically in fig.1. (See the review by Traub and Piez, 1971, for a more detailed description.) The packing arrangement of molecules in the fibril was deduced by correlating the banding patterns observed in electron micrographs of native fibrils with that of aggregates in which all of the molecules are parallel to one another, with their ends in register (the segment long spacing aggregates) (Hodge and Petruska, 1963). However, the two-dimensional "quarter stagger" arrangement cannot be regularly extended to three dimensions, as shown by spatial

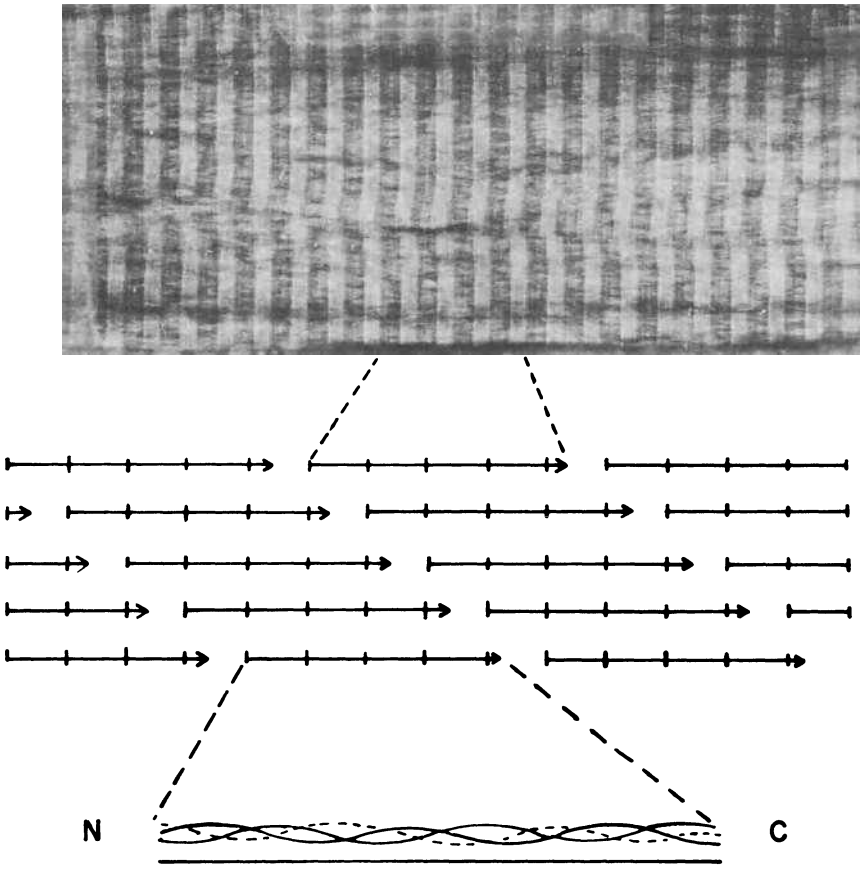


Figure 1. Negatively stained native fibril of calf skin collagen (top) showing the 670 A repeat. Schematic representation of the quarter-stagger packing structure (middle) and a single collagen molecule (bottom).

and statistical analysis (Smith, 1968). As a result several modifications of the scheme for molecular packing have been proposed (Cox et al., 1967; Miller and Parry, 1973; Veis et al., 1967; Veis and Yuan, 1975) in which oligomeric aggregates act as units in the fibril self-assembly process. Such unit microfibrils are thought to contain from four to seven molecules in a quarter-stagger arrangement; presumably they then assemble in a specific manner into fibrils. As shown in fig. 1, there is also a regular pattern of gaps, or "holes," in the packing arrangement. These spaces probably serve as the site of crystal nucleation in mineralized tissues (Krane and Glimcher, 1968).

Once assembled, the fibrils are stabilized by intermolecular crosslinks as shown by studies using specific inhibitors of crosslink formation such as β-aminopropionitrile (BAPN). Experimental animals treated with BAPN or similar compounds develop connective tissues which have markedly diminished tensile strength and from which a much greater proportion of the collagen can be extracted. Narayanan et al. (1972) demonstrated that BAPN can bind irreversibly to lysyl oxidase, the enzyme which catalyzes the first step in crosslink formation. This binding inhibits the conversion of the ε-amino group of specific lysine and hydroxylysine residues to their corresponding aldehyde analogs.

CROSSLINKING COMPOUNDS

As mentioned above, the initial stage in the formation of crosslinks in collagen is the oxidative deamination of specific lysyl and hydroxylysyl residues by a copper requiring enzyme, lysyl oxidase. The enzyme acts preferentially on collagen fibrils rather than collagen molecules (Siegel, 1974). Once formed, the aldehydes spontaneously condense with certain reactive groups of amino acids on adjacent molecules, thus forming a highly stable network. This network appears to become progressively more resistant to environmental distortions, perhaps as a result of additional changes in the nature of the aldehydic crosslinks.

Allysine, the aldehyde form of lysine, undergoes an aldol condensation reaction with another allysine residue to produce an aldol condensation product which occurs as an intramolecular crosslink (Kang et al., 1969). Allysine and δ-hydroxyallysine combine with the ε-amino group of lysine or hydroxylysine, resulting in the formation of aldimine crosslinks, which in the reduced form are lysinonorleucine, hydroxylysinonorleucine, and dihydroxylysinonorleucine (Tanzer and Mechanic, 1970; Bailey and Peach, 1968; Mechanic and Tanzer, 1970). Two allysine residues appear to combine with the ε-amino group of hydroxylysine, generating hydroxymerodesmosine (reduced form) (Tanzer et al., 1973), they also combine with the

side chain of a histidine residue producing aldolhistidine (reduced form) (Fairweather et al., 1972), or with both histidine and hydroxylysine to give histidinohydroxymerodesmosine (reduced form) (Tanzer, et al., 1973; Hunt and Morris, 1973). The probable pathways for these reactions are depicted in fig. 2. See the review by Tanzer (1976) for a more detailed description of the chemistry of these crosslinks.

The crosslinking compounds described above all share the common feature that they contain a C=N or C=C double bond and, with the exception of dehydrodihydroxylysinonorleucine, are relatively unstable to nonphysiological conditions. The double bonds can be chemically reduced with sodium borohydride under relatively mild conditions (Tanzer, 1968; Balian et al., 1969), rendering the crosslinks stable to the hydrolytic procedures used for their isolation. Advantage was taken of this property by using tritium-labeled $NaBH_4$ so that the crosslinks incorporated a radioactive label, facilitating purification of the small amounts of these compounds from the mixture of common amino acids. The assumption is made that the only chemical modification which occurs during reduction, hydrolysis and purification involves reduction of the double bond.

The question of whether the products of acid or base hydrolysis occurred as such in situ or were degradation products resulting from hydrolysis conditions has been addressed by Bensusan (1972) for acid-soluble calf skin collagen and by Housley et al. (1974) for insoluble calf skin collagen. In both studies, collagen which had been reduced with NaB^3H_4 was enzymatically hydrolyzed and the elution positions of the ^3H-labeled compounds were compared with the positions of the crosslinks which had been obtained from acid and base hydrolysates. All of the reduced crosslinks which had been identified in acid or base hydrolysates of calf skin collagen were present in the enzymic hydrolysates. These data indicated that the major crosslinks did not arise as artifacts of acid or base hydrolysis. However, the amount of ^3H-labeled crosslinks liberated by enzymic hydrolysis of insoluble calf skin collagen was somewhat less than that obtained by acid or base hydrolysis. This observation may be explained by one or more possibilities. Hydrolysis by the enzyme mixture could be incomplete so that some crosslinks remain in peptide linkage. Another factor is that sugars are known to be glycosidically attached to the hydroxylysine residues in some of the crosslinks and these sugars probably remain intact during proteolytic hydrolysis. It is also possible that some of the crosslinks are part of more complex structures which are insensitive to enzymic hydrolysis but are released under more strenuous hydrolytic conditions. It is likely that all of these possibilities occur.

Fujii et al. (1975) isolated a crosslinked tripeptide from an alkaline hydrolysate of insoluble calf bone collagen. The peptide

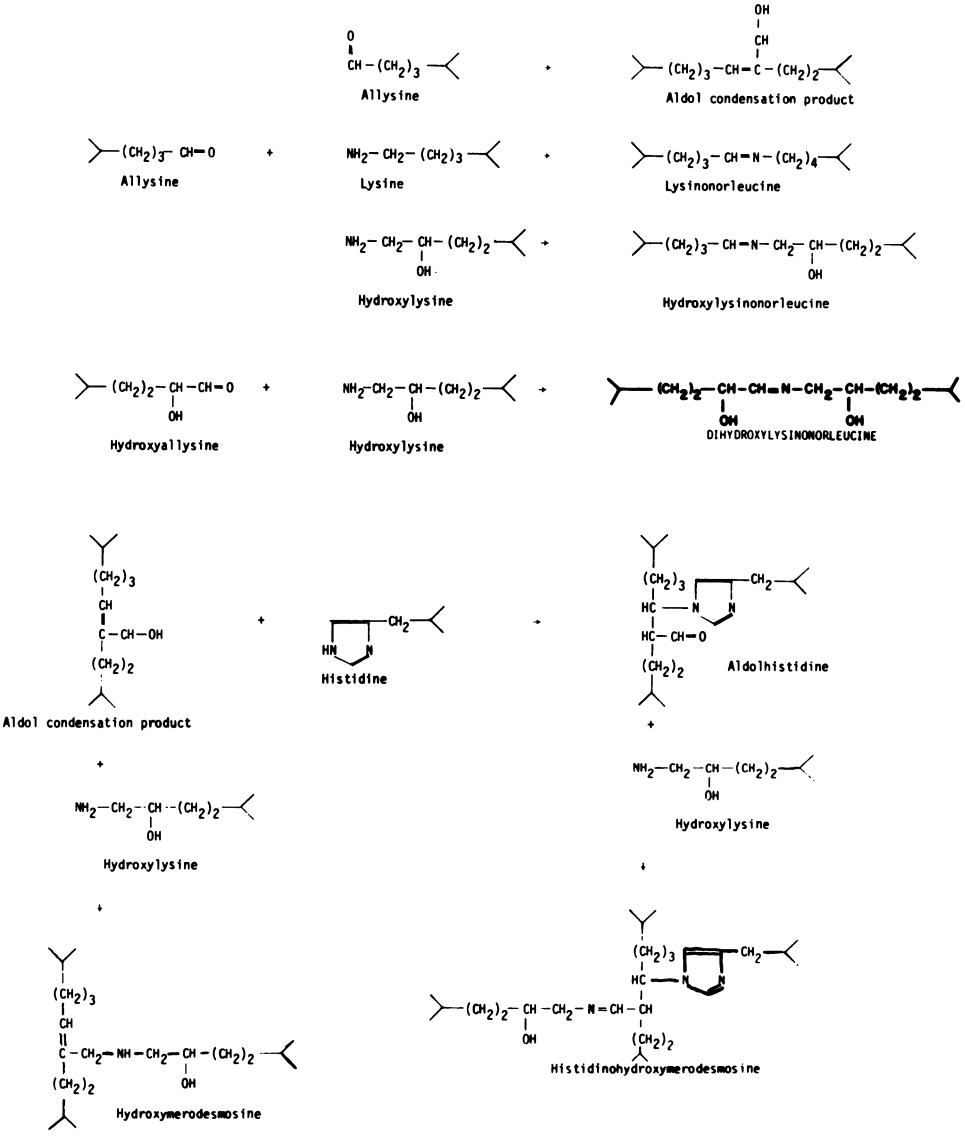


Figure 2. Proposed pathways for the biosynthesis of the reducible crosslinks of collagen.

was unusually stable to hydrolytic conditions that are normally sufficient to cause complete hydrolysis. In addition, it is known that some amino acid sequences, especially those containing proline, are particularly resistant to digestion (Hill, 1965).

The presence of glycosidic residues bound to collagen crosslinks has not been extensively investigated. It is known that varying proportions of the hydroxylysine residues are glycosylated, depending on the tissue and animal species (Spiro, 1969), but since most of these studies were carried out using soluble collagen it is not possible to determine a general relationship of carbohydrate to crosslinking. That carbohydrates can occur in crosslinks was demonstrated by Eyre and Glimcher (1973) who isolated a crosslinked peptide from insoluble calf bone collagen following digestion with crude bacterial collagenase; this peptide contained one residue each of galactose and glucose. It is possible that a significant proportion of all the crosslinks are bound to hexoses in situ since hydroxylysine is the most frequent component of crosslinks. Since the glycosidic bond is labile in acid, care must be taken to avoid cleavage of that bond during the purification processes.

It is well known that during in vivo maturation, and also during in vitro incubation under physiological conditions, native collagen fibers display a progressive increase in tensile strength and insolubility. Paralleling the changes in these properties is a progressive decrease, in most tissues, in the content of the borohydride-reducible crosslinks. Studies of such components in tissues from a wide range of ages also showed that no new reducible compounds are formed during the aging process (Deshmukh et al., 1971; Fujii and Tanzer, 1974). To account for these facts, it was proposed that the reducible crosslinks act as intermediates and are converted to more stable, non-reducible, forms during the maturation process.

The possibility that the crosslinks become reduced in vivo, by natural processes has been investigated by several labs and is still a controversial issue. Mechanic et al. (1971), using isotope dilution of $NaBD_4$ in combination with mass spectroscopy, showed that 25 -50% of the hydroxylysinonorleucine and dihydroxylysinonorleucine in bovine bone collagen became reduced in vivo. These data were supported by studies of reconstituted fibrils from rat skin collagen which previously had been labeled with ^{14}C-lysine in vivo (Deshmukh and Nimni, 1972). Four non-reducible crosslinks progressively appeared, two of which co-chromatographed with lysinonorleucine and hydroxylysinonorleucine. Robins et al. (1973) on the other hand, attempted unsuccessfully to detect the reduced crosslinks in large amounts of protein. They also did isotope dilution studies in which several ^{3}H- labeled crosslinks were added to hydrolysates of bovine tendon collagen and reisolated with no detectable change in specific radioactivity. It is possible that tissue differences

could account for these discrepancies, with natural reduction occurring in mineralized tissues but not in soft tissues.

