0163-4992/87/1002-0169$04.20

Osmotic Properties of the Rabbit Corneal Endothelium and Their Relevance to Cryopreservation

D. E. PEGG,* C. J. HUNT, AND L. P. FONG†

MRC Medical Cryobiology Group, University Department of Surgery, Douglas House, Trumpington Road, Cambridge, UKCB2 2AH

Received July 21, 1986; Accepted January 30, 1987

ABSTRACT

The process of cryopreservation subjects cells to gross changes in the composition of the solution that surrounds them, changes that cause the cells first to shrink and then to swell by an osmotic mechanism. Empirical methods have been developed that permit many cells to survive freezing and thawing, but the cornea, which is crucially dependent upon the function of its endothelial monolayer, has proved quite refractory. In this paper we explore the osmotic response of the corneal endothelium of the rabbit to solutions ranging in osmolality from 0.25 to 8.6 × isotonic. Boyle van't Hoff behavior was observed between 0.43 and 8.6 × isotonic, and there was an apparent nonosmotic volume of 33.6%. However, ultrastructural damage was observed at the limits of this range, and it appeared that the tolerated range was 0.64–4.4 × isotonic. We show the extent to which dimethyl sulfoxide (Me_2SO) would be expected to moderate changes in volume during freezing and suggest that its initial concentration should be at least 2*M* to prevent excessive shrinkage. We also show that cell swelling during removal of Me_2SO is especially likely to be hazardous.

Index Entries: Corneal endothelium; Boyle van't Hoff response; cryopreservation; osmotic properties of corneal endothelium.

†Present Address: Hilles Immunology Laboratory, Harvard Medical School, 243 Charles Street, Boston, MA 021114

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

When cells are frozen in the presence of a penetrating cryoprotectant (CPA), stored at ~ −200°C, thawed, and the CPA removed (that is, they are subjected to the now commonplace process of cryopreservation), they experience gross changes in the concentration of the solution that surrounds them. The separation of water to form ice during the freezing process concentrates the solutes that are present, thawing results in their dilution, and procedures for the removal of CPA once thawing is complete subject the cells to further changes in their environment. Such changes have long been implicated in freezing injury (*1,2*), and their modulation by CPAs is generally accepted as a most important mechanism by which these compounds exert their beneficial action (*3,4*). These cycles of increase and decrease in concentration of impermeant solutes produce corresponding, but inverse, changes in the volume of the cells as they maintain osmotic equilibrium, and it has been proposed that such changes may be the fundamental mechanism whereby cells are damaged by freezing and thawing (*5,6*). Thus, an understanding of the volume response of cells to changing external concentrations of impermeant solutes, and of their ability to tolerate such changes, is vital for any attempt to design rational cryopreservation procedures. In fact, techniques for the effective cryopreservation of isolated cells are remarkably well developed, and there are few mammalian cell-types that have been studied in detail without evolving an effective storage method. However, this optimistic statement does not apply to most tissues, and, in particular, the cornea has proved quite refractory (*7,8*).

It is known that the functional integrity of the cornea is crucially dependent upon the survival and continued function of the endothelium (*9*). This delicate monolayer of cells on the internal surface of the cornea controls the hydration of the stroma, which in turn controls the transparency of the cornea. Thus, any effective cryopreservation method must preserve a viable endothelial cell layer, and this has been recognized in published experimental studies (*10–12*). The purpose of the present study was to measure the osmotic response of the endothelium of rabbit cornea to both hypo- and hypertonic conditions and to determine the limits of osmotic tolerance as indicated by ultrastructural damage, then, using these data, to calculate the changes in volume that accompany standard techniques for the addition of the favored CPA, dimethyl sulfoxide (Me_2SO), and subsequent freezing, thawing, and removal of Me_2SO. These calculations place limits within which cryopreservation schedules should be drawn up if the endothelium is to avoid damaging excursions in volume. Such limits are necessary, although they may not be sufficient, for the effective cryopreservation of the cornea. A comparable study on preimplantation mouse and bovine embryos, but using a rather different analytical approach, has recently been published by Mazur and Schneider (*13*).

THEORY

The interpretation of the osmotic responses of cells has been the subject of much controversy that has centered on the nature of intracellular water and the properties of cell membranes (*14,15*). In the ensuing discussion we shall reject the association-induction hypothesis of Ling (*16*), largely for the reasons elegantly discussed by Garlid (*17*); we will assume that most or all of the intracellular water has the properties of bulk water, to which the classical attributes of dilute aqueous solutions apply. We will, however conclude that a second, smaller phase, "abnormal" water in Garlid's terminology, may exist, which does not respond to changes in the osmolality of the suspending medium. Such "nonosmotic" water is not necessarily "nonsolvent" with respect to all solutes. Nevertheless, we shall assume that it is not accessible to Me_2SO; in the case of rat liver mitochondria there is specific evidence that the distribution volume of Me_2SO is indeed equal to the volume of "normal" water (*17*).

