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Vitrification as an Approach to Cryopreservation[1]

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Recent developments have opened the possibilty that the problems of freezing and thawing organs might eventually be overcome by an alternative approach to organ cryopreservation, namely, vitrification. Here we will review some of the principles of vitrification, describe the current state of the art, consider how a practical vitrification scheme might work, and conclude by noting how the principles of vitrification relate to and illuminate the principles and practices of freezing.

VITRIFICATION OF ORGANS

Vitrification can be and has been defined in many ways (12, 19, 34, 42), but essentially it is the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during cooling. During vitrification the solution is said to become a glass; translational molecular motions are significantly arrested, marking the effective end of biological time but without any of the changes brought about by freezing. An organ capable of being vitrified need no longer satisfy classical constraints of optimal cooling and warming rates, but instead can neatly escape both "solution effects" injury and the dangers of intracellular freezing.

Vitrification of relevant aqueous solutions using cooling rates that are realistic for whole organs requires the presence of high concentrations of a cryoprotective agent (41, 44, 64). The primary challenge that must be met in order to successfully vitrify organs, therefore, is to make the required concentrations of cryoprotectant nontoxic to the organ. Although this is a demanding task, it must be kept in mind that freezing ultimately exposes organs to even higher (41, 44, 64) and probably even more damaging (17) concentrations of cryoprotectant than are required for vitrification because of the concentrating action of ice separation on the residual unfrozen liquid.

The basic concepts of vitrification are best described by reference to a supplemented phase diagram such as the generalized one shown in Fig. 1. T_m is the equilibrium freezing or melting point curve. Solutions normally supercool to some point between T_m and T_h, the homogeneous nucleation temperature, before they actually nucleate or begin to freeze, as represented here by X's. T_g is the glass transition temperature, at which supercooled liquid vitrifies. And finally, T_d is the devitrification curve, at which the previously vitrified solution freezes upon rewarming.

There are certain rather well-defined regions on the phase diagram in which different types of vitrification behavior ap-

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pear. In the relatively dilute Region I, vitrification is for all practical purposes impossible because both heterogeneous and homogeneous nucleation are unavoidable (47, 49). In the more concentrated Region II both types of nucleation are inhibited, as is crystal growth, and it becomes possible, typically at a concentration which brings T_h to within 20 (46) to 40° (65) of T_g, for emulsified (45, 46) or quenched bulk samples (3, 4, 67) to be cooled through the homogeneous nucleation curve without seeming to freeze and form what are referred to as doubly unstable glasses. Doubly unstable glasses formed in this way, however, are now thought to be heavily nucleated (46) and normally are inevitably fated to freeze or devitrify upon warming (45). This fact plus the impossibility of cooling samples the size of organs rapidly enough to avoid freezing in this concentration regime makes doubly unstable glasses ostensibly unsuitable for organ preservation. However, we will later consider an apparent exception to this rule.

At still higher concentrations (Region III), T_h becomes equal to and then actually falls below T_g. In this region it is possible to slowly cool even bulk liquids directly to T_g without experiencing any detectable freezing events, despite the presence of heterogeneous nucleating agents (19, 65). The intersection between the T_h curve and the T_g curve therefore establishes the threshold or lowest possible concentration of cryoprotectant that might be used for organ vitrification.

Although it is possible to vitrify organs in this region without forming detectable quantities of ice, the existence of devitrification upon heating has been interpreted as evidence for significant heterogeneous nucleation during cooling (47). However, the amount of ice formed during this heterogeneous nucleation is of course minute, and, as we shall see, it should be possible to heat organs in this region at rates sufficient to prevent any appreciable growth of the existing ice, thereby avoiding devitrification.

Finally, at Region IV, the devitrification curve vanishes even at slow warming rates. Here all nucleation is prevented, and the system is virtually stable. Although ideal in principle for organ preservation, this region is presently well beyond reach due to overwhelming problems with cryoprotectant toxicity, leaving Region III as the main focus of interest for practical preservation. Note that, although nucleation normally does not occur spontaneously in Region IV, preexisting ice can still grow. Therefore, slowly frozen systems tend to follow the dashed extension of the T_m curve until it intersects T_g (41, 44, 64). Obviously, the resulting cryoprotectant concentrations far exceed those needed to vitrify an unnucleated sample.

MAKING IT PRACTICAL

I. Reducing the Concentration of Cryoprotectant Needed for Vitrification

It is apparent from Fig. 1 that the threshold concentration of cryoprotectant needed for vitrification is nevertheless quite high. Although a few biological systems are known to tolerate the high concentrations required (14, 20, 43, 61–63), the organs of

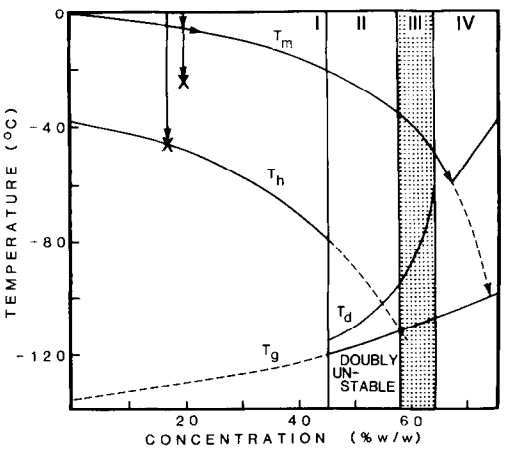

FIG. 1. Supplemented phase diagram of a hypothetical cryoprotectant. For discussion, see text.

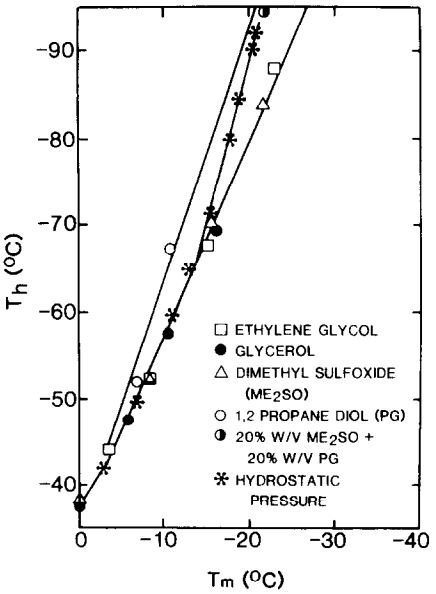

FIG. 2. Relationship between T_m and T_h when T_m and T_h are depressed either by cryoprotective agents or by hydrostatic pressure. The effects of pressure are similar to those of cryoprotective agents. After MacKenzie (48). Data from Refs. (2, 29, 30, 45, 65), and unpublished data.

greatest interest and, indeed, most biological systems probably are not so tolerant. However, there are at least three ways of reducing the effective amount of cryoprotectant required for vitrification.