It has also been suggested that the reducible crosslinks mature into more complex, non-reducible crosslinks by the addition of one or two amino acid side chains to the double bond. Davis et al. (1975) have presented indirect evidence for the role of lysine and/or hydroxylysine in this process. When the ε-amino groups were chemically modified there was no age dependent decrease in the content of reducible crosslinks in insoluble calf bone and dentin collagen. When histidine, arginine, glutamate or aspartate residues were modified the crosslinks decreased at the same rate and to the same extent as in unmodified tissues. These results were interpreted as indicating that collagen maturation involves the nucleophilic

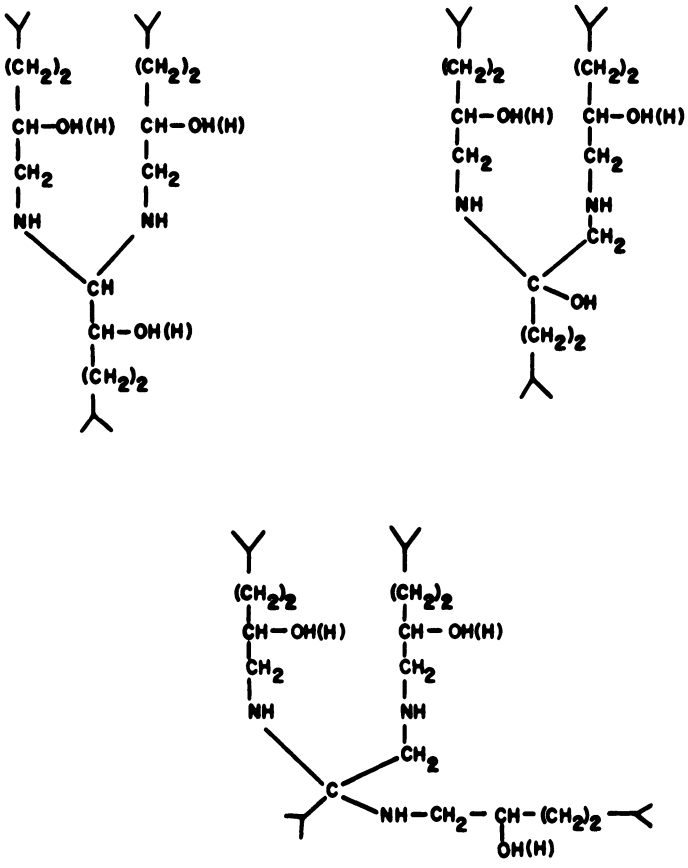


Figure 3. Proposed structures for the "mature," non-reducible collagen crosslinks.

addition of lysine and/or hydroxylysine residues to the double bond of the reducible crosslinks. The structures of these proposed compounds are shown in fig. 3. Unfortunately no direct evidence is available to confirm the existence of these compounds even though they are predicted to be considerably more stable than their reducible precursors and should be abundant in older tissues.

Direct evidence bearing on this point has been provided by Housley et al. (1975) who isolated a stable, non-reducible crosslink, hydroxyaldol-histidine, from insoluble cow skin collagen. The structure of this compound was determined by proton magnetic resonance spectroscopy and by low and high resolution mass spectroscopy of the trifluoroacetyl methyl ester derivative. Prior reduction of the protein with $NaBH_4$ had no apparent effect on the abundance of the compound. The evidence that this new compound serves as a crosslink in collagen is based on its existence in stoichiometric amounts in a homogeneous peptide, consisting of three polypeptide chains, which was isolated from a tryptic digest of insoluble cow skin collagen. The proposed structure is consistent with a condensation of allysine, hydroxyallysine and histidine as shown in fig. 4.

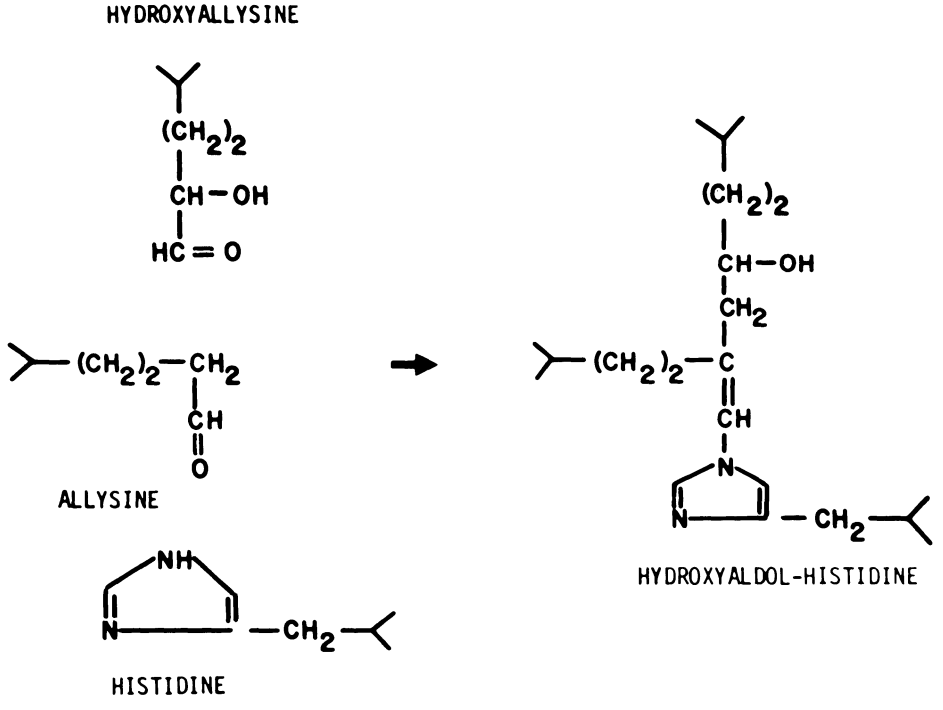


Figure 4. Postulated origin of hydroxyaldol-histidine.

Evidence that other stable crosslinking compounds exist was provided by Becker et al. (1975) who isolated crosslinked peptides from a tryptic digest of insoluble cow skin collagen without prior chemical reduction. Five peptides were purified, one of which had a three chain structure; the other four each contained two chains. As noted above, the triple-chain peptide was joined by a residue of hydroxyaldol-histidine whose presence in stoichiometric amounts was interpreted as confirmation of the function of this new crosslink. The nature of the crosslinks connecting the four remaining double-chain peptides has not been determined although it is evident that they are stable to chromatography in the mildly acidic buffers employed for their purification.

CROSSLINK LOCATION

The tactical approach which has been taken for locating the crosslinking sites in collagen fibrils is much the same as that taken for virus particles and ribosomes to determine which of the constituent proteins are nearest neighbors. Namely, the intact structures are exposed to bifunctional reagents under conditions which produce crosslinks between adjacent proteins. The assumption is necessarily made that the reaction conditions are sufficiently mild such that the native conformation of the proteins involved is preserved. The total structure is then disrupted and the constituent components are characterized and compared with the uncrosslinked components which have been obtained in the same manner. The newly generated, crosslinked species which appear in place of the uncrosslinked components is an indication that they are close together in their native state. This method has enabled investigators to construct a topological map of ribosomal proteins (Clegg and Hayes, 1974).

In insoluble collagen fibrils the crosslinks are already present and the task becomes one of selecting those peptides which are present in digested insoluble material but not in similar digests of uncrosslinked protein. Or, as previously noted, a tactic which is often employed is to reduce the crosslinks with tritiated sodium borohydride, thereby introducing a tritium label into the crosslinked peptides and at the same time stabilizing labile crosslinks. One can then look for the appearance of radioactive peptides in chromatograms of digested collagen fibrils. This approach has been used in several investigations and can provide valuable insights into the packing arrangement of collagen molecules in the fibril.

Using these tactics, Kang (1972) isolated a crosslinked peptide from rat tail tendon collagen. In this study purified rat tail tendon collagen, reconstituted native fibrils and intact tail tendons were separately reduced with NaB^3H_4 and then cleaved at methionyl

residues with cyanogen bromide. The resulting peptides were fractionated by ion exchange chromatography and gel filtration. Fifteen specific peptides were obtained from the soluble collagen as expected, nine from the α1 chain and six from α2, (see fig. 6) none of which contained known crosslinking compounds. Digests of the reduced reconstituted native fibrils and the intact tendon contained significantly decreased amounts of the amino-terminal peptide α1-CB1 and the carboxy-terminal peptide α1-CB6. Quantitation of the recovery of the other peptides was complicated by significant amounts of cross-contamination. Isolation of tritium-labeled material eluting in the region of α1-CB6 yielded a pure peptide whose amino acid composition agreed with the sum of the amino acids of the two peptides, α1-CB1 and α1-CB6. The crosslink which joined these two peptides was hydroxylysinonorleucine.

Lysine occurs only once in α1-CB1, at position 9^N (1) in the amino-terminal non-helical region and is known to be incompletely hydroxylated. Hydroxylysine occurs at two positions in α1-CB6, at residues 915 and 927 in calf skin collagen. This particular sequence has not been determined for rat skin collagen but is predicted by sequence homologies.

Volpin and Veis (1973) characterized the products if CNBr digestion of insoluble bovine skin and dentin collagens without using prior $NaBH_4$ reduction. In addition to the expected peptides, both skin and dentin collagens showed the presence of several new components which were not present in the CNBr peptides from soluble collagen. One of these peptides was identified as originating from α1-CB0,1 crosslinked to α1-CB6, the same peptide as the one isolated from rat tail tendon collagen. (Bovine collagen has an amino acid substitution of leucine for the methionine at position 2^N, thus the nomenclature α1-CB0,1.) Since this peptide was isolated without the preliminary $NaBH_4$ reduction step, the crosslink is therefore stable to the conditions of cyanogen bromide digestion, which were incubation in 70% formic acid at 37° for eight hours. This stability was interpreted as an indication of in vivo reduction of the crosslink (which was assumed to be hydroxylysinonorleucine) since the aldimine crosslinks were believed to be unstable in acidic solvents. In light of the controversy over in vivo reduction and because the identity of the crosslink in this peptide was not determined, it is equally possible that the lysine at position 9^N in bovine collagens is hydroxylated, making the crosslink dehydrodihydroxylysinonorleucine, which is known to be stable at low pH (Jackson et al., 1974).

---

(1) The nomenclature used is adopted from Fietzek and Kuhn (1976) in which amino acids in the amino- and carboxy-terminal nonhelical regions are numbered 1^N-16^N and 1^C-25^C. In the helical region amino acid residues are numbered sequentially from amino- to carboxy-terminus.

Alternatively, it is possible that dehydrohydroxylysinonorleucine in peptide linkage is stable to the conditions of CNBr cleavage. In fact Bailey et al. (1974), citing unpublished data, have reported that dehydrohydroxylysinonorleucine, when it arises from condensation of δ-hydroxyallysine and lysine, can be isolated from hydrolysates without prior reduction.

deLuque, et al. (1970) isolated three small crosslinked peptides from reconstituted calf skin collagen which previously had been reduced with NaB^3H_4 and then sequentially digested with bacterial collagenase, trypsin and papain. The peptides contained one residue each of hydroxylysinonorleucine and their amino acid compositions were consistent with their derivation from the same locus of the collagenous network. Although at that time it was not possible to determine the precise locus of these peptides within the collagen sequence, it is now apparent that they must arise from the crosslinking site between lysine 9^N and hydroxylysine 927 in α1-CB0,1 x α1-CB6 (fig. 5). Assuming that the same crosslinks form at the same positions in bovine skin and dentin and in rat tail tendon, this means that the hydroxylysinonorleucine which links α1-CB0,1 (α1-CB1 in rat) and α1-CB6 originates from lysine at position 9^N and hydroxylysine at position 927.

Dixit and Bensusan (1973) isolated the same peptide, α1-CB0,1 x α1-CB6, from the precipitate obtained after cooling a solution of peptides obtained from a CNBr digest of insoluble bovine skin collagen. They also purified peptides whose compositions were consistent with two α1-CB6 peptides linked to one α1-CB0,1 and with one α1-CB6 linked to a small peptide whose identity is not clear but is different from α1-CB0,1. The identity of these crosslinked peptides was based on amino acid composition and molecular weight but the crosslinks were not identified.

Kuboki et al. (1973) reported the isolation of two peptides containing dihydroxylysinonorleucine which were obtained from NaB^3H_4 reduced insoluble bovine dentin collagen which had been sequentially digested with cyanogen bromide and trypsin. Neither peptide contained tyrosine, indicating that they did not arise from the amino-terminal region of the α1 chain. Both peptides contained two residues of histidine per residue of dihydroxylysinonorleucine. None of the three possible histidine-containing tryptic peptides from the α1 chain could generate the reported amino acid compositions of the isolated peptides. The tryptic peptide encompassing residues 51-62 in α2-CB4 could contribute one side of each peptide. The other side appears to be different for each peptide and may arise from an unsequenced region of the α2 chain.