Ideal Boyle van't Hoff behavior is exhibited by cells in which the plasma membrane remains impermeant to the internal and external solutes, all the intracellular water is osmotically active, and the internal solutes behave in an ideal manner. Under these conditions, it is acceptable to postulate a number of internal osmoles, N_i, that remains constant, and since osmotic equilibrium is maintained across the plasma membrane, then:

$$M_e = \frac{N_i}{V_i} \qquad [1]$$

where M_e is the external osmolality, and V_i is the volume of intracellular water. If we measure volume and osmolality on relative scales (V_i^{rel} and M_e^{rel}), such that both isotonic values = 1, then:

$$\frac{M_e}{M_e^{iso}} = \frac{N_i}{V_i} \times \frac{V_i^{iso}}{N_i}$$

$$\frac{M_e}{M_e^{iso}} = \frac{V_i^{iso}}{V_i}$$

$$V_i^{rel} = \frac{1}{M_e^{rel}} \qquad [2]$$

Hence, the Boyle van't Hoff plot of V_i^{rel} on $1/M_e^{rel}$ would be a straight line of slope = 1, passing through the origin. It is common to find that the volume of intracellular water taking part in osmotic responses appears to be less than the total water content measured by drying or isotopic means (*14,18*); that is, there is a positive intercept on the V_i^{rel} axis of the Boyle van't Hoff plot. Such a circumstance is illustrated in Fig. 1, which is drawn from data for human platelets obtained by Armitage in this laboratory (*19*). Note that the line still passes through the point (1,1) since the