The first method is to apply high hydrostatic pressure. High pressures lower T_h and elevate T_g, thus shifting the point of intersection to a lower concentration of cryoprotectant (29). Once the temperature has been brought to below T_g, the pressure can be released without danger of crystallization (19). As can be seen from Fig. 2, which is a plot of T_h vs T_m, hydrostatic pressure is at least as effective as ordinary cryoprotectants at depressing the T_h of pure water. The effects of pressure on the T_h of dilute solutions of propylene glycol or dimethyl sulfoxide are shown in Fig. 3. Although there is a peculiar lack of additivity of cryoprotectant and pressure effects on T_h for 20% solutions at higher pressures, for more concentrated solutions pressure has both a significant additive effect on T_h and an appreciable effect on T_g. When the data shown in this figure are presented in the form of a conventional phase diagram, we see (Fig. 4) that 1000 atm shifts the T_h/T_g intersection point from 44% w/v at 1 atm down to 39%, concentrations which agree well with the concentrations needed to vitrify (or the CNVs) as found by visual inspection of slowly cooled bulk samples (represented by the circled V's in Fig. 4). The CNV at 1500 atm is expected to be even lower, although at present the intersection shown is only an extrapolation.

In order to evaluate the usefulness of simple visual determination of CNV as a function of pressure, the relationship between CNV as defined by the intersection of the T_h and the T_g curves and CNV as defined visually was checked further as shown in Fig. 5. The visual CNV was defined by the absence of even a single tiny ice sphere in a visible volume of about 6–8 ml after cooling at ~5–30°C/min. Here data for the T_h of aqueous glycerol, ethylene glycol, and dimethyl sulfoxide solutions have been extrapolated based on the known relationship between T_h and T_m (Fig. 2) until intersections are obtained with the

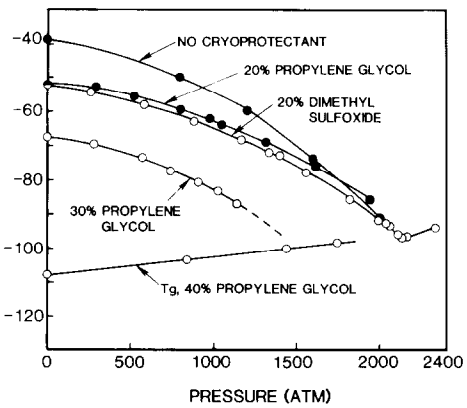

FIG. 3. Effect of pressure on T_h and T_g of dilute propylene glycol–water and dimethyl sulfoxide–water solutions. Additional data have appeared elsewhere (45) showing results for more concentrated solutions. Not shown here are indications that dimethyl sulfoxide promotes the formation of ice III at lower pressures than normal (45).

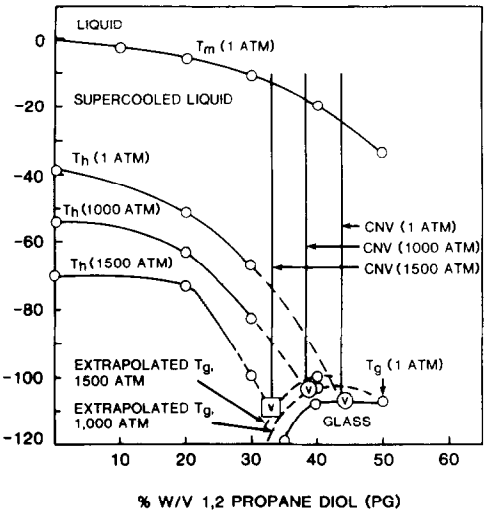

FIG. 4. Nonequilibrium phase diagram for propylene glycol–water showing the effect of pressure on the concentration needed to vitrify (CNV). T_m data from (69) and unpublished results; T_g data from (3) and unpublished results. Dashed lines represent extrapolations. For T_h (homogeneous nucleation temperature) at 1 and 1000 atm, the extrapolation is based on Fig. 2.

T_g's for these systems. The circled V's represent the CNVs as determined visually and are slight underestimates because they were obtained in the presence of physiological carrier solution solutes. As is apparent, the CNVs established by the two methods are essentially synonymous, in agreement with the results already shown for the propylene glycol–water system, despite the fact that the extrapolated portions of the T_h curves are presumably invisible by differential thermal analysis. This rather useful finding allows us to obtain and tentatively interpret CNV data for a variety of systems using the visual technique without the need to define complete phase diagrams for each system.

Data of this sort are shown in Table 1 for both 1 and 1000 atm. Here we see the glass forming tendencies of a number of cryoprotectant systems in the presence of our carrier solutions, Rδ (19) or RPS-2 (8). In general, the effect of 1000 atm on CNV is significant and, as we will see, of practical value.

The second method for reducing CNV is outlined in Fig. 6. Here we note (A, B, C) that cells naturally contain high concentrations of protein, which should be able to facilitate vitrification, so that, for example, only 40% rather than 46% penetrating cryoprotectant (PCP) might be needed for intracellular vitrification (compare B to A). To prevent freezing of the extracellular solution, 40% penetrating agent alone is not adequate (C), but 40% penetrating plus 6% nonpenetrating agent (for example, polyvinyl pyrrolidone (PVP)) is effective (B) and avoids the unnecessary intracellular exposure produced by 46% penetrating agent (A).

In order to test the applicability of this approach in the case of kidney, slices were

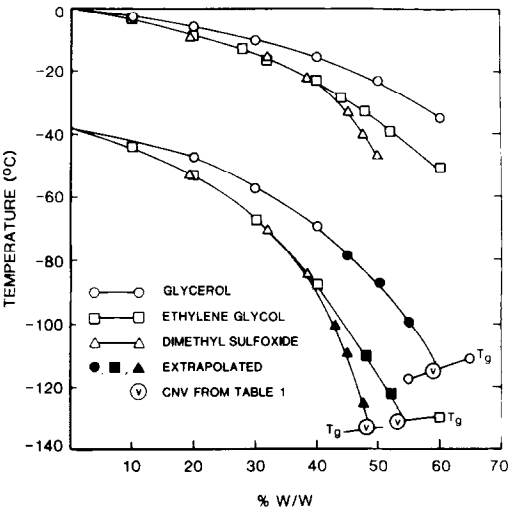

FIG. 5. Extrapolation of the T_h's of cryoprotectant–water solutions to T_g based on the T_m–T_h relationship given in Fig. 2. CNVs as listed in Table 1 were used to calculate CNVs on a %w/w basis and the results are plotted on the appropriate T_g curves for comparison (circled V's). This calculation assumes that the 4.1% w/v concentration of carrier solution solutes in Table 1 makes no direct contribution to the depression of T_h. Taking the contribution of carrier solution solutes into account would give higher CNVs for the pure cryoprotectant–water systems, causing all (V) points to fall outside of the doubly unstable ranges for these systems and perhaps to coincide even more closely with the T_h–T_g intersections. Data from (44, 45, 65), and unpublished results.