A second crosslinking site on the α1 chain was located by Eyre and Glimcher (1973a) who purified a crosslinked peptide from calf

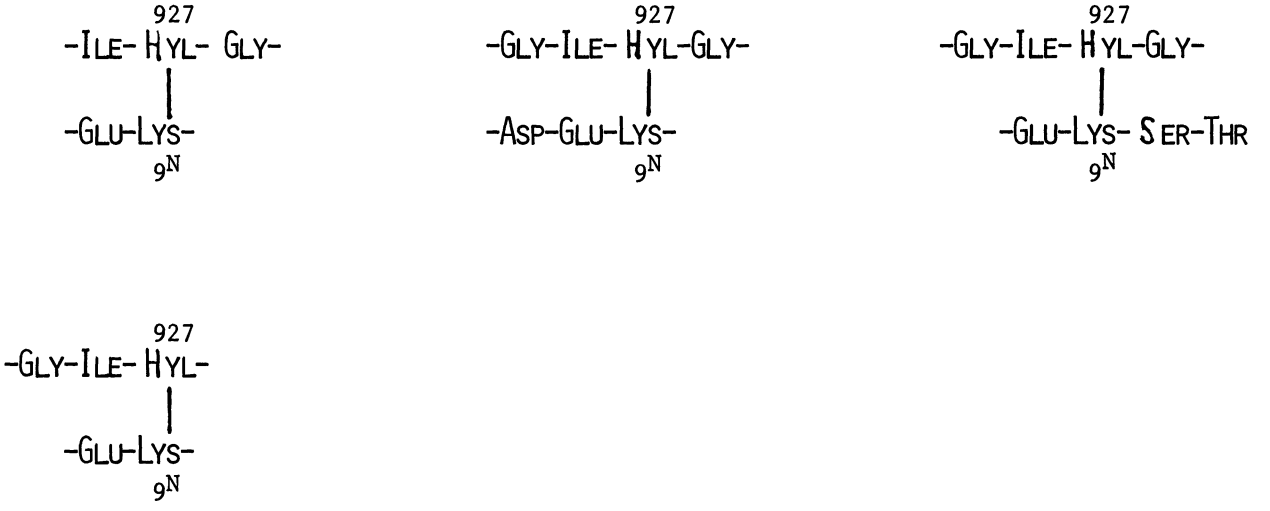


Figure 5. Postulated amino acid sequences and location of the crosslinked peptides isolated from reconstituted calf skin collagen by deLuque et al. (1970).

bone collagen following digestion with crude bacterial collagenase. The amino acid composition indicated that it probably derived from an intermolecular crosslink between a carboxy-terminal sequence of one collagen molecule and a region near the amino-terminus of another. The peptide could be isolated either with or without prior reduction with NaB^3H_4 and the reduced crosslink was identified as dihydroxylysinonorleucine. Hexose analysis indicated that both glucose and galactose were present, probably attached as a disaccharide to the crosslink. The crosslink was cleaved by periodate oxidation and one of the component polypeptide chains was reisolated. The other was apparently too small to be detected. The isolated peptide had an amino acid composition which was consistent with its derivation from the last eighteen residues of the α1 chain, residues 8^C to 25^C in the carboxy-terminal non-helical region. The composition of the smaller peptide was deduced by comparing the composition of the crosslinked peptide with that of the large peptide released by periodate cleavage. The small peptide was thought to originate from a sequence at the junction of α1-CB4 and α1-CB5, encompassing residues 85-90 in the triple helical region. The crosslink joining the molecules at this site would arise from the condensation of hydroxylysine at position 87 and hydroxyallysine at position 17^C (fig. 6).

A peptide with an identical composition was isolated from chicken bone collagen digested with crude bacterial collagenase, with or without prior NaB^3H_4 reduction (Eyre and Glimcher, 1973).

Fujii et al. (1975) isolated a crosslinked tripeptide, containing dihydroxylysinonorleucine, from an alkaline hydrolysate of NaB^3H_4 reduced calf bone collagen. Determination of the amino acid sequence of this peptide showed that one side arose from residues 670-671 in α1-CB7. The sequence of the other side of the peptide does not occur anywhere in the α1 chain or in any of the known sequences of the α2 chain. It will be necessary to isolate this crosslinking site in a larger peptide in order to define its location more precisely.

Becker et al. (1975) have compared the gel filtration patterns of tryptic digests of denatured insoluble calf skin collagen with similar digests of isolated collagen polypeptides. Three peaks were obtained from the digests of insoluble collagen which were absent in the uncrosslinked preparation. Five different peptides encompassing a size distribution between 87 and 142 amino acid residues were purified from these peaks. Determination of the amino- and carboxy-terminal amino acids indicated that four peptides contained two individual chains and one had three chains. One of the double chain peptides contained antigenic sites from the amino-terminal non-helical region and was presumed to be derived from the first 24 amino acids, residues 1^N-9, of an α1 chain crosslinked to a 63 amino acid peptide from the carboxy-terminal region of an adjacent α2 chain. Two other double chain peptides contained antigenic sites for the carboxy-

terminal non-helical region, indicating that they both contained the carboxy-terminal tryptic peptide, residues 1006-25^{C}, linked to other tryptic peptides whose origins could not be determined. The fourth double chain peptide did not contain antigenic sites for either amino- or carboxy-terminal regions and it is speculated that it arose from a helical region in type III collagen. The three chain polypeptide contained a carboxy-terminal tryptic chain, as determined antigenically, as well as a chain which included residues 76-90 near the amino-terminus. The origin of the third chain has not been identified. This peptide is linked by a nonreducible histidine-containing crosslink, hydroxyaldol-histidine, whose structure is described above.

It is interesting to note that while there is a crosslinking site containing dihydroxylysinonorleucine between residues 87 and 17^{C} in calf and chicken bone collagens, both chains also contain a histidine which is two residues to the carboxy-terminal side of the crosslink (fig. 6). One of these histidines may react to form the stable crosslink, hydroxyaldol-histidine.

Scott and Veis (1976) have recently reported the isolation of a peptide from a CNBr digest of insoluble bovine dentin collagen whose molecular weight and composition indicate its origin from α1-CB6 x α2-CB4. The material was not reduced with $NaBH_4$ prior to digestion, so the crosslink is relatively stable but it was not identified.

The idea that other crosslinking sites are present in the collagen fibril is supported by the observation that tritium-labeled components co-elute with non-crosslinked peptides throughout chromatograms of digests of NaB^3H_4 reduced insoluble collagens and by the fact that no histidinohydroxymerodesmosine or aldolhistidine containing peptides have been isolated, although it is known that these compounds are the predominant crosslinks in bovine skin collagens (Tanzer et al., 1973).

DYNAMICS OF CROSSLINK FORMATION

It has been predicted that a number of heritable connective tissue disorders are the result of improper or diminished crosslink formation due to inhibition at some point in the biosynthetic pathway. Some evidence has recently been published which supports this idea.

The hereditary mutation of cattle, dermatosparaxis, in which the collagen precursor molecules in the skin of affected aminals are incompletely converted to collagen, results in tissues with markedly diminished tensile strength. It has been proposed (Bailey and Lapiere, 1973) that the procollagen extensions impair the proper alignment of

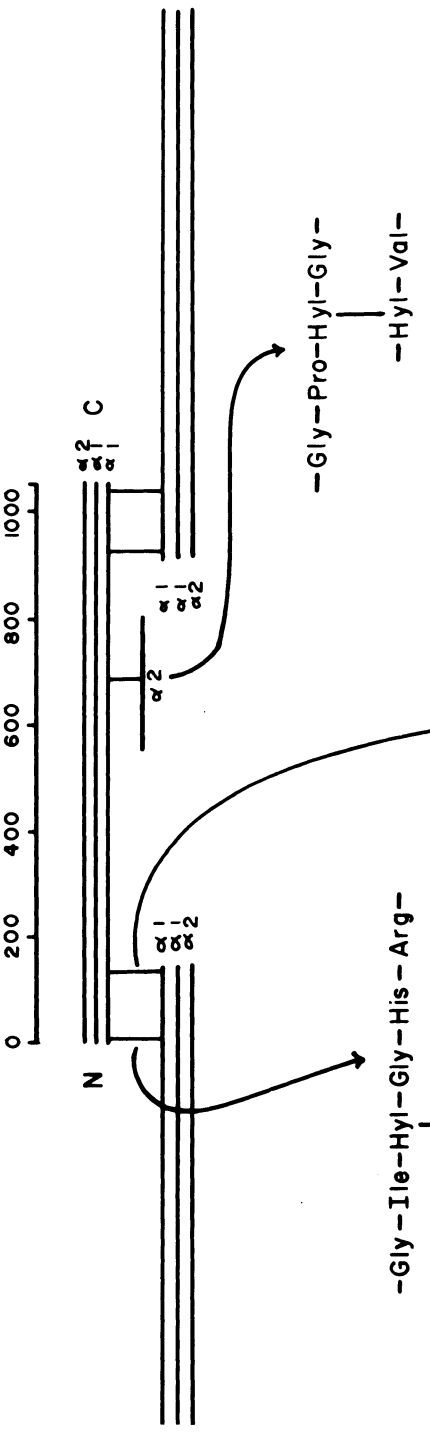


Figure 6. Schematic representation of crosslinked type I collagen showing the location of the known crosslinking sites and the amino acid sequences around them.

molecules in the fibrils, thus preventing the formation of crosslinks. Development of the aldehyde-mediated crosslinking compounds in dermatosparaxic fibrils was reported to be markedly diminished. In contrast, it has recently been reported (Fujii et al., in press) that highly purified procollagen from dermatosparaxic calf skin does form crosslinked fibrils in vitro but contains a quantitatively different crosslinking pattern than normal fibrils. There was a significant increase in the hydroxylysinonorleucine content of dermatosparaxic fibrils compared to normal collagen fibrils and a concomitant drop in the content of histidinohydroxymerodesmosine. The reason for this difference was not apparent.

A similar defect in the conversion of procollagen to collagen has been described in the human disorder, Ehlers-Danlos syndrome type VII (Lichtenstein et al., 1973). The activity of procollagen peptidase in cultured skin fibroblasts from patients with the disease was only 10-20% of normal.

Uitto and Lichtenstein (1976) have suggested that several connective tissue disorders which are biochemically characterized by increased extractability of the collagen and decreased tensile strength of the affected tissue are the result of defects in the enzyme lysyl oxidase. Among the disorders described are Ehlers-Danlos syndrome type V, cutis laxa, homocystinuria, and Menkes kinky hair syndrome. Differences in clinical manifestations would be explained by the occurrence of separate enzymes for collagen and elastin and for lysine and hydroxylysine in collagen. There is no evidence yet regarding this possibility. The observation that most of these diseases are tissue specific indicates that there may also be distinct isoenzymes of lysyl oxidase in different tissues.

A deficiency in lysine hydroxylation results in Ehlers-Danlos syndrome type VI (Pinnell et al., 1972) and the crosslinking pattern of the connective tissued is drastically altered. The contents of hydroxylysinonorleucine and histidinohydroxymerodesmosine in skin and of dihydroxylysinonorleucine in bone and cartilage are markedly diminished. A new reducible compound, which elutes after histidinohydroxymerodesmosine on the amino acid analyzer is present in large quantities in the hydroxylysine deficient skin. The elution position is consistent with the formation of an unhydroxylated analog of histidinohydroxymerodesmosine. It is not clear at this time whether the clinical manifestations are the result of the alteration in crosslinking compounds or the lack of sites for glycosylation. (Eyre and Glimcher, 1972).

Fujii and Tanzer (in press) have also reported an altered crosslinking pattern in the collagen of patients affected by the hereditary disease, osteogenesis imperfecta. In this study bone collagen from normal and affected individuals of the same age and sex was compared

for their amino acid compositions and content of reducible crosslinks. Osteogenesis imperfecta collagen incorporated more tritium into reducible crosslinks and showed an increased content of dihydroxylysinonorleucine compared with normal collagen. The data were interpreted as suggesting delayed maturation in the crosslinking pathway.

Similarly, the crosslinking patterns in bone collagen in experimental rickets (Mechanic et al., 1972, 1975) and in dermis in scar formation (Forrest et al., 1972; Bailey et al., 1973) were reported to be increased in dihydroxylysinonorleucine. In all three cases it has been suggested that the observed changes are the result of the failure of the fibroblasts to synthesize normal amounts of type I collagen and the crosslink profiles obtained reflect the synthesis of embryonic collagen.

CONCLUSION

As the three dimensional structure of the collagen matrix becomes better defined it will be possible to incorporate other connective tissue components in their proper positions. For example, the enzyme lysyl oxidase may be a component of every unit microfibril. Its molecular weight is such that it could easily fit into the "hole" zones (fig. 1) between adjacent collagen molecules (Siegel, 1970). Its presence in that position would also explain the predominant formation of aldehydes at the ends of the molecules.

Furthermore, it is likely that the tissue differences in crosslinking compounds reflect differences in the packing of microfibrillar units into collagen fibrils (Katz and Li, 1973) making the formation of histidine-containing crosslinks more favorable in skin collagen than in bone. In this scheme, the histidine-containing crosslinks may preferentially join molecules in adjacent microfibrils in skin collagen. It is not clear whether these tissue differences are inherently derived from the collagen molecules (controlled for example by the level of glycosylation, which is known to vary) or are the result of other components of the extracellular matrix, such as the presence of type III collagen in skin or of the non-collagenous proteins in bone, which account for about 10% of total bone protein.

Possibly, many of the enzymes involved in the posttranslational modifications of collagen differ in individual tissues and may be specific for different collagen types. Since the different collagen types are known to be genetically distinct and since the cellular requirement for the posttranslational enzymes must parallel the requirement for collagen, one can speculate that all of the posttranslational enzymes are under the control of the same promoter region of the DNA as the unique collagen chains; i.e., each collagen chain type and all its posttranslational modification enzymes may be

included on the same operon. This scheme would provide for more precise regulation of collagen expression, especially if each collagen type is located on a separate chromosome.

ACKNOWLEDGEMENTS

The electron micrograph was provided by Dr. P. Cooke. This work was supported by grants from the NIH, AM-12683, and the American Heart Association, AHA 75773.