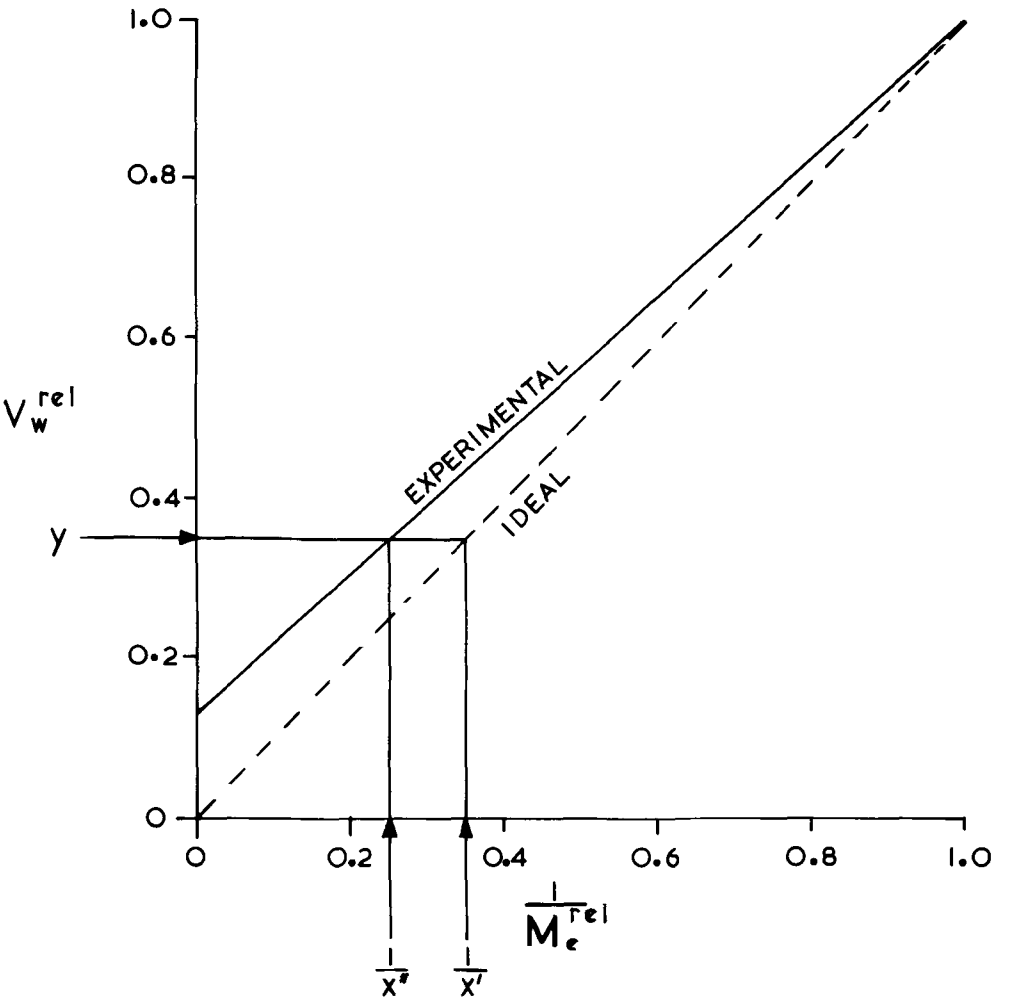

Fig. 1. Boyle van't Hoff data for platelets [data of Armitage (24)] plotted as a solid line relating relative cell water (V_w^{rel}) against reciprocal relative osmolality ($1/M_e^{rel}$). Here, the ordinate intercept represents apparent nonosmotic cell water. The broken line illustrates ideal behavior, where there is no apparent nonosmotic water. If the intercept is related to nonideal behavior of internal solutes, then $\phi^{rel} = x''/x'$.

cells must, by definition, have an isotonic volume in an isotonic solution, and, consequently, the slope of the line is fixed by the intercept.

Thus, by elementary geometry:

$$V_i^{rel} = V_i^{inf} + \frac{1 - V_i^{inf}}{M_e^{rel}} \quad [3]$$

where V_i^{inf} is the volume of intracellular water at infinite osmolality. It is useful to note that the sum of the ordinate intercept and the slope, when the units are relative volume and relative inverse osmolality, must always be 1.

The concept of a second phase of "abnormal" or "nonosmotic" water provides an appealing explanation for this anomaly; it is simple, and one can readily visualize that water in close contact with macromolecular surfaces might have abnormal properties. Indeed, there is strong evidence that this is so (*17*). There is, however, an alternative explanation; namely, that appropriate non-ideal behavior of the impermeant internal solutes could produce such a volume response without invoking a second phase. In the case of the data shown in Fig. 1, this would require that the internal solutes behave in an extremely nonideal fashion at high concentration [as indeed is so for proteins (*20*)]. The relative osmotic coefficient, ϕ^{rel} (that is, relative to ϕ^{iso}) required to produce such behavior can be calculated from Eqs. [2] and [3], which describe ideal and experimentally observed behavior. In Fig. 1, when $V_i^{rel} = y$, then the corresponding ideal relative osmolality would be x', but the corresponding, experimentally observed relative osmolality is x''.

From Eq. [2]:
$$y = \frac{1}{x'}$$

and from Eq. [3]:
$$y = V_i^{inf} + \frac{1 - V_i^{inf}}{x''}$$
$$\frac{1}{x'} = V_i^{inf} + \frac{1 - V_i^{inf}}{x''} \qquad [4]$$
$$\frac{1}{x''} = \frac{\frac{1}{x'} - V_i^{inf}}{1 - V_i^{inf}}$$
$$\frac{x'}{x''} = \frac{1 - x'V_i^{inf}}{1 - V_i^{inf}}$$

Since internal osmolality is equal to external osmolality under all equilibrium conditions:

$$\phi^{rel} = \frac{x''}{x'} = \frac{1 - V_i^{inf}}{1 - x'V_i^{inf}}$$

or, generally:

$$\phi^{rel} = \frac{1 - V_i^{inf}}{1 - \nu m^{rel} V_i^{inf}} \qquad [5]$$

where m^{rel} is molality relative to isotonic, and ν is the number of dissociated species.

Alternatively, ϕ^{rel} can be obtained in terms of osmolality by multiplying Eq. [4] by x''.

Thus:

$$\phi^{rel} = \frac{x''}{x'} = x''V_i^{inf} + 1 - V_i^{inf} = 1 - V_i^{inf}(1 - x'')$$

or, generally:

$$\phi^{rel} = 1 - V_i^{inf}(1 - M^{rel}) \quad [6]$$

where M^{rel} is osmolality relative to isotonic, as before.

The dependence of ϕ^{rel} on $\nu\, m^{rel}$ and M^{rel} for the platelet data is shown in Fig. 2. Note that ϕ has an hyperbolic dependence on the molality of internal solutes similar to that exhibited by hemoglobin (*18*), whereas the relationship to external, and hence to internal, osmolality is linear. Although such an extreme dependence is by no means inconceivable, the rival concept of nonosmotic water seems more plausible and has independent supporting evidence (*17*). We shall make this latter assumption throughout this paper.

