Aging in Germ-Free Mice: Life Tables and Lesions Observed at Natural Death[1]

Helmut A. Gordon, M.D.,[2] Edith Bruckner-Kardoss, M.T.,[3] and Bernard S. Wostmann, Ph.D.[3]

THE RATIONALE for the use of germ-free animals in aging studies is that they permit a clear differentiation between (a) alleged deleterious effects of microbial associates on the host and (b) the impairment in performance of the aging organism by endogenous and other non-microbial environmental agents. Although these issues were recognized for many years, work in this area was not undertaken because germ-free techniques were not developed to the point of usefulness for studies on aging. These and related problems were reviewed several years ago (Gordon, 1959a). To date, the oldest reported age for germ-free rats is 1045 days (Wistar strain, l.c.), for germ-free mice 600 days (C_3H strain, Reyniers & Sacksteder, 1958).

In 1958 a colony of germ-free and conventional control mice was initiated at Lobund Laboratory with the intent to explore the practicability of germ-free methods in gerontological work and to study the effects of the normal microbial flora on aging processes. A brief interim report on this work has previously been given (Gordon, Bruckner-Kardoss, & Wostmann, 1964). The purpose of this communication is to present life tables, major lesions observed at natural death, along with other observations made in these animals.

MATERIALS AND METHODS

Animals.—The animals reported in this study originated from Swiss-Webster mice which were obtained from the Harlan Farms (Harlan Industries, Cumberland, Indiana) in 1955. From these we reared in the same year to maturity a single couple of germ-free, cesarean-delivered mice. Both the germ-free and conventional breeding colonies which supplied the mice for the present work were derived from these parent animals. The germ-free breeders maintained their bacteriologically sterile status in an uninterrupted line. The ancestors of conventional breeders were germ-free mice which soon after forming the original nucleus were removed from the isolators and allowed to become contaminated with the microbial flora of conventional quarters. From then on, close genetic similarity between the two colonies was maintained by introducing germ-free males into the conventional colony at four- to five-month intervals. The actual groups of aging animals were formed by randomly selecting germ-free and conventional mice from the breeding colonies at the age of 12 months and transferring them into their special quarters. All mice were 14th to 19th generation normal-born, mother-suckled descendants of the original germ-free cesarean-delivered ancestors. Both males and females were used; the females were allowed a maximum of four previous gestation periods. Only groups numbering more than 30 mice were included in the life tables of this study. The time which was needed for assembling the animals included in this report extended over a period of approximately five years.

Housing, germ-free.—Individual housing was in one-gallon churn jars, three or four animals of the same sex in each. The jars were contained in flexible plastic isolators of Trex-

[1] This work was supported by grants from the U.S. Public Health Service.
[2] Department of Pharmacology, University of Kentucky, Lexington, Kentucky.
[3] Lobund Laboratory, University of Notre Dame, Notre Dame, Indiana.

ler's design (1959) (24x24x48 in.) with a maximum of 12 jars in each isolator. The jars were restocked only after they became empty by death of all previous inhabitants. Each isolator contained both "male" and "female" jars. Air flow was maintained at 3 to 4 cfm. The temperature within the isolators was held at 26 ± 1 C.; the humidity of the air was approximately 45%. Detailed description of the operation of the germ-free isolators and of periodic sterility testing are given by Reyniers, Trexler, and Ervin (1946) and by Wagner (1959).

Housing, conventional.—Individual housing of animals was the same as described for the germ-free group. The jars were held in open environment. Animal quarters, until 1960, were in Lobund's Quonset Building, in an air-conditioned room. After 1960, the conventional animal quarters were in a completely isolated, air-conditioned room where the same temperature and humidity were maintained as that for the germ-free group. Bedding consisted of sterilized wood shavings for both germ-free and conventional groups. Clean-up operations were once a week. The animal curators were the same throughout the period of this work.