REFERENCES

Bailey, A.J. and Peach, C.M. (1968) "Isolation and Structural Identification of a Labile Intermolecular Crosslink in Collagen" Biochem. Biophys. Res. Commun. 33 812-819

Bailey, A.J., Bazin, S., and Delaunay, A. (1973) "Changes in the Nature of the Collagen During Development and Resorption of Granulation Tissue," Biochim. Biophys. Acta 328 383-390

Bailey, A.J. and Lapiere, C.M. (1973) "Effect of an Additional Peptide Extension of the N-Terminus of Collagen from Dermatosparactic Calves on the Crosslinking of Collagen Fibres," Eur. J. Biochem. 34 91-96

Bailey, A.J., Robins, S.P., and Balian, G. (1974) "Biological Significance of the Intermolecular Crosslinks of Collagen," Nature 251 105-109

Balian, G.A., Bowes, J.H., and Cater, C.W. (1969) "Stabilization of Crosslinks in Collagen by Borohydride Reduction," Biochim. Biophys. Acta 181 331-333

Becker, U., Furthmayr, H., and Timpl, R. (1975) "Tryptic Peptides From the Crosslinking Regions of Insoluble Calf Skin Collagen," Hoppe-Seyler's Z. Physiol. Chem. 356 21-32

Bensusan, H.B. (1972) "Investigation of Crosslinks in Collagen Following an Enzymic Hydrolysis," Biochim. Biophys. Acta 285 447-452

Boedtker, H. and Doty, P. (1956) "The Native and Denatured States of Soluble Collagen," J. Am. Chem. Soc. 78 4267-4280

Clegg, C. and Hayes, D. (1974) "Identification of Neighbouring Proteins in the Ribosomes of Eschericia coli: A Topographical Study with the Crosslinking Reagent Dimethyl Suberimidate," Eur. J. Biochem. 42 21-28

Cox, R.W., Grant, R.A., and Horne, R.W. (1967) "The Structure and Assembly of Collagen Fibrils. I. Native Collagen Fibrils and Their Formation from Tropocollagen," J. Roy. Micro. Soc. 87 123-142

Davis, N.R., Risen, O.M., and Pringle, G.A. (1975) "Stable, Non-reducible Crosslinks of Mature Collagen," Biochemistry 14 2031-2036

deLuque, O. Mechanic, G. and Tanzer, M.L. (1970) "Isolation of Peptides Containing the Crosslink Hydroxylysinonorleucine, from Reconstituted Collagen Fibrils," Biochemistry 9 4987-4993

Deshmukh, A., Deshmukh, K., and Nimni, M.E. (1971) "Synthesis of Aldehydes and Their Interactions During the in Vitro Aging of Collagen," Biochemistry 10 2337-2342

Deshmukh, K. and Nimni, M.E. (1972) "Identification of Stable Intermolecular Crosslinks Present in Reconstituted Native Collagen Fibers," Biochem. Biophys. Res. Commun. 46 175-182

Dixit, S.N. and Bensusan, H.B. (1973) "The Isolation of Crosslinked Peptides of Collagen Involving α1-CB6," Biochem. Biophys. Res. Commun. 52 1-8

Eyre, D.R. and Glimcher, M.J. (1972) "Reducible Crosslinks in Hydroxylysine Deficient Collagens of a Heritable Disorder of Connective Tissue," Proc. Nat. Acad. Sci., USA 69 2594-2598

Eyre, D.R. and Glimcher, M.L. (1973a) "Analysis of a Crosslinked Peptide from Calf Bone Collagen: Evidence that Hydroxylysyl Glycoside Participates in the Crosslink," Biochem. Biophys. Res. Commun. 52 663-671

Eyre, D.R. and Glimcher, M.L. (1973d) "Collagen Crosslinking. Isolation of Crosslinked Peptides from Collagen of Chicken Bone" Biochem. J. 135 393-403

Fairweather, R. Tanzer, M.L., and Gallop, P.M. (1972) "Aldol-histidine, a New Trifunctional Collagen Crosslink," Biochem. Biophys. Res. Commun. 48 1311-1315

Fietzek, P.P. and Kuhn, K. (1976) "The Primary Structure of Collagen," Int'l. Rev. of Conn. Tiss. Res. 7 1-60

Forrest, L., Shuttleworth, A., Jackson, D.S., and Mechanic, G.L. (1972) "A Comparison Between the Reducible Intermolecular Crosslinks of the Collagens from Mature Dermis and Young Dermal Scar Tissue of the Guinea Pig," Biochem. Biophys. Res. Commun. 46 1776-1781

Fijii, K. and Tanzer, M.L. (1974) "Age Related Changes in the Reducible Crosslinks of Human Tendon Collagen," FEBS Letters 43 300-302

Fujii, K., Corcoran, D., and Tanzer, M.L. (1975) "Isolation and Structure of a Crosslinked Tripeptide from Calf Bone Collagen," Biochemistry 14 4409-4413

Fujii, K. and Tanzer, M.L. (in press) "Osteogenesis Imperfecta: Altered Crosslinking of Bone Collagen," Clin. Orthopaed.

Fujii, K., Tanzer, M.L., and Cooke, P. (in press) "Collagen Fibrogenesis and the Formation of Complex Crosslinks," J. Mol. Biol.

Gallop, P.M. and Paz, M.A. (1975) "Posttranslational Protein Modifications, With Special Attention to Collagen and Elastin," Physiol. Revs. 55 418-487

Hill, R.L. (1965) "Hydrolysis of Proteins," Adv. Prot. Chem. 20 37-107

Hodge, A.J. and Schmitt, F.O. (1960) "The Charge Profile of the

Tropocollagen Macromolecule and the Packing Arrangement in Native-type Collagen Fibrils," Proc. Nat. Acad. Sci., USA 46 186-197

Hodge, A.J. and Schmitt, F.O. (1963) "Recent Studies With the Electron Microscope on Ordered Aggregates of the Tropocollagen Molecule," in Aspects of Protein Structure (ed. G,N, Ramachandran) pp 289-300 Academic Press, London

Housley, T.J., Tanzer, M.L., and Bensusan, H.B. (1974) "Release of Reducible Crosslinks of Collagen by Total Enzymic Hydrolysis," Biochim. Biophys. Acta 365 405-414

Housley, T.J., Tanzer, M.L., Henson, E., and Gallop, P.M. (1975) "Collagen Crosslinking: Isolation of Hydroxyaldol-histidine, a Naturally Occurring Crosslink," Biochem. Biophys. Res. Commun. 67 824-829

Hunt, E. and Morris, H.R. (1973) "Collagen Cross-Links. A Mass-Spectrometric and ^{1}H- and ^{13}C-Nuclear Magnetic Resonance Study," Biochem. J. 135 833-843

Jackson, D.S., Ayad, S., and Mechanic, G. (1974) "Effect of Heat on Some Collagen Crosslinks," Biochim. Biophys. Acta 336 100-107

Kang, A.H., Faris, B., and Franzblau, C. (1969) "Intramolecular Cross-link of Chick Skin Collagen," Biochem. Biophys. Res. Commun. 36 345-349

Kang, A.H. (1972) "Studies on the Location of the Intermolecular Cross-links in Collagen. Isolation of a CNBr Ppetide Containing δ-Hydroxylysinonorleucine," Biochemistry 11 1828-1835

Katz, E.P. and Li, S.T. (1973) "The Intermolecular Space of Reconstituted Collagen Fibrils," J. Mol. Biol. 73 351-369

Krane, S.M. and Glimcher, M.J. (1968) "The Organization and Structure of Bone, and the Mechanism of Calcification," in Treatise on Collagen (ed. G.N. Ramachandran) Vol 2B pp 67-251 Academic Press, London

Kuboki, Y., Tanzer, M.L., and Mechanic, G.L. (1973) "Isolation of Peptides Containing the Intermolecular Crosslink δ,δ'-Dihydroxylysinonorleucine from Dentin Collagen," Arch. Biochem. Biophys. 158 106-115

Lichtenstein, J.R., Martin, G.R., Kohn, L.D., Byers, P.H., and McKusic, V.A. (1973) "Defect in Conversion of Procollagen to Collagen in a Form of Ehlers-Danlos Syndrome," Science 182 298-300

Mechanic, G. and Tanzer, M.L. (1970) "Biochemistry of Collagen Crosslinking. Isolation of a New Crosslink, Hydroxylysinohydroxynorleucine, and its Reduced Precursor, Dihydroxynorleucine, From Bovine Tendon," Biochem. Biophys. Res. Commun. 41 1597-1604

Mechanic, G., Gallop, P.M., and Tanzer, M.L. (1971) "The Nature of Crosslinking in Collagens from Mineralized Tissues," Biochem. Biophys. Res. Commun. 45 644-653

Mechanic, G.L., Toverud, S.U., and Ramp, W.K. (1972) "Quantitative Changes of Bone Collagen Crosslinks and Precursors in Vitamin D Deficiency," Biochem. Biophys. Res. Commun. 47 760-765

Mechanic, G.L., Toverud, S.U., Ramp, W.K., and Gonnerman, W.K. (1975) "The Effect of Vitamin D on the Structural Crosslinks and Maturation of Chick Bone Collagen," Biochim. Biophys. Acta 393 419-425

Miller, A. and Parry, D.A.D. (1973) "Structure and Packing of Microfibrils in Collagen," J. Mol. Biol. 75 441-447

Narayanan, A.S., Siegel, R.C., and Martin, G.R. (1972) "On the Inhibition of Lysyl Oxidase by β-aminopropionitrile," Biochem. Biophys. Res. Commun. 46 745-751

Piez, K.A. (1968) "Crosslinking of Collagen and Elastin," Ann. Rev. Biochem. 37 547-570

Pinnell, S.R., Krane, S.M., Kenzora, J.E. and Glimcher, M.J. (1972) "A Heritable Disorder of Connective Tissue. Hydroxylysine Deficient Collagen Disease," N. Engl. J. Med. 286 1013-1020

Ramachandran, G.N. (1967) "Structure of Collagen at the Molecular Level," in Treatise on Collagen (ed. G.N. Ramachandran) Vol. 1 pp 103-183, Academic Press, New York

Robins, S.P., Shimokomaki, M. and Bailey, A.J. (1973) "The Chemistry of the Collagen Crosslinks. Age-related Changes in the Reducible Components of Intact Bovine Collagen Fibres," Biochem. J. 131 771- 780

Scott, P.G., Veis, A., and Mechanic, G. (in press) "The Identity of a Cyanogen Bromide Fragment of Bovine Dentin Collagen Containing the Site of an Intermolecular Crosslink," Biochemistry

Siegel, R.C., Pinnell, S.R., and Martin, G.R. (1970) "Cross-Linking of Collagen and Elastin. Properties of Lysyl Oxidase," Biochemistry 9 4486-4492

Siegel, R.C. (1974) "Biosynthesis of Collagen Crosslinks: Increased Activity of Purified Lysyl Oxidase with Reconstituted Collagen Fibrils," Proc. Nat. Acad. Sci., USA 71 4826-4830

Smith, J.W. (1968) "Molecular Pattern in Native Collagen," Nature 219 157-158

Spiro, R.G. (1969) "Characterization and Quantitative Determination of the Hydroxylysine-Linked Carbohydrate Units of Several Collagens," J. Biol. Chem 244 602-612

Tanzer, M.L. (1968) "Intermolecular Cross-Links in Reconstituted Collagen Fibrils. Evidence for the Nature of the Covalent Bonds," J. Biol. Chem. 243 4045-4054

Tanzer, M.L. (in press) "Crosslinking," in Treatise on Collagen (ed. G.N. Ramachandran) Academic Press, New York

Tanzer, M.L. and Mechanic, G. (1970) "Isolation of Lysinonorleucine from Collagen," Biochem. Biophys. Res. Commun. 39 183-189

Tanzer, M.L., fairweather, R., and Gallop, P.M. (1973) "Isolation of the Crosslink, Hydroxymerodesmosine, from Borohydride-reduced Collagen," Biochim. Biophys. Acta 310 130-136

Tanzer, M.L., Housley, T., Berube, L., Fairweather, R., Franzblau, C., and Gallop, P.M. (1973) "Structure of Two Histidine-Containing Crosslinks From Collagen," J. Biol. Chem. 248 393-402

Traub, W. and Piez, K.A. (1971) "The Chemistry and Structure of Collagen," Adv. in Prot. Chem. 25 243-352

Uitto, J. and Lichtenstein, J.R. (1976) "Defects in the Biochemistry of Collagen in Diseases of Connective Tissue," J. Invest. Dermatol. 66 59-79

Veis, A. and Yuan, L. (1975) "Structure of the Collagen Microfibril. A Four-Strand Overlap Model," Biopolymers 14 895-900

Veis, A., Anesey, A., and Mussell, S. (1967) "A Limiting Microfibril Model for the Three-Dimensional Arrangement Within Collagen Fibres," Nature 215 931-934

Volpin, D. and Veis, A. (1973) "Cyanogen Bromide Peptides from Insoluble Skin and Dentin Bovine Collagens," Biochemistry 12 1452-1464

38

THE QUALITATIVE AND QUANTITATIVE CROSSLINK CHEMISTRY OF COLLAGEN MATRICES

Gerald L. Mechanic
Dental Research Center and
Department of Biochemistry and Nutrition
University of North Carolina
Chapel Hill, North Carolina 27514

All normal mammalian collagen, whether it is from different fetal or mature tissues, exhibits the same physical chemical properties, such as shrinkage temperature, molecular weight, etc. Likewise, the protein fibrils, regardless of tissue origin, exhibit the same X-ray diffraction pattern and the same electron microscopic appearance. The amino acid compositions have characteristic contents, such as 1/3 glycine, approximately 1/3 imino acids, and in all, the combined hydroxylysine and lysine totals are the same. Some, however, vary in carbohydration and in the per cent of hydroxylation of the lysine; these hydroxylation variations are found in fetal tissues, mature bone and cartilage. In all cases, the collagen fibrils consist of collagen molecules arranged in a specific three dimensional array, although the collagen in the different tissues serve varied physiological functions. Despite the specific quarter-stagger configuration, another order of specificity might exist in each tissue collagen. This would direct the organization, packing and macromolecular stabilization (intermolecular covalent crosslinks) to specifically serve a tissue's unique function.

Chemistry of Collagen Crosslinking

Selected lysyl or hydroxylysyl residues are converted to their respective aldehydes: α-amino-adipic acid δ-semialdehyde (allysine) and δ-hydroxy-α-amino-adipic acid δ-semialdehyde (hydroxyallysine). The aldehydes, in concert with other lysyl, hydroxylysyl, histidyl, allysyl and hydroxyallysyl residues in the same or adjacent collagen molecules, combine in specifically defined manners to produce characteristic crosslinks composed of two, three or four amino acid components. Crosslink formation

occurs solely when the collagen molecules exist as 670 Å fibrils (Tanzer and Mechanic, 1968). The intermolecular linkages therefore appear to involve the crosslinking of molecular regions displaced from each other and consistent with the specific quarter-stagger packing array. Unstructured fibrils do not contain crosslinks (Tanzer and Mechanic, 1968). Crosslink loci involve the NH_2- and COOH-terminal non-helical regions as well as helical regions of the molecule (Deshmukh and Nimni, 1971, 1971a; Kuboki et al. 1973; Scott et al. 1976).

The intermolecular covalent crosslinks are directly responsible for the stabilization of the macromolecular matrices of support tissues. Crosslink maturational changes occur relative to the age of the animal (Mechanic and Tanzer, 1970; Robins et al. 1973), and between the same tissue in the prenatal and postnatal animal (Mechanic, 1971; Mechanic et al. 1971). The quantitative and qualitative varieties of crosslink chemistry exhibited in collagen obtained from different tissues suggest, as was proposed earlier (Kuboki and Mechanic, 1974), that the crosslinking phenomena in different tissues are under the fine biochemical control of that tissue and are related to collagen fibril structure and function.

Quantitative and qualitative crosslink relationships have been found to be altered in collagen obtained from pathological tissues as will be discussed.

Vitamin D-Deficiency in Chicks

In an early study by Mechanic et al. (1972) it was demonstrated that the ratio of dihydroxylysinonorleucine (DHLNL) to hydroxylysinonorleucine (HLNL) was elevated in $[^3H]NaBH_4$-reduced diaphyseal bone collagen of 4-week-old vitamin D-deficient chicks.