TABLE 1
Concentrations Needed to Vitrify (CNV) at 1 and 1000 atm: Penetrating Agents[a,b]

Agent(s)	1 atm				1000 atm				
	Q^c	m^c	M	%w/v	Q^c	m^c	M	%w/v	$\%\Delta^d$
Individual agents									
Ethylene glycol	3.3	18	8.9	55	2.6	15	7.9	49	−20
1,3-propanediol	3.1	17	7.5	57	—	—	—	—	—
Glycerol	2.7	15	7.1	65	2.3	13	6.5	60	−15
Dimethyl sulfoxide (D)	2.1	12	6.3	49	1.8	10	5.8	45	−14
1,2-propanediol (propylene glycol; P)	1.9	10	5.7	44	1.5	8.5	5.1	39	−19
2,3-dihydroxybutane	1.8	9.9	5.1	46	—	—	—	—	—
TMAA[e]	1.1	6.0	3.4	41	~0.88	~4.9	~3.0	~36	~−19
DMAE–acetate[f]	1.0	5.7	3.0	45	~0.91	~5.0	~2.8	~42	~−12
Mixture of penetrating agents									
D + formamide (DF) (2 mol:1 mol)	3.2	18	8.3	56	2.5	14	7.5	50	−20
D + urea (3 g:1 g)	3.1	17	8.1	59	~2.7	~15	~7.6	~55	~−13
D + acetamide (DA) (1 mol:1 mol)	2.7	15	7.7	53	2.4	13	7.1	49	−14
DA + 10% P (DAP_{10})	2.6	15	7.4	52	—	—	—	—	—
D + propionamide (D Pr) (1 mol:1 mol)	≤2.6	≤14	≤7.0	≤53	—	—	—	—	—
DA + P (1 g:1 g)	2.4	13	6.9	50	2.0	11	6.2	45	−17
D + P (1 g:1 g)	2.0	11	6.0	46	1.7	9.3	5.4	42	−15

[a] In the presence of carrier solution ($R\delta^{2-}$ or $RPS\text{-}2^{2-}$; the superscript 2− refers to the absence of Ca^{2+} and Mg^{2+}).

[b] Q = moles of agent per 10 mol of water; m = molality; M = molarity

[c] Figured assuming a measured volume of 2.0 ml of carrier solution solutes per 100 ml; values in Ref. (19) neglected all but 0.5 ml of this volume.

[d] Percentage change based on Q at 1000 atm vs Q at 1 atm. Figured using nonrounded values of Q.

[e] Trimethylamine acetate; at 1000 atm, container wall seeded the solution.

[f] Dimethylaminoethanol acetate; at 1000 atm, container wall seeded the solution.

made of renal cortex, medulla, and papilla. The slices were then loaded with 6% PVP and enough penetrating cryoprotectant to vitrify at 1000 atm if and only if at least 6% PVP is present, as in Fig. 6B vs C. The slices were then cooled at 1000 atm to below T_g and inspected for evidence of freezing. If inadequte intracellular solute is

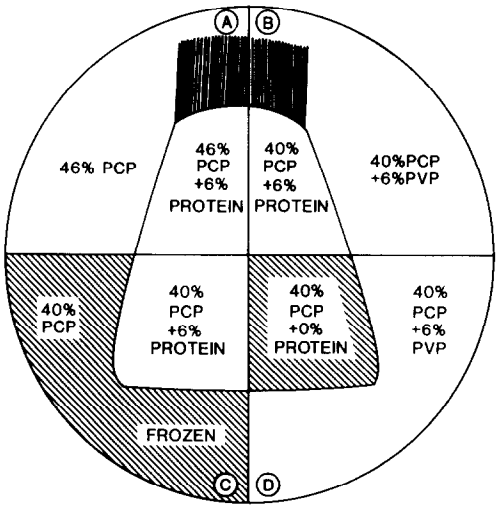

FIG. 6. Effect of penetrating cryoprotectant (PCP) and nonpenetrating cryoprotectant (PVP) on intracellular PCP concentration (A vs B), intracellular vitrification (A, B, and C vs D), and extracellular vitrification (A, B, and D vs C). The scheme assumes vitrification requires either 46% PCP, 40% PCP + 6% PVP, or 40% PCP + 6% intracellular protein. The cell depicted schematically is a proximal tubular cell, with brush border at top.

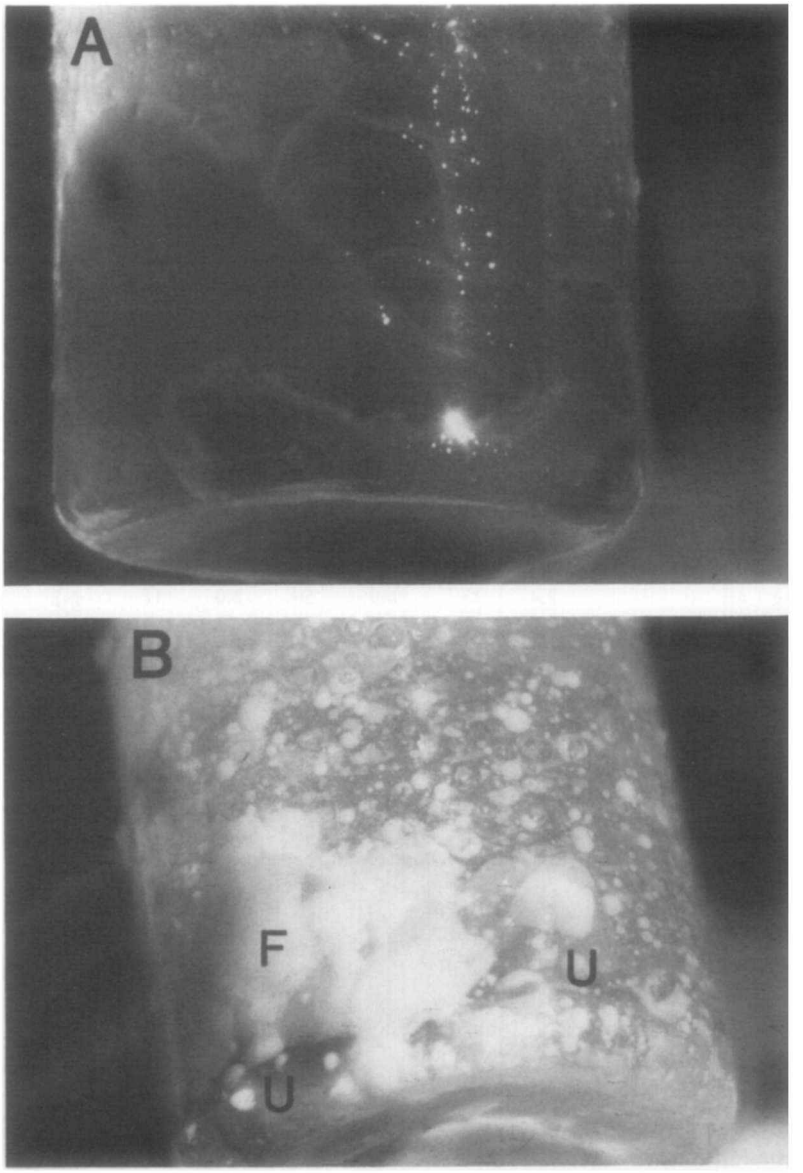

FIG. 7. (A) Kidney slices loaded with 40% DAP_{10} (see Table 3) and 6% PVP, pressurized to 1000 atm, and cooled to T_g. No ice is visible. Areas appearing slightly white are light reflections. (B) Slices treated similarly to those in A but without sufficient time for cryoprotectant permeation and distribution into the extracellular space. Slices themselves as well as the medium they have diluted by their presence show clear evidence of ice formation, demonstrating the visibility of ice when it is present. F, frozen tissue; U, largely unfrozen region of tissue.