Diets.— In this study the following diets were used: (a) fortified, practical, germ-free-type L 462 diet (Wostmann, 1959) sterilized, designation in this paper: *L 426 st;* (b) fortified, practical, germ-free type commercial diet (5010C Purina Mouse Chow, Ralston-Purina, St. Louis), sterilized, designation: *5010C st;* (c) practical, conventional type commercial diet (Purina Mouse Lab Chow, same source) not sterilized, designation: *P non-st.* This, in essence, is a diet similar to 5010C. All diets were in the form of pellets. Sterilization was by steam at 252 F., 16 to 17 psi, over a period of 25 minutes. Diet and tap water were offered *ad libitum.* The mice in this study were divided into the following groups: A. Including both germ-free and conventional mice: (1) From weaning to death on diet *L 462 st;* (2) from weaning to ten months of age on diet *L 462 st*; from then to death on diet *5010C st.* Designation of this group is *L 462 st → 5010C st.* B. Including only conventional mice: (3) From weaning to ten months of age on diet *P non-st,* from then to death on diet *5010C st.* Designation of this group is *P non-st → 5010C st.* (4) From weaning to death on diet *P non-st.*

Terminal procedures.—All animals were kept in their quarters until natural death occurred. After this was ascertained, they were removed as promptly as feasible (in most instances within hours). Once on the outside, the abdominal cavity was opened by a small median incision, and the animal was placed in 10% neutral formol fixative in individual jars. Consecutively the following were observed in a randomly selected group: 1) Body weight, after rinsing the animal in water and blotting off the liquid accumulated in the abdominal cavity and on the fur. The body weights given were corrected by subtraction of the cecal weight. 2) Weight of the full cecal sack after

Table 1. Mean Age at Death of Germ-Free and Conventional Mice Fed Different Diets.

Diet	Germ-free						Conventional					
	Males			Females			Males			Females		
	N[a]	M[b]	S.D.[c]	N	M	S.D.	N	M	S.D.	N	M	S.D.
L 462 st	83	24.13	5.69	144	22.68	4.66	96	16.03	3.41	114	17.24	3.93
L 462 st to 5010C st	35	22.40	5.27	56	20.02	5.16	55	16.25	3.79	41	17.24	3.50
P non-st to 5010C st	—	—	—	—	—	—	—	—	—	42	17.69	3.58
P non-st	—	—	—	—	—	—	—	—	—	35	19.31	3.70

[a] N=number of mice.
[b] M=mean age in months.
[c] S.D.=standard deviation.

Significance of differences among some groups of germ-free and conventional mice.

		p diff.
1. gf *L 462 st* ♂	vs. gf *L 462 st* ♀	= 0.04
	conv *L 462 st* ♂	=<0.01
2. gf *L 462 st* ♀	vs. conv *L 462 st* ♀	=<0.01
	conv *P non-st* ♀	=<0.01
3. conv *L 462 st* ♂	vs. conv *L 462 st* ♀	= 0.02
4. conv *L 462 st* ♀	vs. conv *P non-st* ♀	= 0.01

ligation and excision at the ileo-cecal-colic junction. 3) Major lesions at death. With the necessity of leaving the animals in their original quarters until death and with the delay suffered in transferring the dead animals from the isolators to the outside, the onset of post-mortem changes has often clouded the evaluation. The material offered represents observations made in relatively well preserved specimens.

Losses by accidental contamination.—Germ-free mice housed in isolators that became accidentally contaminated were eliminated from this study. Until 1962 four isolators were lost in this fashion. None became contaminated after that date.

Results

The germ-free state did not impart any readily visible characteristics to the mice. Unless they were affected by particular lesions, old germ-free mice seemed a little fatter and their abdomens were more protruding than in conventional controls. At autopsy the fat depot seemed greater and, invariably, the cecum was considerably enlarged. The odor of these animals, including the intestinal contents, resembled that of caramel.