In an extended study, Mechanic et al. (1975), using diaphyseal bone collagen from 1- to 4-week-old control chicks (1.4 I.U. Vitamin D_3 per g diet) and 1- to 4-week-old chicks on a high but non-toxic amount of Vitamin D_3 (50 times control diet), found that the ratio of DHLNL to HLNL was higher in the reduced collagen of control chicks 1 to 3 weeks of age than that of the high-D chicks of comparable age (See Table 1). The ratios were still higher in collagen of vitamin D-deficient chicks of all 4 ages. The lowest ratio occurred in 3- and 4-week-old high-D and 4-week-old control-D chicks and was identical in the latter 3 groups. These results strongly suggest that the maturation of bone collagen, as determined by its reducible crosslinks was accelerated in the high-D animals and that vitamin D may have a dose-related effect on this phenomenon.

It was also demonstrated in that same study (Mechanic et al. 1975) that the DHLNL/HLNL ratio of reduced bone collagen was the

TABLE 1

EFFECTS OF VITAMIN D ON DIAPHYSEAL BONE COLLAGEN CROSSLINKS (DIHYDROXYLYSINONORLEUCINE AND HYDROXYLYSINONORLEUCINE), LYSINE HYDROXYLATION, SERUM CALCIUM AND BODY WEIGHT IN THE CHICK

Group	Age (weeks)	DHLNL/HLNL	Lysine hydroxylation (%)	Serum calcium (mg/100 ml)	Body weight (g)
D-deficient	1	4.2	29	9.75	77
	2	3.9	28	8.40	140
	3	6.6	30	6.47	168
	4	7.6	29	7.10	176
Control-D*	1	3.6	29	10.50	69
	2	3.0	22	10.67	136
	3	2.2	22	10.33	258
	4	1.8	23	9.55	320
High-D**	1	3.2	30	10.90	77
	2	2.2	24	10.15	147
	3	1.8	22	10.63	237
	4	1.8	23	9.50	238

*1.4 I.U. of vitamin D_3 per g diet.
**70 I.U. of vitam D_3 per g diet.

most sensitive determinent of vitamin D-deficiency in chicks when compared to controls (Table 1). A statistically significant increase in the crosslink ratio was found at one week of age, while other indicators of rickets (such as hypocalcemia and histological changes of bone) were not apparent until 2 weeks. The data also suggested that the increase in ratio between the crosslinks in vitamin D-deficient chick bone was due to the lack of vitamin D and not to increased hydroxylation, growth inhibition, nor low circulating calcium. There was also a reduction in crosslink ratio from 1 to 2 weeks in the vitamin D-deficient group, believed to be due to the fact that the chick is born with residual vitamin D_3 or its metabolites from the egg, which are not depleted until after the second week of age, and, therefore, normal maturation continues until these are depleted.

Vitamin D appears to be necessary for normal physiological maturation of bone collagen. No effect of vitamin D-deficiency or high doses of vitamin D was observed on chick skin collagen crosslinks.

In preliminary work (unpublished observation) we have demonstrated that bone collagen from 3-week-old rachitic chicks that had been reduced with standardized [^{3}H]$NaBH_4$ did not contain diminished amounts of DHLNL but did contain less HLNL (See Table 2).

TABLE 2

QUANTITATIVE DETERMINATION[1] OF REDUCIBLE LABILE AND STABLE KETO-IMINE CROSSLINKS IN CONTROL AND RACHITIC CHICK BONE COLLAGEN

3-week-old Chicks

Collagen	mole DHLNL / mole collagen	% DHLNL as Keto-imine	mole HLNL / mole collagen	% HLNL as Keto-imine
Control	1.001		0.747	
Control		46		63
(Ac_2O/HOAc)	0.464		0.47	
Rachitic	0.993		0.373	
Rachitic		45		14
(Ac_2O/HOAc)	0.447		0.051	

[1] Method of Mechanic et al. (1974)

Furthermore, there was no change in the amount of stable reducible crosslink 5-keto-5'-hydroxylysinonorleucine (reduced to DHLNL) while a smaller portion of HLNL was present as 5-keto-lysinonorleucine (reduced to HLNL).

Recently, it was also demonstrated (Gonnerman et al., 1976) that the lysyl oxidase activity was elevated in extracts of bone from vitamin D-deficient chicks. These data, combined with that described above on the quantification of the crosslinks that indicate a decreased amount of aldehydic crosslink precursor, suggest that the increased production of lysyl oxidase (Table 3) might be a primary response to tissue insult or traumatization by vitamin D deficiency. It is proposed that the increase in enzymic activity was not related to the quantitative biochemical crosslink data in the rachitic bone collagen matrix.

Osteogenesis Imperfecta (O.I.)

Biopsy material was obtained from the iliac crest of a number of osteogenic imperfecta patients, and site- and age-matched controls were obtained from normal patients undergoing other surgical procedures. Analysis showed (Mechanic and Bullough, 1975) that the samples of O.I. bone collagen had higher ratios of DHLNL to HLNL than the normal (Table 4). These ratios are reminiscent of those found in rachitic and fetal bovine bone collagens (Mechanic et al., 1971), and might be due to an insufficient maturation of the bone collagen matrix in O.I.

TABLE 3

BODY WEIGHT, PLASMA Ca AND PLASMA INORGANIC PHOSPHATE (P) LEVELS AND ACTIVITY OF LYSYL OXIDASE FROM BONE AND AORTA OF CHICKS MAINTAINED ON CONTROL AND VITAMIN D-DEFICIENT DIETS

	Body wt (g)	Plasma Ca (mg/100 ml)	Plasma P (mg/100 ml)	Lysyl Oxidase (CPM/mg Prot) Bone	Aorta
D-deficient	136	7.64	7.04	23,000	4,100
Control D	147	10.91	5.50	12,400	5,330
High D	145	10.91	5.27	13,900	4,680
SE	±3	±0.19	±0.17	±1,900	±1,280

TABLE 4

RATIOS OF DHLNL TO HLNL IN HUMAN NORMAL AND OSTEOGENESIS IMPERFECTA BONE COLLAGEN

Age Years	Clinical Evaluation	Ratio	Hydroxylysine / lysine
5	Moderate to Severe	2.5	0.15
3	Normal	1.9	0.13
8	Severe	3.1	0.21
13	Normal	1.7	0.13
19	Severe	1.7	0.17
18	Normal	1.4	0.13
13	Moderate	2.0	0.15
13	Normal	1.7	0.13

The results indicate that the quantitative crosslink chemistry of the bone collagen matrix of O.I. is different than the normal, and further suggest that a younger type of collagen is present in the pathological tissue because of the increased amount of hydroxylysine in the bone of O.I. (This is supported by earlier findings [Miller et al. (1967) and Barnes et al. (1972)], that increased hydroxylation of lysine was noted in younger tissue.) The data suggesting a younger type collagen are also supported by scanning electron microscopic studies on bones from O.I. patients that indicate more woven bone or younger type of tissue than their age-matched controls (Dr. S. Titelbaum, personal communication).

Scar Tissue

In an unpublished study from this laboratory, skin scar tissue (normal hypertrophic and keloid) collagens, obtained from the same site (cheek area) and of the same age (2 years from time of injury), were reduced with $[^3H]NaBH_4$ and analyzed for reducible crosslinks, and compared to normal human dermal collagen that was obtained during surgical correction of the keloid scar. The percentage of counts present in the known radioactive component of the normal skin and the scar tissues are presented in Table 5.

All scar tissue contained a large amount of DHLNL while the normal dermal collagen contained little. The most abundant crosslink in the normal was histidinohydroxymerodesmosine (HHMD), and while HHMD was present in all three scar tissues as the least prevalent crosslink, significant amounts were present. The normal scar crosslink pattern was very similar to the patterns obtained from 10- 20- and 30-day-old wound collagen of guinea pig skin (Forrest et al. 1972) and silica granuloma collagen (Jackson and Mechanic, 1974). The occurrence of DHLNL in a fibroblast-synthesized collagen seems to be much like a regenerating tissue that contains a more stable matrix (Jackson et al. 1974).

TABLE 5

% RADIOACTIVITY OF TOTAL[1] IN COMPOUND FROM REDUCED DERMAL COLLAGENS

	HNL[2]	DHLNL	HLNL	HHMD
Normal Skin	11.6	12.0	42.0	34.4
Keloid Scar	13.9	40.2	33.4	12.5
Hypertrophic Scar	19.2	34.0	26.0	20.9
Normal Scar	12.0	46.0	26.3	15.6

[1]Total radioactivity in HNL, DHLNL, HLNL and HHMD

[2]HNL-hydroxynorleucine-reduced aldehyde of lysine

TABLE 6

RATIOS OF RADIOACTIVE COMPOUNDS IN DERMAL COLLAGENS

	$\frac{HNL}{HLNL}$	$\frac{HNL}{HHMD}$	$\frac{DHLNL}{HLNL}$	$\frac{DHLNL}{HHMD}$	$\frac{HLNL}{HHMD}$
Normal	0.22	0.34	0.29	0.35	1.22
Keloid	0.42	1.11	1.75	3.22	2.68
Hypertrophic	0.74	0.92	1.31	1.62	1.24
Normal Scar	0.46	0.77	1.75	2.95	1.69

Table 6 contains data from this laboratory (unpublished observations) showing the ratios of the various radioactive substances in the dermal collagens.

A cursory examination of the above data suggests that only regenerative type collagen is being synthesized and turned over in the normal scar while the keloid and hypertrophic scar fibroblasts are synthesizing both the regenerative type collagen as well as normal skin dermal collagen. Apparently there may be a possibility that the latter type is not turning over as quickly and therefore the collagen is accumulating to form the type of lesion observed.

REFERENCES

Barnes, M.J. Constable, B.J., Morton, L.F. and Kodicek, E. (1972) Hydroxylysine in the N-terminal telopeptides of skin collagen from chick embryo and newborn rat. Biochem. J. 125, 925.

Deshmukh, K. and Nimni, M.E. (1971) Characterization of the aldehydes present in the cyanogen bromide peptides from mature rat skin collagen. Biochemistry 10, 1640.

Deshmukh, K. and Nimni, M.E. (1971a) In vitro formation of cross-links in mature rat skin collagen in the absence of N-terminal peptides. Biochem. Biophys. Res. Commun. 43, 416.

Forrest, L., Shuttleworth, A., Jackson, D.S., and Mechanic, G.L. (1972) A comparison between the reducible intermolecular crosslinks of the collagens from mature dermis and young dermal scar tissue of the guinea pig. Biochem. Biophys. Res. Commun. 46, 1776.

Gonnerman, W.A., Toverud, S.U., Ramp, W.K. and Mechanic, G.L. (1976) Effects of dietary vitamin D and calcium on lysyl oxidase activity in chick bone metaphyses. Proc. Soc. Exptl. Biol. and Med. 151, 453.

Jackson, D.S., Ayad, S. and Mechanic, G.L. (1974) Effect of heat on collagen crosslinks. Biochim. Biophys. Acta 336, 100.

Jackson, D.S. and Mechanic, G.L. (1974) Crosslink patterns of collagens synthesized by cultures of 3T6 and 3T3 fibroblasts and by fibroblasts of various granulation tissues. Biochim. Biophys. Acta 336, 228.

Kuboki, Y., Tanzer, M.L. and Mechanic, G.L. (1973) Isolation of polypeptides containing the intermolecular crosslink 5,5'-dihydroxylysinononleucine from dentin collagen. Arch. Biochem. Biophys. 158, 106.

Kuboki, Y. and Mechanic, G.L. (1974) The distribution of dihydroxylysinonorleucine in bovine tendon and dentin. Connec. Tiss. Res. 2,223.

Mechanic, G.L. and Tanzer, M.L. (1970) Biochemistry of collagen crosslinking--isolation of a new crosslink, hydroxylysinohydroxynorleucine, and its reduced precursor, dihydroxynorleucine, from bovin tendon. Biochem. Biophys. Res. Commun. 41, 1597.

Mechanic, G.L. (1971) The intermolecular crosslink precursors in collagens as related to function. Identification of δ-hydroxy-α-amino adipic-δ-semialdehyde in tendon collagen. Israel J. Med. Sci. 7, 458.

Mechanic, G.L., Gallop, P.M. and Tanzer, M.L. (1971) The nature of crosslinking in mineralized tissues. Biochem. Biophys. Res. Commun. 45, 644.

Mechanic, G.L., Toverud, S.U., and Ramp, W.K. (1972) Quantitative changes of bone collagen crosslinks and precursors in vitamin D deficiency. Biochem. Biophys. Res. Commun. 47, 760.

Mechanic, G.L., Kuboki, Y., Shimokawa, H., Nakamoto, K., Sasaki, S. and Kawanishi, Y. (1974) Collagen Crosslinks: Direct quantitative determination of stable structural crosslinks in bone and dentin collagens. Biochem. Biophys. Res. Commun. 60, 756.

Mechanic, G.L. and Bullough, P. (1975) The quantitative and qualitative crosslink structure of human normal and osteogenesis imperfecta bone collagens. In: Physico-Chimie et Cristallographie des Apatite D'Interet Biologique. Editions Du Centre National De La Recherche Scientifique Paris, France. pg.226.

Mechanic, G.L., Toverud, S.U., Ramp, W.K. and Gonnerman, W.A. (1975) The effect of vitamin D on the structural crosslinks and maturation of chick bone collagen. Biochim. Biophys. Acta 393, 419.

Miller, E.J., Martin, G.R., Piez, K.A. and Powers, M.J. (1967) Characterization of chick bone collagen and compositional changes associated with maturation. J. Biol. Chem. 272, 5481.

Robins, S.P. Shimokamaki, M. and Bailey, A.J. (1973) The chemistry of the collagen crosslinks. Biochem. J. 131, 771.

Scott P.G., Veis A. and Mechanic G. (1976) The identity of a cyanogen bromide fragment of bovine dentin collagen containing the site of an intermolecular cross-link. Biochemistry 15, 3191.

Tanzer, M.L. and Mechanic, G. (1968) Collagen Reduction by Sodium Borohydride: effects of reconstitution, maturation and lathyrism. Biochem. Biophys. Res. Commun. 32, 885.