TABLE 2
Concentrations Needed to Vitrify (CNV) at 1 and 1000 atm: Penetrating and Nonpenetrating Agents[a,b,c]

Agent(s)	1 atm				1000 atm				
	Q^d	m^d	M	%w/v[e]	Q^d	m^d	M	%w/v[e]	%Δ[f]
6% w/v PVP[g] plus									
Dimethyl sulfoxide (D)	2.1	11	5.9	46	1.7	9.4	5.2	41	−18
1,2-propanediol (P)	—	—	—	—	1.5	8.1	4.7	36	—
D + P (1 g:1 g)	—	—	—	—	1.7	9.3	5.2	40	—
D + acetamide (1 mol:1 mol; DA)	2.3	13	6.6	46	2.0	11	6.2	42.5	−13
DA + 10% P (DAP_{10})	2.3	13	6.6	46	1.8	10	5.7	40	−21
DA + P (1 g:1 g)	—	—	—	—	1.7	9.4	5.4	39	—
D + formamide (2 mol:1 mol) (DF) + 10% P (DFP_{10})	2.5	14	7.0	48	2.0	12	6.2	43	−18
DF + P (1 g:1 g)	—	—	—	—	1.9	10	5.7	41	—
Miscellaneous solutions									
8% PVP + DAP_{10}	—	—	—	—	1.8	9.8	5.5	39	—
6% HES[h] + DA	2.6	14	7.3	50	2.1	12	6.4	44	−20
6% HES + DAP_{10}	2.5	14	7.0	49	1.9	11	6.0	42	−24
6% sucrose + DA	2.5	14	7.2	48	2.2	12	6.7	45	−11
6% sucrose + DAP_{10}	2.4	13	6.9	47	2.0	11	6.2	42	−18
4% PEG[i] 600 + DFP_{10}	—	—	—	—	2.1	11	6.2	43	—
6% PEG 8000 + DAP_{10}	—	—	—	—	1.8	10	5.7	40	—
6% PEG 8000 + P	—	—	—	—	1.5	8.5	4.9	37	—

[a,b,d,f] See Table 1 footnotes for explanations.
[c] Q, m, and M include contribution of polymer.
[e] Percentage concentration of penetrating agents only.
[g] Polyvinyl pyrrolidone K30.
[h] Hydroxyethyl starch.
[i] Poly(ethylene glycol).

available to mimic the effects of at least 6% PVP, the cells would be expected to freeze as in Fig. 6D. Control slices were also cooled which had been treated for times too short for adequate cryoprotectant equilibration. These slices were expected to freeze. As is apparent in Fig. 7, the fully equilibrated slices (A) did in fact vitrify, in contrast to the poorly equilibrated slices, which, as expected, clearly froze. It appears, then, that extracellular agents can be helpful at reducing CNV in kidney and, in all likelihood, in other organs as well (23).

Vitrification data for polymer solutions are shown in Table 2. Figure 8 presents a visual representation of the effects of both pressure and polymer, separately and combined, on CNV. Based on the number of moles of agent per 10 mol of water, pressure reduces the concentration needed to vitrify by an average of 17%, polymer reduces CNV by 8%, and together they reduce CNV by 24%.

Table 3 provides a specific example for our least toxic cryoprotectant solution, consisting of an equimolar mixture of dimethyl sulfoxide and acetamide (referred to as DA). Pressure drops CNV by 14%, polymer by 16%, and the combination by 26%. Also shown here is the third maneuver for reducing CNV, i.e., the inclusion of small concentrations of propylene glycol (PG). This agent is an excellent glass former (Tables 1 and 2) but is too toxic to use at concentrations above 10%. PG by itself is able to reduce the CNV of DA solutions by only about 4%. But when combined with the other modalities its effects are much more significant, so that all together CNV is reduced by a dramatic 34%.

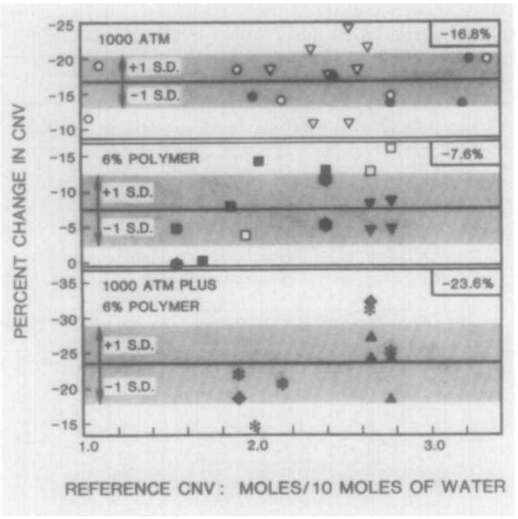

FIG. 8. Effect of 1000 atm (top), 6% polymer (middle), and both combined (bottom) on CNV. Data are from Tables 1 and 2. Top: inverted open triangles, solutions containing penetrating agents plus polymer; open circles, individual agents; solid circles, mixtures of penetrating agents. Middle: filled inverted triangles, HES and sucrose, 1 atm; hexagons, HES, sucrose, and PEG, 1000 atm; open boxes, PVP, 1 atm; solid boxes, PVP, 1000 atm. Bottom: triangles, HES and sucrose; asterisks, PVP; diamonds, PEG 8000.

The cryoprotectant mixture containing both DA and 10% propylene glycol is referred to as DAP_{10}. The composition shown here, 40% DAP_{10} + 6% PVP, is of particular interest, as we will soon see.

II. Reducing Baroinjury and Cryoprotectant Toxicity

Although additional methods may exist for reducing the cryoprotectant concentration needed for vitrification, further reduction of CNV may not be useful owing to the increasingly inexorable prospect of devitrification at progressively lower cryoprotectant concentrations (3, 4). Hence, the task that remains is to find ways of reducing the damaging effects of pressure (or baroinjury) and of those concentrations of additive which are presently required for vitrification.

As shown in Table 4, several investigators have found a variety of agents, including glycerol, to be baroprotective in a rather miscellaneous array of other systems. It would thus seem that there may be many possibilities for coping with baroinjury. It is also encouraging to note that in the absence of cryoprotectant, dog kidneys (32) and hearts (68) can survive 2- and 30-min exposure, respectively, to 1000 atm, as tested by transplantation in both cases.

Some of our own results with baroinjury and baroprotection (19) are summarized in Table 5. The index of viability is the steady-state K^+:Na^+ ratio achieved by rabbit kidney slices after restoration of active metabolism under "physiological" conditions (16, 19). Here we see that although pressures of 670–1000 atm are severely damaging in the absence of cryoprotectants, the presence of dimethyl sulfoxide, propylene glycol, or both in combination is strongly protective, so that pressures of at least 1000 atm can be tolerated with little or no damage. We have also obtained similar baroprotection at 1000 atm with 40% DAP_{10} + 6% PVP, but those results require confirmation. Overall, prospects would seem fa-

TABLE 3
Reduction of CNV for Dimethyl Sulfoxide/Acetamide (DA) Solutions

Modality:	None	1000 atm	6% PVP	PVP + 1000 atm	10% PG	PG + PVP + 1000 atm
Q[a]	2.7	2.4	2.3	2.0	2.6	1.8[b]
% Change[c]	0	−14	−16	−26	−4	−34

[a] Moles of cryoprotectant per 10 mol of water
[b] Equivalent to 30% w/v DA + 10% w/v PG (40% DAP_{10}) + 6% PVP K30.
[c] Based on nonrounded values of Q.