Comparing (a) percentage of survivors at successive time intervals (Fig. 1) and (b) mean age at death (Table 1) in germ-free and conventional mice fed *L 462 st* diet, it was shown that the germ-free group of this series survived significantly longer than the conventional controls. In the dietary variant group *L 462 st → 5010C st* the same trend was indicated. In both *L 462 st* and *L 462 st → 5010C st* groups the germ-free males showed higher mean age at death than germ-free females. In case of the former diet *P* of this difference was 0.04, in the latter, 0.02. In the groups of conventional mice this condition appeared reversed. In the other dietary variant group *P non-st → 5010C st* (which included only conventional females) the mean age at death was similar to that of the conventional controls in the *L 462 st* and *L 462 st → 5010C st* groups. Finally, among the females of the dietary group *P non-st* an elevation of mean age was indicated (approximately by two months) in comparison with the conventional controls of the other three groups fed sterilized diets. In two of these comparisons *P* was 0.02 or less; in the third *P* was nonsignificant.

Table 2 lists the body weight and the weight of the cecum of mice fed *L 462 st* diet after formol fixation. These are offered as the best available information, in lieu of data taken in the customary fashion, i.e., after sacrifice. In separate observations it was ascertained that

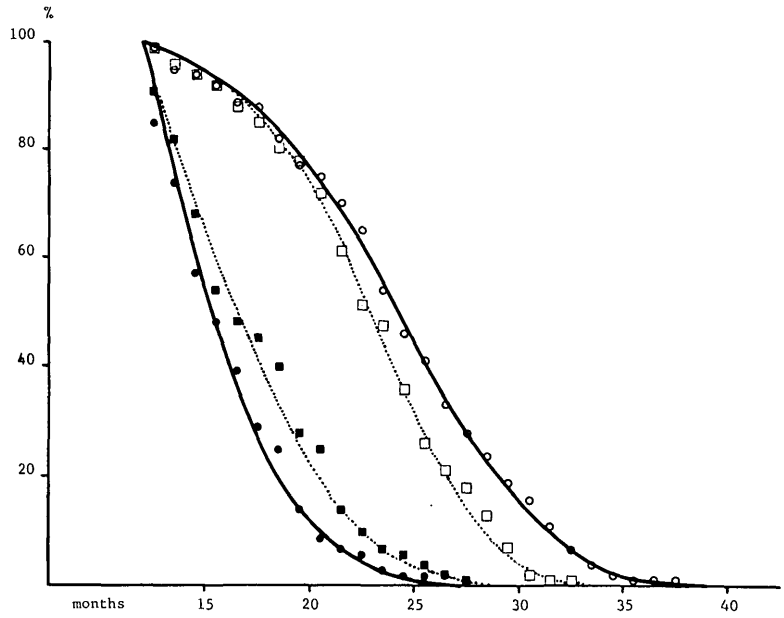

Fig. 1. Survival curves of germ-free male (o——o) and female (□·····□) and conventional male (●——●) and female (■·····■) mice fed *L 462 st* diet. The number of survivors was estimated at the end of each month but in the graph the point is plotted at the mid-point of the month.

formol fixation did not alter the original weight values. The body weights of the germ-free mice were higher than those of conventional animals. This seemed to be due in part to the large fat depots observed in the former group. The weight of the cecum of germ-free mice averaged at death approximately 15 times the conventional value. Germ-free females had considerably larger ceca than males (by about 30% on absolute weight basis or by over 40% when expressed per unit).

Table 3 shows the lesions observed at natural death. (a) Respiratory infections. Pneumonitis, occasionally combined with bronchiectasis, was observed only in conventional animals. Even in apparently normal lungs of old conventional mice there was consistently an elevated number of RE cells in the walls of the alveoli. In comparison, the germ-free alveolar wall seemed to be reduced to the non-defensive functional essentials. (b) Circulatory failure. Animals showing pronounced venous engorgement in the lungs and in various organs, without other readily detectable lesions, were included in this group. (c) Intestinal atonia. This prevalent injury of germ-free animals was manifested by greatly reduced or absent muscle tone and excessive filling of the lower bowel. In approximately one-half of the cases listed, the loss of tone extended over the entire gastrointestinal tract, which resulted in an apparent standstill of gut movements. Most conspicuous in this change was the inordinately enlarged cecum, which was present in all observed germ-free mice as mentioned before (Fig. 2). Germ-free mice often displayed a considerable enlargement of the gall bladder (Fig. 3). The bile always appeared clear and free from precipitates. Conventional controls did not display similar changes. (d) Intestinal volvulus. This lesion was found predominantly at the ileo-cecal-colic junction in germ-free animals. In the past, volvuli of this nature have repeatedly been observed in colonies of germ-free rats and mice even at a young age. This change leads to bowel occlusion and death. (e) Intestinal spasm. Spastic contractions (single or more often multiple) of the small intestine preventing normal propulsion of the gut contents have been observed in conventional animals only. (f) Hepatomegaly and (g) liver atrophy designate merely observation of excessively large (3 to 4 times the normal weight) and very small livers. (h) Lesions of kidneys. In germ-free animals these were mainly progressive degeneration of the tubular epithelium. In conventional controls the same changes appeared to occur accompanied, occasionally, by signs of inflammation. (i) Inflammations of the genital tract, (j) peritonitis, and (k) ear infection were observed only in conventional and not in germ-free mice. In the course of postmortem examinations the impression was gained that the incidence of neoplasia, often affecting the lungs and the female reproductive organs, was at least as frequent among germ-free mice as it was among conventional controls. This question is currently under investigation.