39

STUDIES ON CROSS-LINKED REGIONS OF ELASTIN

R.A. Anwar, G.E. Gerber and K.M. Baig

Department of Biochemistry
University of Toronto
Toronto, Canada M5S 1A8

ABSTRACT

Elastin, a protein of unique elasticity and tensile strength, is a connective tissue component. Like most elastomers, it consists of randomly coiled polymer chains, joined together by cross-links into an extensible, three dimensional network. The major cross-links of elastin are formed as the result of the deamination of three out of four lysine side chains which subsequently condense to give desmosine or isodesmosine cross-links. We made a novel use of Edman degradation in the study of desmosine and isodesmosine containing elastolytic peptides of mature elastin. This permitted the isolation and sequence studies of peptides C-terminal to the desmosine cross-links in bovine, porcine and human aortic elastin as well as bovine ligamentum nuchae elastin. This identifies the lysines in the tropoelastin (soluble precursor of elastin) which give rise to the desmosine cross-links. The sequences of the C-terminal peptides were found to fall into two distinct classes, one starting with hydrophobic residues, the other starting with alanine. The study of lysine sequences of tropoelastin from a lathyritic calf, with the use of Myxobacter AL-I Protease II, suggests that essentially all lysines occur in pairs separated by two or three small amino acid residues. The majority of the lysines occur in the sequence -Lys-Ala-Ala-Lys- and -Lys-Ala-Ala-Ala-Lys-. It is proposed that two such pairs meet to form desmosine or isodesmosine cross-link and that the hydrophobic residue at the carboxyl end of lysine prevents the enzymic oxidative deamination of the adjacent lysine ε-amino group and this then contributes the nitrogen to the pyridinium ring of the cross-link.

INTRODUCTION

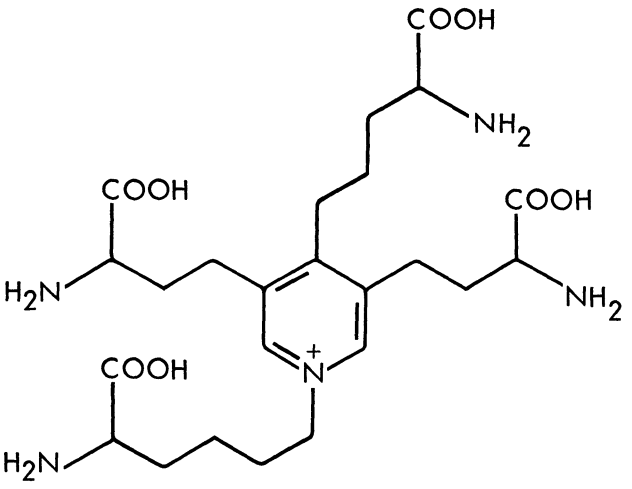
The protein elastin is concentrated in those tissues (ligaments, alveoli, large blood vessels) which require rapid extension and complete recovery, but is only a minor component of skin, tendons and loose connective tissue (Partridge, 1962). It is insoluble in all non-hydrolytic solvents. Hydrated elastin fibers exhibit the unusual properties of a typical elastomer (Hoeve et al., 1958; Weis-Fogh and Anderson, 1970); thus they stretch rapidly under a small load and retract rapidly and completely to their original dimensions (with little loss of energy as heat) upon removal of the load. All typical synthetic elastomers consist of long polymeric chains which are freely mobile with respect to one another except at points of cross-linking; these cross-links must be sufficiently widely spaced to allow considerable extension without breaking covalent bonds, but must be sufficiently close to give the fiber its high tensile strength and modulus when fully stretched. Therefore, it was argued (Partridge, 1962) that elastin must consist of an amorphous system of peptide chains covalently cross-linked at intervals, to account for its elastic properties and insolubility. This realization led to the search for elastin cross-links.

ELASTIN CROSS-LINKS

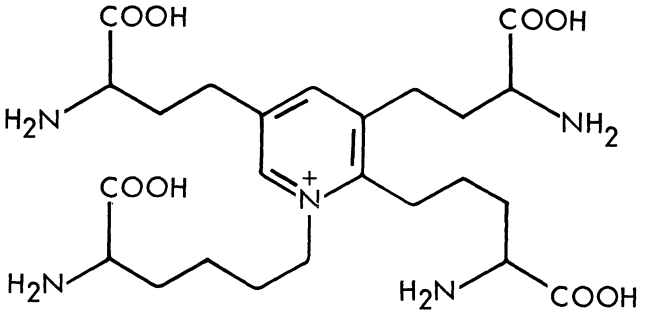
The search for elastin cross-links culminated in the isolation of two polyfunctional amino acids named desmosine and isodesmosine from bovine ligamentum nuchae elastin (Thomas et al., 1963). The structures of these compounds are shown in Fig. 1. Each compound has a pyridinium nucleus and 4 α amino and carboxylic groups. Desmosine is 1,3,4,5 substituted pyridine and isodesmosine is 1,2,3,5 substituted pyridine. Thus these compounds are capable of cross-linking four polypeptide chains. The isolation of these two new amino acids was later extended to elastin preparations from different tissues and species (Anwar, 1966). After the isolation of these compounds, the attention naturally turned to the problem of their biosynthesis.

BIOGENESIS OF DESMOSINE CROSS-LINKS

The structures of desmosine and isodesmosine suggested that each could be derived from four lysine molecules. In support of this suggestion, it was shown that ^{14}C label was incorporated into desmosine and isodesmosine isolated from aortas of rats given [^{14}C]-lysine (Partridge et al., 1964, 1966) and chicken aortas grown in a medium containing [^{14}C]-lysine (Miller et al., 1964, 1965). The degradation of isolated desmosine and isodesmosine



Desmosine



Isodesmosine

Fig. 1 Structure of desmosine and isodesmosine

from chick aortas grown in the presence of [^{14}C]-lysine clearly demonstrated that these isomers are in fact derived from four lysine residues and that the intact carbon skeleton of lysines is incorporated into the isomers (Anwar and Oda, 1966, 1967).

Hantzsch in 1886 showed that N-methyl nicotinic acid when heated in strong alkali (KOH) yields methylamine: Huff and Perlzweig (1943) on similar treatment of N-methylnicotinamide were able to recover CH_3-N≡ group from the ring as methylamine in

97-98% yield. This is a degradation of 1-substituted pyridine derivative with the liberation of ring nitrogen attached to the side chain as primary amine. As desmosine and isodesmosine are 1-substituted pyridine derivatives, therefore, alkali degradation of these compounds was attempted. This type of degradation with desmosine and isodesmosine would be expected to yield lysine (side chain attached to the ring N) as shown in Fig. 2. This was indeed found to be the case; one of the compounds liberated by the alkali degradation of both desmosine and isodesmosine was identified as lysine (Anwar and Oda, 1966b).

It is apparent from Fig. 2 that if intact carbon skeletons of four lysines condense to form desmosine and isodesmosine then only 1/4th of the radioactivity incorporated into desmosine and isodesmosine from [^{14}C]-lysine would appear in the lysine liberated by the alkali degradation of these compounds. Further, if precursor lysine were specifically labeled in position one ([1-^{14}C]-lysine) then all the radioactivity incorporated into desmosine and isodesmosine would be in the carboxyl carbons and thus would be liberated as CO_2 with the use of the ninhydrin reaction. This was found to be the case as shown in Table I and Table II. Table I shows that the lysine liberated by the alkali degradation of desmosine and isodesmosine contained only 1/4th of

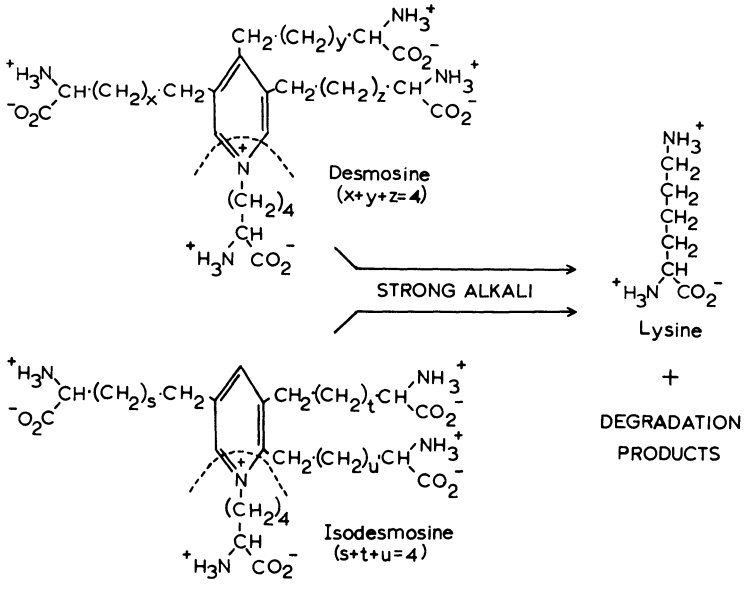


Fig. 2 Alkali degradation of desmosine and isodesmosine

TABLE I

Alkali Degradation of Labeled Desmosine and Isodesmosine Synthesized from [1-^{14}C] Lysine by Chick Embryo Aortae

Source	Specific Activity of the Compound taken for Degradation (c.p.m./μmole) x 10^{-3}	Specific Activity of the Liberated Lysine	Ratio of Specific Activity Entire Molecule/ Lysine Liberated
Desmosine	10.7	2.8	3.8
Isodesmosine	32.8	8.1	4.0

TABLE II
Ninhydrin Decarboxylation of Labeled Desmosine and Isodesmosine Synthesized from [1-^{14}C] Lysine by Chick Embryo Aortae and Lysine

Source	Total Activity Taken	Total Activity Recovered as CO_2	% Recovery
	c.p.m.		
Desmosine	307	297	96.8
Isodesmosine	339	319	94.2
Lysine (added to medium)	16,127	14,730	91.5
Lysine (isolated from elastin)	2,035	1,916	94.2

the radioactivity of the parent molecule. Table II shows that all the radioactivity incorporated into desmosine and isodesmosine from [1-^{14}C]-lysine was liberated as CO_2 by the ninhydrin reaction. These results clearly show that intact carbon skeletons of four lysines condense to form desmosine or isodesmosine molecule. The results of pulse labeling experiments (Miller et al., 1965; Partridge et al., 1966) suggested that lysine is first incorporated into the polypeptide chains and that the lysine side chains in the polypeptides serve as the precursors of desmosine and isodesmosine.

The formation of desmosine or isodesmosine from four lysine side chains requires the loss of three ε-amino groups, presumably through oxidative deamination. The reaction then proceeds through aldol and Schiff base type of condensations. The studies with model aldehydes and amines suggested that, after the enzymic oxidative deamination of ε-amino groups, the reactions occur spontaneously leading to the formation of desmosine and isodesmosine (Davis and Anwar, 1970). Based on these studies a detailed mechanism for the formation of desmosine and isodesmosine was proposed and is shown in Fig. 3. Appropriate aldol and Schiff base condensations result in the formation of substituted dihydropyridines. The dihydropyridinium ion is readily oxidized by molecular oxygen, to the aromatic structure. All these reactions can occur under physiological conditions.

Several other lysine-derived amino acids have been isolated from elastin and are shown in Fig. 4. Most of these are intermediates in the formation of desmosine and isodesmosine.