TABLE 4
Baroprotection in Systems other than Rabbit Kidney

Treatment or agent	Biological System	Ref.
1. Glycerol	*Gammarus oceanicus* (euryhaline amphipod);	(54)
	microtubules	(71)
2. Concentrated seawater	*G. oceanicus;*	(54)
	Mytilus edulis (mussel) gill	(58)
3. Elevated Ca^{2+}	*Eupagurus zebra* (hermit crab)	(73)
4. Magnesium	*Escherichia coli* phages	(21)
5. ATP	*Amoeba proteus*	(77)
	egg cell (cleavage)	(37)
6. GTP	Microtubules	(71)
7. pH adjustment	*M. edulis* gill	(59)
8. Temperature adjustment		
Elevation	*M. edulis* gill	(58)
Elevation	Turtle atria	(28)
Reduction	Most microorganisms	(78)
Reduction	*Crangon crangon* (decapod shrimp)	(54)
9. D_2O	Microtubules	(50)
10. Prevention of oxidation?	Bacteria, various	(78)
	free radicals form	(24)

vorable for successful avoidance of pressure-related injury.

Substantial progress has also been made toward circumventing cryoprotectant toxicity. Based on the glass forming ability of the standard cryoprotectants and preliminary information concerning their toxicities and rates of permeation, dimethyl sulfoxide was selected as the agent most likely to be useful as the primary ingredient of a vitrification solution. Figure 9 shows some early results which illustrate both the difficulties of using this agent and some of the keys to avoiding these difficulties.

TABLE 5
Baroinjury and Baroprotection in Rabbit Renal Cortex at 0°C

Cryoprotectant[a]	Steady-state K^+:Na^+ ratio[b] after exposure to			
	1 atm	670 atm	1000 atm	1600 atm
None	5.53 ± 0.25 (5)	1.78 ± 0.14 (6)	0.94 ± 0.05 (8)	
20% Me_2SO	5.21 ± 0.22 (4)	5.50 ± 0.18 (8)		
30% Me_2SO	5.60 ± 0.27 (5)		5.17 ± 0.27 (6)	2.99 ± 0.37 (5)
15% Me_2SO + 15% PG	5.60 ± 0.07 (5)			3.01 ± 0.22 (5)
30% PG	4.26 ± 0.15 (4)		3.59 ± 0.15 (5)	2.00 ± 0.11 (5)

[a] Percentages are w/v.
[b] Measured at 25°C at 1 atm.

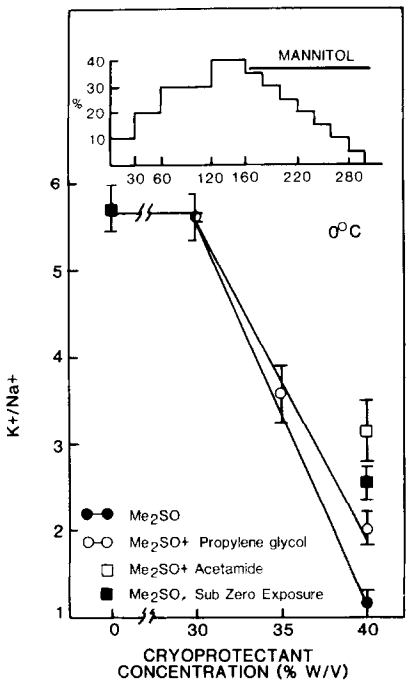

FIG. 9. Effect of cryoprotectant concentration at 0°C and, in one case, at subzero temperatures, on viability (K^+:Na^+ ratio) of rabbit renal cortex. Data are from Ref. (15). Inset at top shows the introduction and removal protocol for 40% solutions; washout was in the presence of 300 m*M* mannitol throughout. This protocol has been shown to avoid osmotic stress, so the effects observed represent intrinsic cryoprotectant toxicity (18). For further details concerning the protocol for subzero treatment see Ref. (15) and the accompanying discussion. One gram of Me_2SO per gram of PG or one mole of Me_2SO per mole of acetamide.

As can be seen, the toxicity of both dimethyl sulfoxide (Me_2SO) alone and of a mixture of Me_2SO and propylene glycol is intensely concentration dependent. The damage can be reduced greatly by temperature reduction (filled box) (1, 7, 14, 15, 20, 22, 33, 56, 61), but since kidneys appear to be damaged by exposure to high subzero temperatures (27, 57), we have restricted our attention to 0°C, which for the time being appears optimal.

Apparently dilution of Me_2SO by PG is beneficial in this instance, but we have found that in general mutual dilution of cryoprotectants cannot be relied upon. Even in this example, a great deal of damage is evident even though the individual concentrations of Me_2SO and PG do not exceed 20%, a normally rather innocuous concentration. The damage is unrelated simply to the total molarity of the solutions, since the total molarity is slightly higher with the mixture than with Me_2SO alone.

The use of acetamide, however, represents a different and more powerful principle than mere dilution, and is the second direct method we have identified for reducing cryoprotectant toxicity. Acetamide is thought to specifically complex with Me_2SO, preventing it from denaturing fructose diphosphatase and similar enzymes while Me_2SO by the same token also reduces any damaging effects of the acetamide (1). As a result of this specific cryoprotectant toxicity neutralization, the recovery at 40% concentration is trebled despite a 16% increase in the total molarity of the solution.

Another agent which may act as a specific toxicity neutralizer for Me_2SO is dextrose (7, 70). Recent studies have shown that dextrose prevents irreversible binding of Me_2SO to proteins (7) and that carrier solutions which contain 140 (22) to 180 m*M* (7) dextrose seem to reduce Me_2SO toxicity compared to extracellular type solutions or a solution high in K^+ and Mg^{2+}. This points up the fact that proper choice of carrier solution can be a direct factor in reducing cryoprotectant toxicity (7), as has been indicated by earlier studies as well, which showed the importance of using impermeant species (14).

A fourth direct method for reducing cryoprotectant toxicity is illustrated in Fig. 10. Here we see that by reducing and ultimately eliminating the time spent at high intermediate concentrations of DA during introduction and removal of 40% (or 5.8 *M*) DA, the toxicity of 40% DA can be entirely abolished, even though the exposure to 40% DA itself is not shortened.

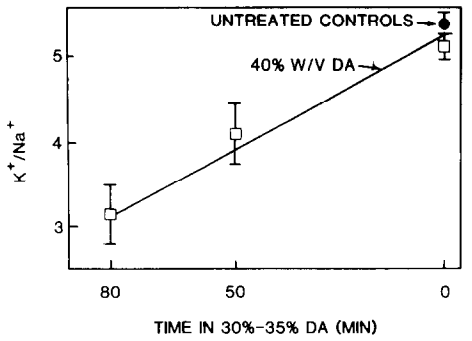

FIG. 10. Effect of time spent at 30–35% DA (1 mole of dimethyl sulfoxide per mole of acetamide) during introduction and removal of 40% DA on K^-:Na^- ratio maintained by kidney slices after cryoprotectant washout and restoration of "physiological" conditions. Data from Refs. (15) and (18). Temperature: 0°C.