Table 2. Body Weights[a] and Cecal Weights[a] of Germ-Free and Conventional Mice[b].

		on *L 462 st* Diet					
		Males			Females		
		N	M	S.D.	N	M	S.D.
Body weight[c], Gm.	gf	30	39.6	5.0	29	35.7	5.1
	conv	19	32.1	3.9	19	28.7	2.8
Cecum, Gm. %[d]	gf	30	16.3	5.2	29	23.6	6.9
	conv	19	1.3	0.5	19	1.4	0.6

[a] After formol fixation.
[b] Animals taken at random from the groups listed in Table 1.
[c] Corrected for cecal contents.
[d] Gm./100 Gm. corrected body weight.

Table 3. Lesions Observed at Natural Death in Mice Fed *L 462 st* Diet.

Lesion	Germ-free N=80[a] %	Conventional N=80[a] %
Respiratory infection	0	38
Circulatory failure	4	2
Intestinal atonia	36	0
Intestinal volvulus	9	0
Intestinal spasm	0	8
Hepatomegaly	6	5
Liver atrophy	0	4
Lesions of kidneys	12	14
Inflammations of the genital tract	0	2
Peritonitis	0	4
Ear infection	0	1
Unidentified	33	22

[a] Both sexes

Discussion

The genetic similarity and randomization of our stock of germ-free and conventional animals seem to be adequately assured in the present work. The decision to start out with mice aged 12 months aimed at the elimination of the effect of early losses on the results and reflected also, in part, the limited availability of germ-free animals in the initial stages of this study. The same reason also caused the gradual, rather

than simultaneous, buildup of some groups of mice included in this study. Mortality in young and young adult germ-free mouse populations is, by common experience, essentially similar to that of conventional controls. However, the onset of increased mortality in the adult before 12 months of age takes place somewhat earlier in the conventional groups than in the germ-free animals.

Conventional control mice were maintained in the open environment because previous experience suggested that holding conventional animals in isolators might result in pronounced changes of the intestinal microflora. It was shown since that by improvement of the hygienic conditions in the isolators, conventional animals may be housed in them without patent ill effect over a period of several months (Gordon, Bruckner-Kardoss, Staley, Wagner, & Wostmann, 1966). However, more work is needed to clarify the issues of the "locked host and locked flora," especially for long-range studies.

Originally *L 462 st* was selected as the only diet for this experiment on the basis that it offers good and comparable results in both germ-free and conventional mice, as generally agreed by workers in this area. However, this diet had been tested primarily in young and young adult animals. As the progress of this work indicated that aging conventional mice fed *L 462 st* were dying at a younger age than comparable mice fed non-sterile rations reported from other laboratories (e.g., Silberberg, Jarrett, & Silberberg, 1962), other dietary groups (as listed under Materials and Methods) were added.

With due consideration of these limiting factors, we assume that germ-free and conventional animals of this study depict primarily the effects of absence and presence of the normal flora on aging of these animals.

Characteristics of the germ-free animal: inconspicuous defenses, anomalies of the lower bowel.—In absence of a contaminating flora, the germ-free animal has been observed to display a number of characteristics that are different from those of the conventional animal.