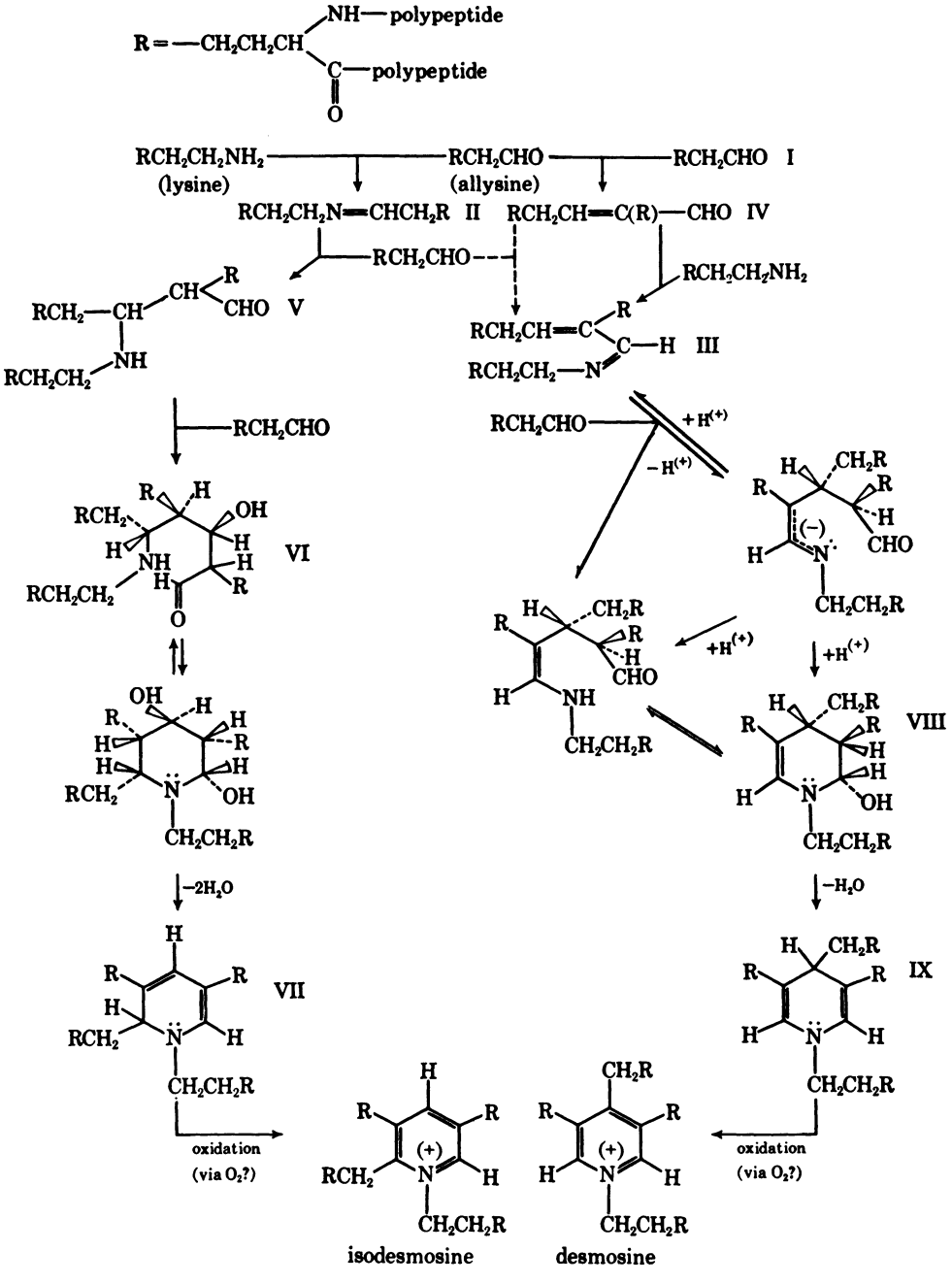


Fig. 3 Mechanism for the formation of desmosine and isodesmosine

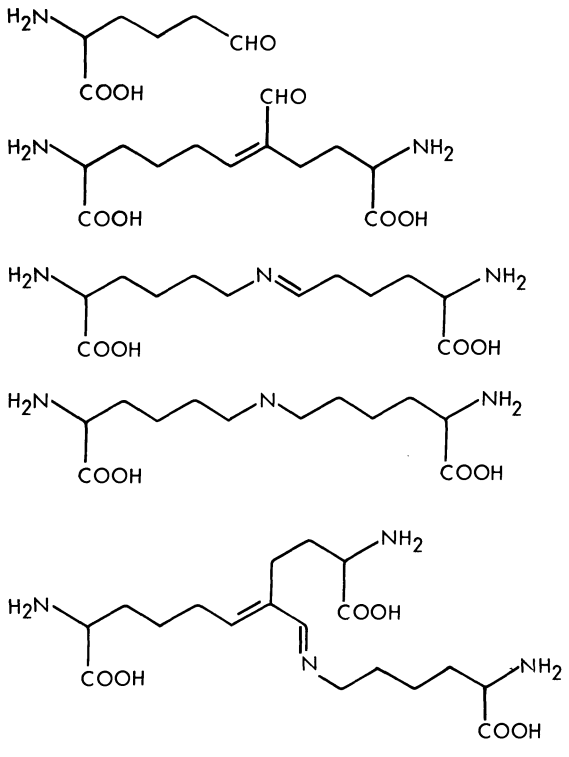


Fig. 4 Amino acids derived from lysine in elastin

AMINO ACID SEQUENCES AROUND DESMOSINE CROSS-LINKS

The obvious next step in understanding the biosynthesis of elastin cross-links was the identification of the lysines which give rise to desmosine and isodesmosine cross-links. For this purpose a study was undertaken to determine the amino acid sequences around the desmosine cross-links.

The classical methods of protein chemistry are not readily applicable to the study of mature elastin owing to its high degree of cross-linking and its insolubility in all nonhydrolytic solvents. Therefore, elastin was exhaustively digested with pure elastase and the elastolytic cross-linked peptides were isolated by chromatographic procedures (Gerber and Anwar, 1974). In order to determine the amino acid sequences, it was necessary that the cross-linked chains be resolved into single chain peptides. Initially, the release of single chain peptides from the cross-links was attempted by means of chemical cleavage of the pyridine rings (Shimada et al., 1969). The characterization

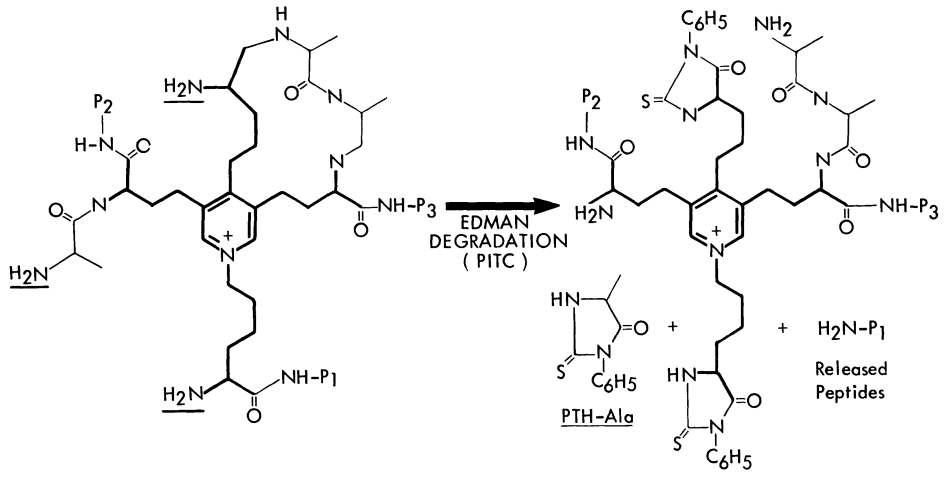


Fig. 5 The use of the Edman degradation for the release of C-terminal peptides from the desmosine-containing peptides

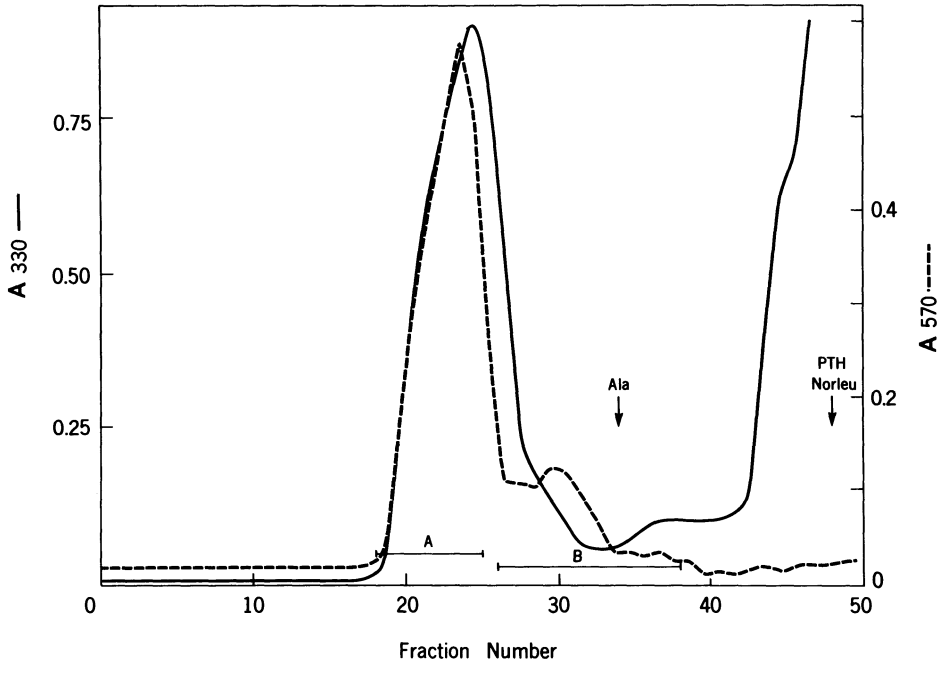


Fig. 6 Chromatography of peptides on Sephadex LH-20 after Edman degradation

of peptides thus released from the cross-links indicated that elastase was cleaving at and very close to the NH_2-terminals of the cross-links. In retrospect this was not an unexpected result. The pure elastase has a strong specificity for alanyl peptides (Narayanan and Anwar, 1969) and the cross-linked regions are rich in alanine content (Shimada et al., 1969; Keller et al., 1969).

The NH_2-terminal analysis of cross-linked peptides showed desmosine and isodesmosine as one of the NH_2-terminals. It was, therefore, thought possible to release C-terminal peptides from the desmosine and isodesmosine cross-links by Edman degradation. To illustrate the rationale of this approach, the degradation of only one of the many possible structures present is shown in Fig. 5. At those positions where the cross-link itself is the NH_2-terminal, Edman degradation will release the peptides attached to the α carboxyl groups (e.g. P_1 in this case). The released peptides can then be separated from the cross-linked ones on the basis of size. The peptide P_2 will be released after a second cycle of Edman degradation. The peptide P_3 will not be released until the cross-link NH_2-terminal immediately preceding P_3 becomes available (i.e. P_3 will be released after four cycles of Edman degradation). This approach was used to study the amino acid sequences C-terminal to the desmosine containing peptides from bovine, porcine and human aortic elastin as well as bovine ligamentum nuchae elastin (Gerber and Anwar, 1975).

After each cycle of Edman degradation the released peptides were separated from the cross-linked ones by chromatography on Sephadex LH-20 in 50% pyridine (Gerber and Anwar, 1974). A typical elution profile is shown in Fig. 6. The released peptides were further purified by chromatography on a column of Beckman AA-15 resin. A typical elution profile of peptides released from the C-terminal of the desmosines of bovine elastin is shown in Fig. 7.

The amino acid sequences of the pure released C-terminal peptides from the various sources are given in Table III. More recent studies (Baig, 1976) with different desmosine containing fraction from bovine ligamentum nuchae elastin have further confirmed these results and have extended the sequence of peptide B6 from Ala-Gln to Ala-Gln-Gly-. These sequences (Table III) of the released C-terminal peptides should correspond to and are thus compared with the NH_2-terminal sequences of tryptic peptides of porcine aortic tropoelastin (Gray et al., 1973; tropoelastin is the soluble precursor of elastin and contains unmodified lysines). The peptides obtained from bovine ligamentum nuchae elastin were identical with those obtained from bovine aortic elastin (Column 3). Therefore, the differences between these sequences and the NH_2-terminal sequences of tryptic peptides of porcine aortic tropoelastin (Column 1) or the sequences of the released C-terminal peptides from porcine aortic elastin

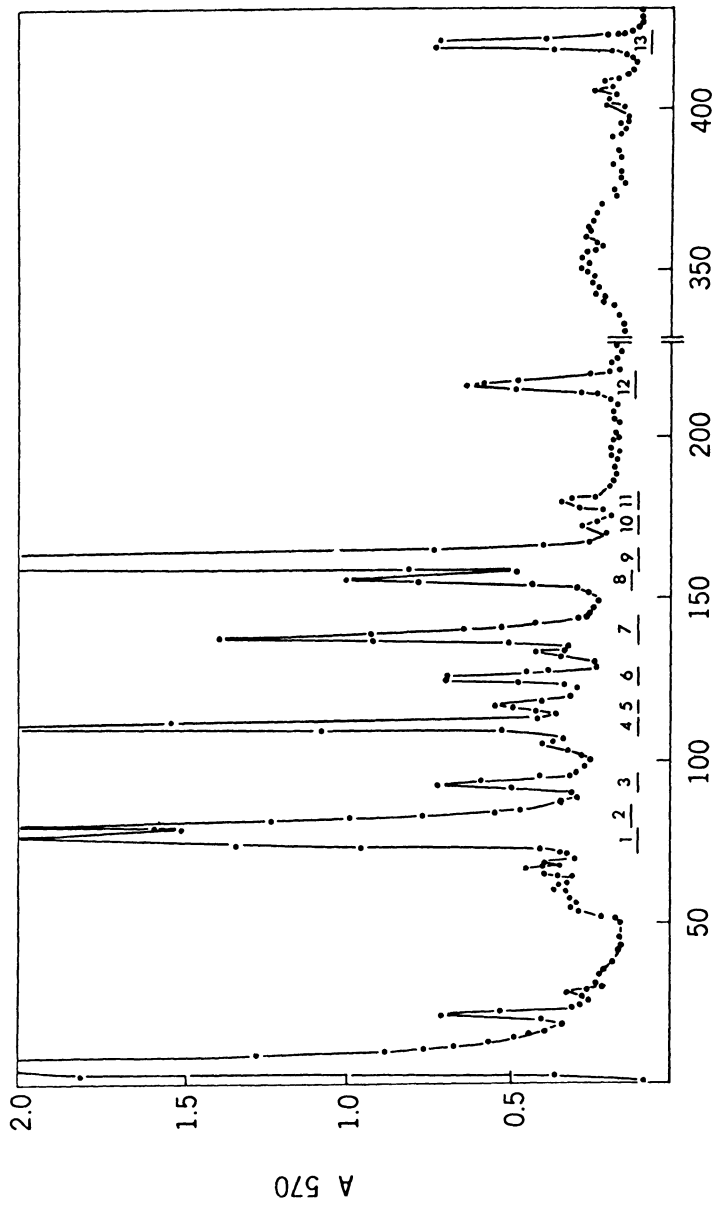


Fig. 7 Purification of peptides released from the C-terminals of the desmosine cross-links of bovine ligamentum nuchae elastin on a column of Beckman AA-15 resin

TABLE III

Comparison of the sequences of peptides C-terminal to the desmosines in elastin from porcine aorta, bovine aorta and ligament and human aorta with the N-terminal sequences of tryptic peptides of porcine tropoelastin.

Porcine tropoelastin*		Porcine aorta elastin		Bovine aorta and ligament elastin		Human aorta elastin	
T1	Tyr-Gly-Ala-Ala-Gly-Gly-Leu-						
		PA12b	Tyr-Gly-Ala-Ala	B7b	Phe-Gly-Ala-Ala	HA10a	Tyr-Gly-Ala-Ala
T9c	Tyr-Gly-Ala-Ala-Gly-Ala-Leu-						
T4	Tyr-Gly-Ala-Pro-Gly-Ala-Gly-	PA2	Tyr-Gly-Ala-Pro-Gly-Ala-Gly				
T2	Ala-Ala-Gln-Phe-Gly-Leu-						
		PA9	Ala-Gln	B6	Ala-Gln	HA6a	Ala-Gln
T9b	Ala-Ala-Glu-Phe-Gly-Val-						
T14b	Ala-Pro-Gly-Gly-Gly-Gly-Ala-	PA1	Ala-Pro-Gly-Gly-Gly-Gly-Ala				
T7b	Ala-Gly-Tyr-Pro-Thr-Gly-	PA3	Ala-Gly-Tyr-Pro-Thr	B1a	Ala-Gly-Tyr-Pro-Thr	HA2a	Ala-Gly-Tyr-Pro-Thr
	Tyr-Gly-Ala-Arg	PA15	Tyr-Gly-Ala-Arg	B13	Phe-Gly-Ala-Arg		
	Ser-Ala-Lys	PA12c	Ser-Ala	B9b	Ser-Ala	HA10c	Ser-Ala
	Ala-Ala-Lys	PA12a	Ala-Ala	B9a	Ala-Ala	HA10b	Ala-Ala
T6	Ala-Gly-Ala-Gly-Leu-Gly-	PA8	Ala-Gly-Ala	B3a	Ala-Gly-Ala-Gly	HA9	Ala-Gly
				B1c	Leu-Gly-Ala-Gly-Gly-Ala		
		PA5	Leu-Gly-Ala-Ala	B1b	Ile-Gly-Ala-Gly-Gly-Val	HA8	Phe-Gly-Ala-Gly
				B2	Phe-Gly-Pro-Gly-Gly-Val		

* Taken from Gray *et al.* (1973).