TABLE 6
Methods of Reducing Cryoprotectant Toxicity

Primary (direct) methods

1. Maintain temperature as low as possible
2. Select appropriate carrier solution
3. Keep exposure time at higher concentrations to a minimum
4. When possible, employ specific cryoprotectant toxicity neutralizers

Secondary (indirect) methods

1. Avoid osmotic injury
2. Mutual dilution of cryoprotectants may be helpful in some instances
3. Use extracellular cryoprotectant to reduce exposure to intracellular cryoprotectant when possible

Table 6 summarizes both direct and indirect methods of reducing cryoprotectant toxicity.

Although 40% DA has now been made innocuous, vitrification requires the inclusion of propylene glycol and the addition of PVP, i.e., the use of 40% DAP_{10} + 6% PVP. As seen in Fig. 11, inclusion of PG is harmless. Unfortunately, addition of 6% PVP is damaging, although it is signficantly less damaging than addition of another 6% penetrating agent. In fact, as illustrated in this example, an encouraging overlap is often seen of the ranges of the control values and the values obtained from slices treated with this vitrifiable solution.

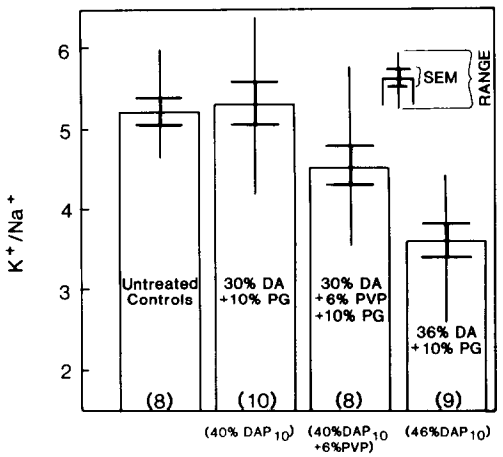

FIG. 11. Tolerance of 40% DAP_{10} by rabbit renal cortex and failure to tolerate 40% DAP_{10} + 6% PVP K30 (General Aniline and Film Corp.). 6% PVP is, however, less damaging than an additional 6% DA. Note overlap of ranges of control values and values for 40% DAP_{10} + 6% PVP treatment. Introduction and removal protocol: 10% for 30 min; 20% for 60 min; 40% or 40% + 6% PVP for 40 min; 20% plus 300 m*M* mannitol for 20 min; remainder of procedure as in Fig. 9. Temperature: 0°C.

Unfortunately, despite considerable effort, it has not as yet been possible to abolish the remaining toxicity. Something about the characteristics of this toxicity are known, however. Surprisingly, its kinetics are quite rapid (Fig. 12), damage going almost to completion as soon as the polymer contacts the cells or as water is osmotically withdrawn. The injury is clearly not osmotic in nature, however (Table 7). The toxicity seems nonspecific in that using different polymers in place of PVP, whether alone or in combination with each other, gives identical results, and the injury is independent of "pH" between pH 7.0 and 11.0. Studies with model systems (55) suggest that PVP and other colloids may interact with membranes hydrophobically and thereby destabilize them. Despite our present inability to abolish the residual

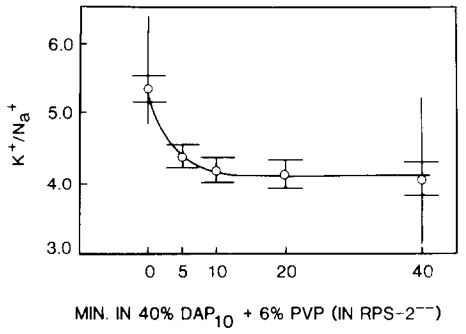

FIG. 12. Kinetics of damaging effect of 6% PVP in the presence of 40% DAP_{10}. Introduction and removal as in Fig. 11.

damage, the progress made so far is encouraging. At the moment the overall concentration required to vitrify amounts to 1.8 mol per 10 mol of water, whereas the concentration yielding complete recovery of viability is 1.7 mol/10 mol of water.

III. Avoiding Devitrification

Assuming that an organ could be successfully vitrified, it would still face the challenge of being warmed without devitrifying. Much less is currently known about this problem than about others we have considered, but better understanding and some useful information is beginning to emerge.

A priori one would expect that aqueous cryoprotectant glasses formed under high pressure conditions would require reapplication of pressure prior to warming above T_g, since at 1 atm these glasses are presum-

TABLE 7
Interventions Having No Effect on (0) or Augmenting (−) the Toxicity of PVP/Penetrating Cryoprotectant Solutions

1. Avoidance of osmotic stress by equilibrating 20 min in 40% DAP_{10} before transfer to 40% DAP_{10} + 6% PVP for 20 min (0)
2. Replacing 6% PVP with
   6% HES (0), 4% PEG 600 (0), 6% PEG 8000 (0), 2% HES + 2% PEG 8000 + 2% PVP (0), 6% dextrose[a] (0/−), 6% bovine albumin (−), 4% trimethylamine oxide[b] + 2% PEG 8000 (−), 6% trehalose[c] (−), 6% tricarballylate[d] (−), 3% PVP + 3% proline[b] (−)
3. Attempting to stablize cellular structures with
   altered "pH" from 7.0 through 11.0[e] (0), 70% D_2O[f] (0), 4 × 10^{-5} *M* (0) or 8 × 10^{-4} *M* (−) chlorpromazine[g], 5 m*M* chloroquine[h] (0), 3 m*M* hydrocortisone[h] (−), 0.5 m*M* indoleacetic acid[i,*] (0), 27.5 m*M* sodium citrate plus 27.5 m*M* potassium citrate[j,d] incorporated into modified RPS-2 (−), calcium[g] (1–2 m*M*) plus magnesium (2–4 m*M*) in Hepes-buffered RPS-2 with or without verapamil (0), or a 110 mV decrease in redox potential[k] induced by ascorbic acid (0)
4. Replacing DA with
   $DF^{l,m}$ (0), $DPr^{l,m}$ (−), 10% glycerol[n] (replacing 10% DA) (0/−), DA containing very pure Me_2SO[o] (0), DA containing fully deuterated Me_2SO (0 or slight +), or a mixture of half DA (by weight) and half DMAE-acetate* (−)
5. Favorably altering renal biochemistry by
   preventing magnesium leaching[p] using 30 m*M* Mg^{2+} in modified RPS-2 (0), or adding either 2 m*M* ATP + 2 m*M* $MgCl_2$[q] (0) or 3 m*M* reduced glutathione[r] (0) to the bathing medium used for 25°C viability testing

[a–r] References to rationales for selected interventions: [a](7), [b](76), [c](9, 10), [d](T. Busby and K. Ingham find this substance to be a very strong protein stabilizer (*Biochim. Biophys. Acta,* 1984, in press)), [e](75), [f](50), [g](16), [h](39), [i](H. T. Meryman, R. J. Williams, and M. Douglas have found this substance to be a potent macromolecular stabilizer (unpublished results)), [j](26), [k](6), [l](see Table 1 for formula), [m](1), [n](74), [o](51), [p](35), [q](25), [r](38).
* Experiment done in the absence of PVP.