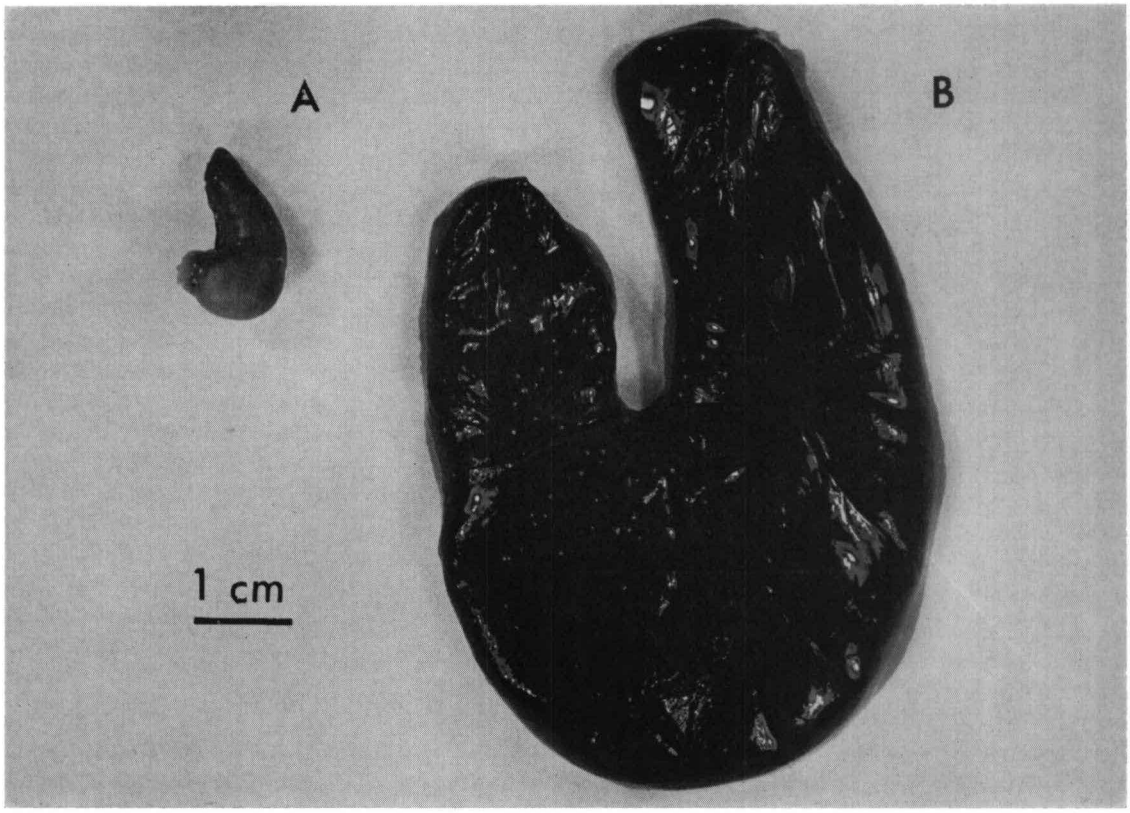

Fig. 2. Formol-fixed ceca from 25-month-old female mice fed *L 462 st* diet.
A. Conventional, 0.5 Gm., corrected body weight 35.5 Gm.
B. Germ-free, 10.5 Gm., corrected body weight 42.5 Gm.

Among these best examined are the singularly low levels of cellular (Gordon, 1959b; Bauer, Horowitz, Levenson, & Popper, 1963) and humoral defensive elements (Wostmann & Gordon, 1958; Gustafsson & Laurell, 1959). Inflammatory lesions which were observed in conventional animals of this series at natural death may be regarded as the cumulative effect of a continuous action and of exacerbations caused by the flora during the host's lifetime. These lesions, in essence, were similar to those described in aging rodents by other workers (e.g., Simms & Berg, 1957).