TABLE IV
Amino Acid Composition of Elastins[a)]

		Soluble		Insoluble
Amino acid	Porcine[b)] aorta	Bovine[c)] Cu-Deficient	Bovine (BAPN)	Bovine ligament
Hydroxyproline	9.9	8	8	5
Aspartic acid	3.7	6	7	6
Threonine	14.4	8	11	8
Serine	11.6	9	10	8
Glutamic acid	16.9	15	16	16
Proline	104	92	103	125
Glycine	326	316	328	344
Alanine	230	220	228	223
Valine	132	147	134	132
Isoleucine	16.0	20	25	26
Leucine	45.2	55	55	64
Tyrosine	16.3	6	8	8
Phenylalanine	26.5	32	27	30
Lysine	43.3	49	46	4
Arginine	4.3	5	6	6

a) Expressed as residues per 1000 residues.
b) Taken from Smith et al. (1972).
c) Taken from Whiting et al. (1974).

(Column 2) must be species rather than tissue specific. The most striking difference involves the occurrence of phenylalanine in bovine elastin and tyrosine in porcine and human elastin C-terminal to the desmosine cross-links. The replacement of tyrosine by phenylalanine is a highly common mutation. All the other differences are also consistent with single point mutation. It may be noted that the sequences of the C-terminal peptides fall into two distinct classes, about one-half starting with alanine and the other starting with hydrophobic residues such as tyrosine, phenylalanine, leucine or isoleucine.

BOVINE TROPOELASTIN

The observed differences in amino acid sequences around the desmosine cross-links of bovine and porcine elastin prompted us to undertake the study of the lysyl sequences of bovine tropoelastin. Bovine tropoelastin was purified from ligamentum nuchae and aorta of a calf fed β-aminopropionitrile (BAPN) in a milk diet. The purification was carried out according to the procedure of Sandberg et al. (1971), which was modified

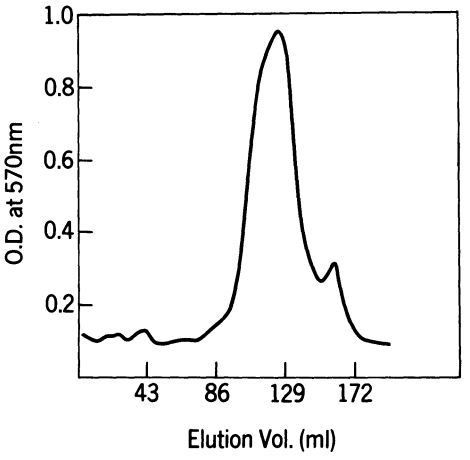


Fig. 8 Chromatography of bovine tropoelastin digest with Myxobacter AL-I Protease II on a Sephadex G-100 column

(Narayanan & Page, 1974) to minimize protein degradation. The molecular weight of the purified protein was estimated to be 70,000 by SDS (sodium dodecyl sulfate) gel electrophoresis. The amino acid composition of the purified bovine tropoelastin is shown in Table IV; also shown is the amino acid composition of porcine tropoelastin (Smith et al., 1972) and bovine tropoelastin isolated from the Cu-deficient calves (Whiting et al., 1974).

The purified bovine tropoelastin was then digested with Myxobacter AL-1 Protease II. This enzyme is very specific and cleaves peptide bonds involving the α-amino group of lysine (Wingard et al., 1972); the lysine appears as the new N-terminal.

The chromatography of the digest on a Sephadex G-100 column showed that the digestion was complete (i.e. no undigested protein was detected). As shown in Fig. 8, the digest separated into two peaks. Each peak was then chromatographed on Sephadex G-25 as shown in Fig. 9. The large molecular size material appeared under the first peak and the second peak was found to contain tri- and tetrapeptides. The material appearing under these two peaks was analysed for total lysine content. As shown in the figure, one-half of the lysines were recovered under the first peak; the second peak contained the same amount of lysine as the first peak. The peptides under the second peak were further purified on a column of Beckman AA-15 resin and characterized. The results are shown in the figure. These results are consistent with the data of Sandberg et al. (1971b) with porcine tropoelastin.

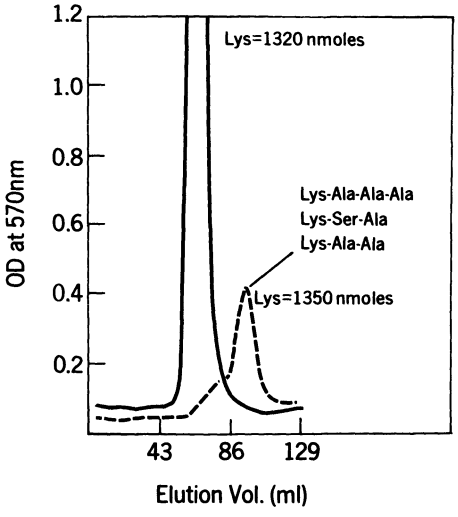


Fig. 9 Chromatography of the two peaks from Sephadex G-100 (Fig. 8) on a column of Sephadex G-25

CONCLUSIONS

The appearance of half of the lysines of tropoelastin as tri- and tetrapeptides and half of the lysines as large peptides after digestion with protease II and the number of different peptides released from the C-terminal of desmosine cross-links suggest that essentially all the lysines in tropoelastin occur in pairs separated by two or three small amino acid residues (e.g. -Lys-Ala-Ala-Lys-, -Lys-Ala-Ala-Ala-Lys-, -Lys-Ser-Ala-Lys-) as shown in Fig. 10. These lysine pairs are uniformly distributed throughout the tropoelastin polypeptide chain. It is proposed that when two such pairs come into alignment after the action of lysyl oxidase they give rise to desmosine or isodesmosine cross-link.

Mainly alanine is found NH_2-terminal to the desmosines. The sequences of C-terminal peptides released from the cross-links suggest that half the pairs are followed by alanine and the others are followed by hydrophobic residues. Thus it seems that three of the four lysines, in two pairs, which give rise to desmosine or isodesmosine, are preceded and followed by alanine or similar small amino acids (e.g. serine) whereas the fourth lysine is followed by a hydrophobic residue. Out of the four lysines that give rise to the desmosine cross-link, three are to be deaminated whereas one retains its ε-amino group. Therefore, it is proposed

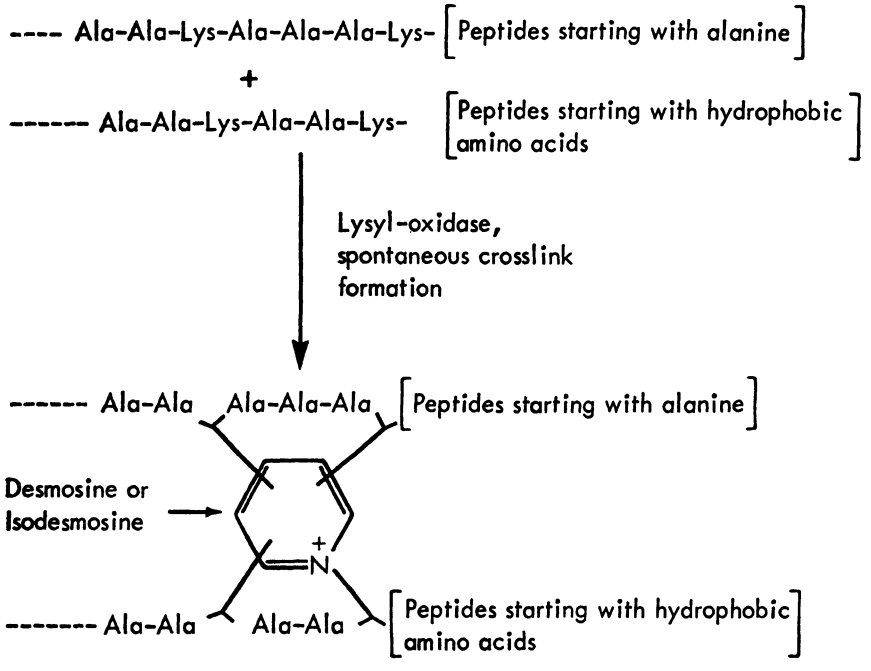


Fig. 10 A scheme for the formation of the desmosine cross-links

that all lysines preceded and followed by alanines or similar small amino acids are oxidatively deaminated by the enzyme (lysyl oxidase) whereas those followed by hydrophobic amino acids retain their ε-amino groups and donate this nitrogen to the pyridine ring as shown in Fig. 10.

ACKNOWLEDGEMENTS

We thank Mrs. Mariana Vlaovic for her excellent technical assistance. We are also grateful to Dr. T. Hofmann for the gift of Myxobacter AL-I Protease II. The work from our laboratory was supported by the Medical Research Council of Canada.

REFERENCES

Anwar, R.A. (1966). Comparison of elastins from various sources. Can. J. Biochem., 44, 725.

Anwar, R.A. and Oda, G. (1966). The biosynthesis of desmosine and isodesmosine. J. Biol. Chem., 241, 4638.

Anwar, R.A. and Oda, G. (1966b). Structure of desmosine and isodesmosine. Nature, 210, 1254.

Anwar, R.A. and Oda, G. (1967). The biosynthesis of desmosine and isodesmosine II. Incorporation of specifically labeled lysine into desmosine and isodesmosine. Biochim. Biophys. Acta, 133, 151.

Baig, K.M. (1976). Studies on amino acid sequences around the cross-links of elastin. M.Sc. Thesis, University of Toronto, Toronto, Canada.

Davis, N.R. and Anwar, R.A. (1970). On the mechanism of formation of desmosine and isodesmosine cross-links of elastin. J. Amer. Chem. Soc., 92, 3778.

Franzblau, C., Sinex, F.M., Faris, B. and Lampidis, R. (1965) Identification of a new cross-linking amino acid in elastin. Biochem. Biophys. Res. Commun., 21, 575.

Gerber, G.E. and Anwar, R.A. (1974). Structural studies on cross-linked regions of elastin. J. Biol. Chem., 249, 5200.

Gerber, G.E. and Anwar, R.A. (1975). Comparative studies of the cross-linked regions of elastin from bovine ligamentum nuchae and bovine, porcine and human aorta. Biochem. J., 149, 685.

Gray, W.R., Sandberg, L.B. and Foster, J.A. (1973). Molecular model for elastin structure and function. Nature, 246, 461.

Hantzsch, A. (1886). Ueber ammoniumderivate von saureathern des pyridins und chinolins. Ber. Chem. Ges., 19, 31.

Hoeve, C.A.J. and Flory, P.J. (1958). The elastic properties of elastin. J. Amer. Chem. Soc., 80, 6523.

Huff, J.W. and Perlzweig, W.A. (1943). N'-methylnicotinamide, a metabolite of nicotinic acid in the urine. J. Biol. Chem., 150, 395.

Keller, S., Levi, M.M. and Mandl, I. (1969). Antigenicity and chemical composition of an enzymatic digest of elastin. Arch. Biochem. Biophys., 132, 565.

Lent, R.W., Smith, B., Salcedo, L.L., Faris, B. and Franzblau, C. (1969). Studies on the reduction of elastin II. Evidence for the presence of α-aminoadipic acid δ-semialdehyde and its aldol condensation product. Biochemistry, 8, 2837.

Miller, E.J., Martin, G.R. and Piez, K.A. (1964). The utilization of lysine in the biosynthesis of elastin cross-links. Biochem. Biophys. Res. Commun. 17, 248.

Miller, E.J., Martin, G.R., Mecca, C.E. and Piez, K.A. (1965). The biosynthesis of elastin cross-links. J. Biol. Chem., 240, 3623.

Miller, E.J., Pinnell, S.R., Martin, G.R. and Schiffman, E. (1967). Investigation of the intermediates involved in desmosine biosynthesis. Biochem. Biophys. Res. Commun., 26, 132.

Narayanan, A.S. and Anwar, R.A. (1969). The specificity of purified porcine pancreatic elastase. Biochem. J., 114, 11.

Narayanan, A.S. and Page, R.C. (1974). Molecular weight heterogeneity of tropoelastin resulting from proteolysis during preparation. FEBS Letters, 44, 59.

Partridge, S.M. (1962). Elastin. Adv. Prot. Chem., 17, 227.

Partridge, S.M., Elsden, D.F., Thomas, J., Dorfman, A., Telser, A. and Ho, P. (1964). Biosynthesis of the desmosine and isodesmosine cross-bridges in elastin. Biochem. J., 93, 30C.

Partridge, S.M., Elsden, D.F., Thomas, J., Dorfman, A., Telser, A. and Ho, P. (1966). Incorporation of labelled lysine into the desmosine cross-bridges in elastin. Nature, 209, 399.

Sandberg, L.B., Zeikus, R.D. and Coltrain, I.M. (1971). Tropoelastin purification from copper-deficient swine: a simplified method. Biochim. Biophys. Acta, 236, 542.

Sandberg, L.B., Weissman, N., Gray, W.R. (1971b). Structural features of tropoelastin related to the sites of cross-links in aortic elastin. Biochemistry, 10, 52.

Shimada, W., Bowman, A., Davis, N.R. and Anwar, R.A. (1969). An approach to the study of the structure of desmosine and isodesmosine containing peptides isolated from the elastase digest of elastin. Biochem. Biophys. Res. Commun., 37, 191.

Smith, D.W., Brown, D.M. and Carnes, W.H. (1972). Preparation and properties of salt-soluble elastin. J. Biol. Chem., 247, 2427.

Starcher, B.C., Partridge, S.M. and Elsden, D.F. (1967). Isolation and partial characterization of a new amino acid from reduced elastin. Biochemistry, 6, 2425.

Thomas, J., Elsden, D.F. and Partridge, S.M. (1963). Degradation products from elastin. Nature, 200, 651.

Weis-Fogh, T. and Anderson, S.O. (1970). New molecular model for the long-range elasticity of elastin. Nature, 227, 718.

Whiting, A.H., Sykes, B.C. and Partridge, S.M. (1974). Isolation of salt-soluble elastin from ligamentum nuchae of copper-deficient calf. Biochem. J. 141, 573.

Wingard, M., Matsueda, G. and Wolfe, R.S. (1972). Myxobacter AL-I protease II: specific peptide bond cleavage on the amino side of lysine. J. Bacteriology, 112, 940.

SUBJECT INDEX