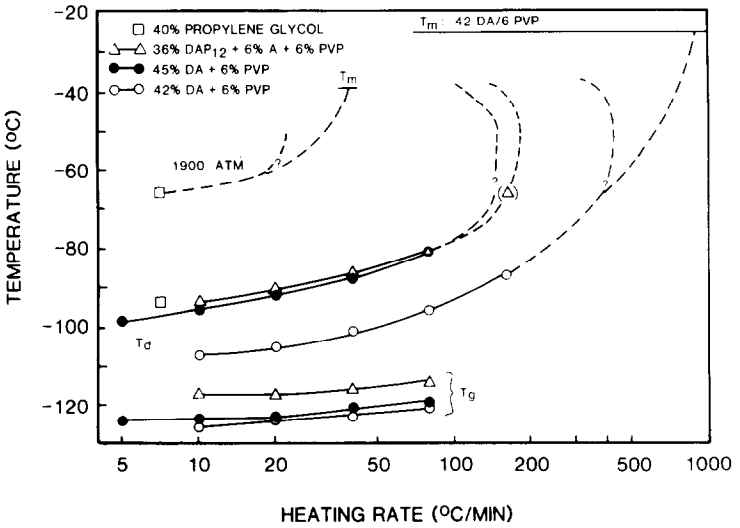

FIG. 13. Effect of heating rate on the temperature of devitrification (T_d). For discussion, see text. Upper open box is for 40% propylene glycol at 1900 atm. Except for the data for propylene glycol, which were obtained using high-pressure differential thermal analysis (45), the data were obtained by differential scanning calorimetry as described in Ref. (45). A = acetamide; other abbreviations as defined previously.

ably doubly unstable and would be expected to devitrify rapidly. However, there is some indication that reapplication of pressure may not be required.

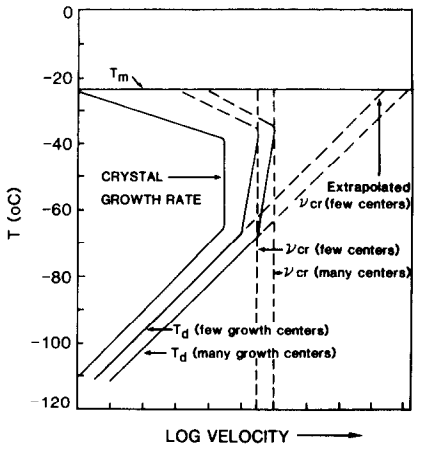

FIG. 14. Speculative scheme explaining heating rate dependence of T_d and the disappearance of T_d at a critical warming rate, ν_{cr}. For discussion, see text. Note how much less ν_{cr} can be than the ν_{cr} as obtained by a straight line extrapolation from lower heating rates as in Refs. (3, 4). A more quantitative consideration of the relationship between crystal growth rate and the T_d vs heating rate curve is given in Ref. (4).

Figure 13 shows the heating rate dependence of the temperature of devitrification of three solutions similar in glass forming ability and composition to 40% DAP_{10} + 6% PVP. These solutions were vitrified by quenching rather than by cooling under high pressure conditions and therefore were more heavily nucleated than would normally be the case. Nevertheless, we see a very interesting disappearance, as well as a near disappearance, of the devitrification curves for two of the three solutions at a heating rate of only 160°C/min. This occurs at temperatures far below T_m and seems to involve a sudden change in the slopes of the devitrification curves (45), as indicated by the speculative extrapolations of the curves.

A speculative but plausible explanation for this behavior is shown in Fig. 14, in which we compare schematically the ice crystal growth rate as described by Luyet and Gehenio (42) and the heating rate dependence of devitrification. Because crystallization velocity stops increasing at some temperature during warming, one would in-

deed expect a change in slope of T_d near this temperature. Furthermore, because crystal growth rate actually decreases above another temperature, one expects the T_d curve to truly vanish at a certain critical heating rate, ν_{cr}. In this region, the heating rate would actually have to decrease in order to observe devitrification (dashed curves extending to the left). Both ν_{cr} and the T_d curve are expected to be functions of the number of nucleation centers.

Even if such a scheme is not valid, Fig. 13 still suggests that heating rates of no more than 400–1000°C/min should be required to suppress devitrification, and these may conceivably be achievable by using microwave heating (5, 31). In addition, techniques to reduce the number of heterogeneous nucleating agents and the inclusion of solutes which inhibit crystal growth in low concentrations may reduce ν_{cr}. Finally, if necessary, reapplication of high pressure should greatly reduce the rate of devitrification.

IV. Application to Whole Organs

One final practical matter must also be considered, namely, the applicability of what has been learned to whole organs. So far, all that is known about this subject, apart from some encouraging but indirect results of earlier investigators (20, 57, 61). is that it is physically possible to vitrify whole organs, cool them to liquid nitrogen temperature without fracturing, and warm them slowly to room temperature at atmospheric pressure without fracturing or devitrification using concentrations of additive which are far too high to be biologically acceptable.

PUTTING IT ALL TOGETHER

Although many uncertainties clearly remain, it will now be instructive to outline how a perfected method might someday actually be applied (Fig. 15). As we now envision it, an organ would be perfused at 0–10°C with gradually increasing concentrations of penetrating cryoprotectant up to a total, safe concentration of 15–25% w/v and held at that concentration until equilibrium is reached. Concentration would then be stepped immediately to whatever is required for vitrification. As soon as the cells become vitrifiable due to osmotic water loss, perfusion can cease. Note that, unlike the situation when organs are being prepared for freezing, it is both unnecessary and, in fact, undesirable to allow time for cells to return to their normal volumes before cooling. Cellular shrinkage is actually beneficial because it concentrates intracellular protein and enhances vitrification. The pressure is then rapidly raised to 1000 atm and the temperature is immediately

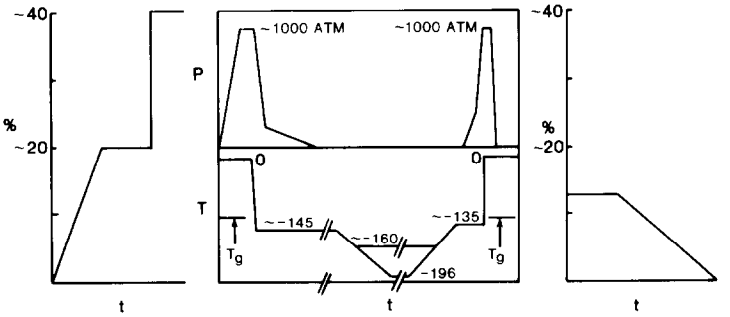

FIG. 15. Schematic representation of how a perfected organ vitrification procedure might be carried out, including cryoprotectant introduction and removal (which differ significantly from what would normally be required for an organ freezing procedure) and avoidance of depressurization-induced, container-induced, and cooling or warming rate-induced fracturing. For discussion, see text.

dropped to about −145°C at the maximum rate possible in order to minimize exposure time, assuming there are no problems with cold shock (53). The initial, rapid temperature descent must not go very far below T_g or the organ will shatter due to thermal stresses (13, 36). At −145°C the pressure is released slowly, since rapid depressurization would also lead to shattering (unpublished observations), and the organ removed both from the pressure bomb and from a peel-away container, yielding a vitreous organ encased in a protective layer of glassy perfusate, much like the kidney on the right in Fig. 16. The kidney on the left has frozen and is shown for comparison. It is necessary to remove the organ from any container because adherence of the perirenal glass to the container as the glass also thermally contracts during further cooling would also lead to shattering (C. T. Moynihan, personal communication, and unpublished observations). After a period of annealing at −150°C or so, the organ is cooled very slowly, again to prevent shattering (11), to −196°C or is stored near −160°C.