In contrast to the more or less anticipated and plausible results of "bacterium freeness," the greatly enlarged cecum of germ-free animals is an unresolved paradox (Wostmann & Bruckner-Kardoss, 1959; Gordon & Wostmann, 1960). It seems that under germ-free conditions the tone of cecal musculature is greatly reduced. The cause of the cecal enlargement is essentially unknown. Recent observations have indicated that the cecum of germ-free rodents contains a considerable excess of substances which demonstrate, among others, musculodepressant properties of hypotensive peptides and which may be implicated in the development of the cecal anomalies (Gordon, 1965; Wiseman & Gordon, 1965). In normal life, it seems, the microbial flora is directly or indirectly engaged in the inactivation of these agents.

Previous observations made in sacrificed germ-free mice suggested that cecal enlargement progresses with age. This was confirmed in the present series by the exorbitant ceca observed at death. A more generalized musculodepressant effect of this nature in older animals was suggested by the spreading of tonus reduction over the entire GI tract, including also the gall bladder.

Diets and mean age of mice.—The comparison of survival and body weight in conventional mice fed sterile and non-sterile rations indicated that both *L 462 st* and *5010C st* when used in conventional conditions were suboptimal for aging animals. This impression was corroborated by the fact that changing of diets

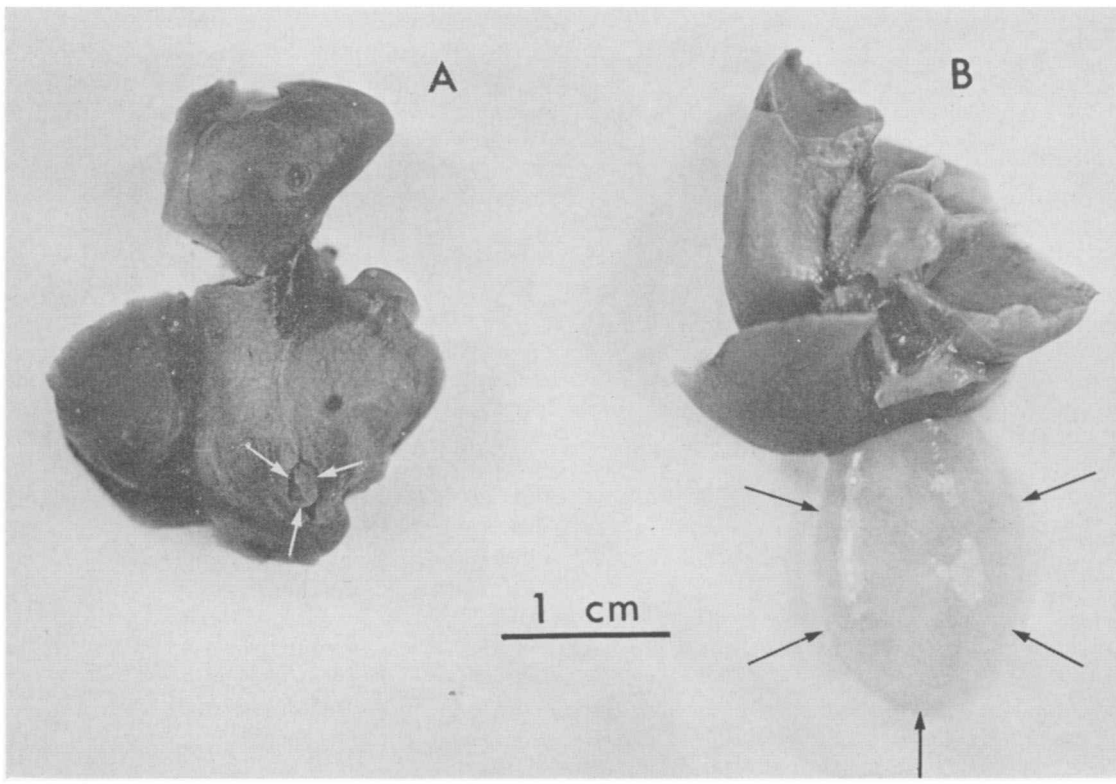

Fig. 3. Formol-fixed livers and gall bladders (arrows) from 23-month-old female mice fed *L 462 st* diet.
A. Conventional, corrected body weight 28.0 Gm.
B. Germ-free, corrected body weight 35.3 Gm.

from non-sterile to sterile form (*P non-st* to *5010C st*) has lowered the animal's mean age to values that were observed among the constant users of sterile diets. In contrast, germ-free animals were thriving on sterile diets as evidenced by their relatively high mean age, body weight, and nutritional status. They were doing much better than the comparable conventional controls, and, for that matter, better than the controls fed non-sterilized ration. These observations suggest that in absence of the challenge of the microbial flora, the requirement of the aging germ-free animal for certain critical nutrients may be reduced. At this point the impression should be avoided that the germ-free status of mice prolonged their life-span in the absolute sense. Actually, the values of mean age of germ-free animals were not higher than those reported in literature for the Swiss-Webster strain in general. Another limiting factor in the interpretation of these results is the relatively low mean age of the conventional controls fed non-sterile diets of this series. Between possible genetic and environmental causes of this shortcoming, the former seems more probable because of the lack of any known cause or symptom that would suggest environmental imperfections.

Mean age of males and females.—The presently used mouse strain indicated that in the more populous conventional groups of this study the mean age of the females was higher than that of the males. In the germ-free groups the males lived at least as long as the females, or perhaps even longer. It might be speculated that this change is caused, in part, by the female's known greater resistance to infections (Wheater & Hurst, 1962). In germ-free life this advantage of the female becomes ineffective. In addition, it must be considered that old germ-free females, for unknown reasons, are penalized by the development of larger ceca than males. This may contribute to the apparent change of the conventional pattern in germ-free life.

Lesions observed at natural death, general conclusions.—A more important consideration in this work is the recognition that germ-free and conventional animals during aging are affected by specific and fundamentally different consequences of their microbial status. Based purely on survival, it seems that the germ-free animal with its cecal anomalies, in comparison to the conventional counterpart with its flora-induced afflictions observed at natural death, is exposed to the lesser of two evils. While this finding is quite unequivocal in our study, it does not permit generalizations. At least theoretically it is entirely conceivable that in future experiments conventional or ex-germ-free animals seeded with a known, more advantageous flora (i.e., synergistic and less antagonistic to the host) may reach or even surpass the life span of germ-free animals that are burdened by more severe forms of intestinal dysfunction. In this context, however, it is also anticipated that in future work the lot of the germ-free host may be improved by reduction of the onera imposed by the lack of microbial synergists.

In general, these findings tend to confirm the ideas of Pasteur (1885) (reconstructed also from other sources, Schottelius, 1908) who speculated that in the course of evolution higher organisms have "learned" to rely on synergistic action of the flora for maintenance of normal body function. Accordingly, we find now that the germ-free host, i.e., an animal "left to its own resources," develops departures from physiological excellence which with advancing age will reach pathological proportions. The conventional control animal, with its repetitive inflammatory episodes yet showing essentially normal bowel function, depicts, in contrast, the action of microbes. Thus, we can view antagonistic and synergistic effects as dual function of the flora.

Summary

Mice may be reared in the germ-free state during their entire life span without undue difficulties. This was illustrated by the present study in which over three hundred germ-free and a comparable number of conventional control mice were held until natural death.

Germ-free animals displayed considerable lack of development of defensive elements throughout life. A counterpart of these, the progressive inflammatory changes imparted by the normal microbial flora in normal life, seemed clearly detrimental to the host.

Contrary to these changes, anomalies in the lower bowel of germ-free animals (cecal enlargement, more liquid intestinal contents, impairment of propulsive movements of the gut) indicated a distinct handicap of germ-free life that progresses with age. In normal life the microbial flora seems to be responsible for elimination of these anomalies.

Groups of germ-free mice showed higher mean age than conventional controls fed the same sterilized ration or non-sterile practical ration. Mean age in groups of germ-free male mice was similar or indicated possibly even higher values than in germ-free female groups. In conventional controls these conditions seemed reversed.

The germ-free animal with its anomalous bowel function, in comparison to the conventional animal with its flora-caused lesions, represents fundamentally different consequences of microbial status. The resulting different types of injury appear to be a major factor (if not the major factor) in the cause of death of the two opposing animal categories.