When the organ is to be retrieved, it is again warmed very slowly (61) to approximately T_g, annealed if necessary, and then warmed rapidly by microwaves (5, 31) or by induction heating (40) with or without being repressurized. At 0°C the pressure is removed and the organ is immediately perfused with a concentration of cryoprotectant amounting to perhaps one-third the level used for vitrification. If the cells have been previously shrunken to perhaps one-half of their normal volumes, then such concentrations, in the presence of moderate concentrations of mannitol, would merely bring the cells back to their normal

FIG. 16. Vitrified (right) and frozen (left) rabbit kidneys. The vitrified kidney was perfused with DFP_{10} (see Table 1) + 6% PVP, reaching a final DFP_{10} concentration of 51% w/v after a direct step from 20%. Perfusion with DFP_{10} + 6% PVP continued for 60 min before cooling to below T_g at 1 atm. This kidney was subsequently split open below T_g using a hammer and chisel to check for any signs of intrarenal ice. No ice could be detected in any part of the kidney. Systematic study of the minimum perfusion time consistent with subsequent vitrification has not been carried out. Measured renal effluent temperature ranged from an initial 2°C up to ~8°C near the end of perfusion in a standard Waters cassette, owing to greatly diminished flow of the cold viscous terminal DFP_{10} solution (1/3 to 1/4 of initial flow).

volumes, avoiding any osmotic stress while abruptly terminating exposure to potentially harmful levels of cryoprotectant.

VITRIFICATION AS BOTH AN ALTERNATIVE AND A GUIDE

In conclusion, although many formidable problems remain to be solved or even addressed, vitrification is an intriguing possibility for indefinite preservation of complex biological systems in general. It has the advantage of presenting problems that are well-defined and limited in number. It also seems to us to be closer to fruition than is organ cryopreservation by freezing.

But we would also like to emphasize that the pursuit of vitrification may lead to improved freezing techniques as well. For example, the problems of cryoprotectant toxicity we must face with this approach may also be at the heart of an explanation for "solution effects" injury in many systems. In fact, the use of cryoprotectant toxicity neutralization has already improved the freeze–thaw recovery of kidney tissue (17). The problem of introducing high concentrations of cryoprotectant must be faced in

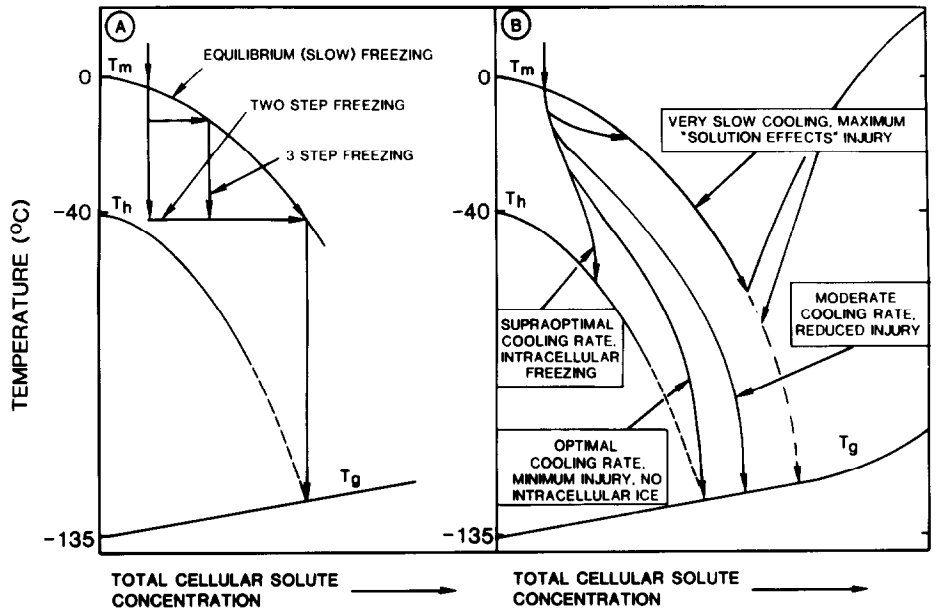

FIG. 17. (A) Relationship between CNV and the temperature from which a slowly frozen cell, or a cell cooled abruptly to and held at high subzero temperatures, can be plunged into liquid nitrogen without intracellular homogeneous nucleation. The safe plunge temperature will be influenced by the choice of cryoprotectant (see Figs. 4 and 5), the starting concentration of cryoprotectant, and the intracellular protein concentration. In general, −40°C should be sufficiently low to render the cytoplasm vitrifiable. (B) Relationship between CNV and the optimal cooling rate. As the cooling rate is progressively elevated, intracellular exposure to high concentrations of cryoprotectant, as well as cellular exposure to volume reduction per se, is progressively diminished. This effect *rather than* (but also in addition to) the postulated role of reduced exposure time (52) probably explains most of the rise in survival seen with increasing cooling rate. At some rate, however, the concentration–temperature trajectory of the cytoplasm begins to intersect the T_h curve, producing intracellular homogeneous nucleation (60, 66). As the cooling rate increases further, this intracellular freezing event becomes more and more damaging until eventually all cells are killed. The optimal cooling rate, therefore, should be the rate which minimizes both cellular shrinkage and intracellular homogeneous nucleation. Note that cells sufficiently damaged by slow-freezing injury may nucleate prematurely (60, 72), removing them from this scheme.

any event in organ freezing procedures as well as in vitrification procedures in order to prevent mechanical damage from ice (57), so the problems of cryoprotectant toxicity are immediate and practical ones for both procedures.

Additional connections between vitrification and freezing are described in Fig. 17. The T_h/T_g intersection point (Fig. 17A) should be relevant for defining the temperature from which a slowly frozen cell or a cell cooled by a step procedure can best survive a plunge into liquid nitrogen, subject, of course, to several secondary considerations. Using this guide, it has in fact recently been possible to document the first substantial recovery of kidney tissue frozen to liquid nitrogen temperature (17). It also appears that the principles of vitrification may, as indicated here, provide a deeper understanding of the optimal cooling rate, which, as suggested here (Fig. 17B), may be that cooling rate which comes closest to bringing the cell's temperature to the cell's T_g at an intracellular concentration just high enough to avoid homogeneous nucleation.

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