The authors are sincerely indebted to Miss Oneida Seevers, Miss Ester Jasicki, Mr. Paul Janiszewski for technical assistance, to Mr. Ervin Zelmer for supervision, to Mr. B. A. Teah for direction of germ-free work and to Dr. Sheldon Rovin for help in the evaluation of the pathological specimens.

References

Bauer, H., R. E. Horowitz, S. M. Levenson, and H. Popper: The response of lymphatic tissue to the microbial flora. Studies on germ-free mice. *Amer. J. Path., 42:* 471-483, 1963.

Gordon, H. A.: The use of germ-free vertebrates in the study of "physiological" effects of the normal microbial flora. *Gerontologia,* 3: 104-114, 1959. (a)

Gordon, H. A.: Morphological and physiological characterization of germ-free life. *Ann. N.Y. Acad. Sci., 78:* 208-220, 1959. (b)

Gordon, H. A.: Demonstration of a bioactive substance in caecal contents of germ-free animals. *Nature, Lond., 205:* 571-572, 1965.

Gordon, H. A., E. Bruckner-Kardoss, and B. S. Wostmann: Aging studies in colonies of germ-free and conventional animals. *In:* P. From Hansen (Editor), *Age with a Future.* Munksgaard, Copenhagen, 1964, pp. 220-223.

Gordon, H. A., E. Bruckner-Kardoss, T. E. Staley, M. Wagner, and B. S. Wostmann: Characteristics of the germ-free rat. *Acta anat., Basel, 64:* 301-323, 1966.

Gordon, H. A., and B. S. Wostmann: Morphological studies in the germ-free albino rat. *Anat. Rec., 137:* 65-70, 1960.

Gustafsson, B. E., and C. B. Laurell: Gamma globulin production in germ-free rats after bacterial contamination. *J. exp. Med., 110:* 675-684, 1959.

Pasteur, L.: Observations à la note de M. Duclaux. *C. R. Acad. Sci., Paris, 100:* 68, 1885.

Reyniers, J. A., and M. Sacksteder: Observations on the survival of germ-free C_3H mice and their resistance to contaminated environment. *Proc. anim. Care Panel, 8:* 41-54, 1958.

Reyniers, J. A., P. C. Trexler, and R. F. Ervin: Rearing of germ-free albino rats. *Lobund Rpt.,—:* (1), 1-86, 1946.

Schottelius, M.: Die Bedeutung der Darmbakterien für die Ernährung. *Arch. Hyg., Berl., 67:* 177-208, 1908.

Silberberg, R., S. R. Jarrett, and M. Silberberg: Longevity of female mice kept on various dietary regimens during growth. *J. Geront., 17:* 239-244, 1962.

Simms, H. S., and B. N. Berg: Longevity and the onset of lesions in male rats. *J. Geront., 12:* 244-252, 1957.

Trexler, P. C.: The use of plastics in the design of isolator systems. *Ann. N.Y. Acad. Sci., 78:* 29-36, 1959.

Wagner, M.: Determination of germ-free status. *Ann. N.Y. Acad. Sci., 78:* 89-101, 1959.

Wheater, D. W. F., and E. W. Hurst: The effect of sex on bacterial infections in mice and on chemotherapy of one of them. *J. Path. Bact., 82:* 117-130, 1962.

Wiseman, R. F., and H. A. Gordon: Reduced levels of a bioactive substance in caecal contents of gnotobiotic rats monoassociated with *Salmonella Typhimurium. Nature, Lond., 205:* 572-573, 1965.

Wostmann, B. S.: Nutrition of the germ-free mammal. *Ann. N.Y. Acad. Sci., 78:* 175-182, 1959.

Wostmann, B. S., and E. Bruckner-Kardoss: Development of cecal distension in germ-free baby rats. *Amer. J. Physiol., 197:* 1345-1346, 1959.

Wostmann, B. S., and H. A. Gordon: Electrophoretic studies on serum proteins of young germ-free, conventional and antibiotic treated conventional chickens. *Proc. Soc. exp. Biol.,* N.Y., *97:* 832-835, 1958.