METHODS

Corneas were obtained from New Zealand white rabbits, weighing 2–2.5 kg, that had been killed by the intravenous injection of pentobarbitone. Corneas were removed from enucleated eyes and mounted on plastic support rings, as previously described (*21*). Each mounted cornea was immersed, endothelial surface uppermost, in 5 mL of glutathione/bicarbonate/Ringer solution (GBR) equilibrated with 5% CO_2 in O_2 to yield a pH of 7.4. The standard GBR solution had a measured osmolality of 322 mosmol/kg (Fiske osmometer) and contained 128.3 mmol/L NaCl

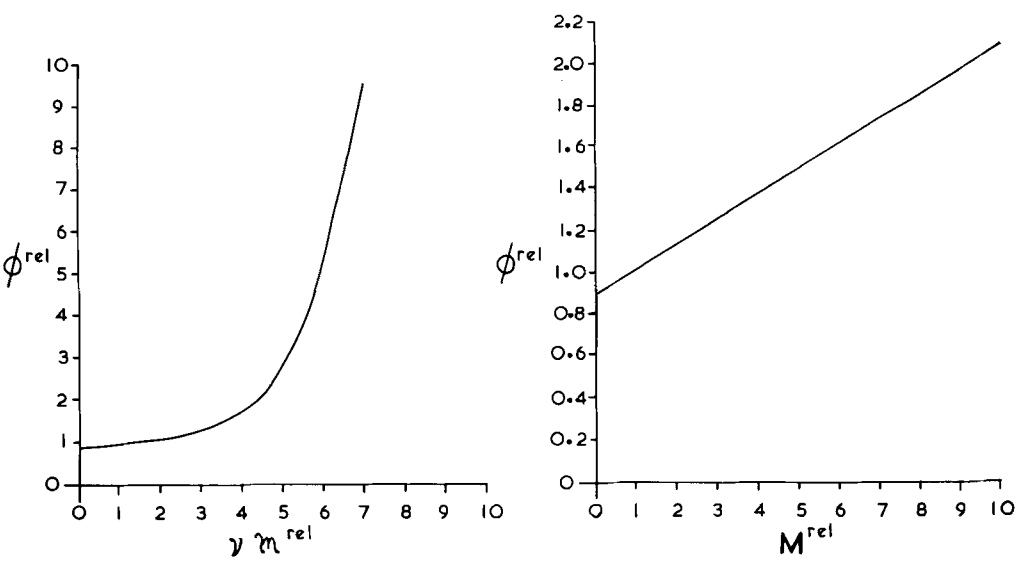

Fig. 2. Calculated relationship between relative osmotic coefficient (ϕ^{rel}) and the molality (left) and osmolality (right) of solutes required to account for the apparent nonosmotic water of Fig. 4 on the basis of nonideal behavior of intracellular solutes. *See* text for a full explanation.

(*10*). Five modified GBR solutions were also used, containing amounts of sodium chloride ranging from 0 to 1305 mmol/L, the other components remaining unchanged from the standard GBR recipe, yielding measured osmolalities of 75, 128, 193, 322, 1317, and 2585 mosmol/kg, respectively. Each of these solutions, and standard GBR, was used to incubate two fresh corneas for 5 min at 20°C: One cornea was then fixed in a GBR of the same osmolality, with the addition of 3% glutaraldehyde (GA) (we ignore the osmolality related to GA and refer to these solutions as isosmolar GBR/GA, *see* Discussion), and the other was returned to the standard GBR (322 mosmol/kg) for 10 min at 20°C before being fixed in 3% GA in 0.16M cacodylate buffer at pH 7.4 (osmolality, 320 mosmol/kg). The pH of the GBR-fixed tissues was maintained at 7.4 by placing them in a CO_2 incubator fitted with a pH-stat (*22*). All corneas were fixed at 20°C for 24 h before being removed from the support rings.

Light Microscopy

Samples of tissue were transferred to isosmolar GBR/GA solutions containing 2 mg/mL of ruthenium red for 2 h at 0°C, then to isotonic-buffered, 1% osmium tetroxide solution with 2.5 mg/mL ruthenium red (*23*) for 1 h. Tissue was then washed in distilled water, and 2–4 areas from the endothelial surface of each cornea were photographed under bright-field illumination at a magnification of ×105. Prints were prepared at a total magnification of ×420, and the number of endothelial cells within a randomly placed rectangle measuring 63 × 42 mm was counted. Under isotonic conditions, there were ~55 cells in this rectangle, which, at the selected magnification, represented 15 × 10^3 μm^2 of the endothelial surface. The mean area of one cell was calculated.

Transmission Electron Microscopy

Samples of tissue that were fixed in isosmolar GBR/GA solutions were diced, washed in isosmolar GBR, and postfixed in 1% osmium tetroxide in isosmolar GBR. Corneas fixed in cacodylate/GA were postfixed in 1% osmium tetroxide in cacodylate buffer. All samples were dehydrated in an ascending series of ethanol solutions (50–100%), embedded in Araldite, sectioned vertically on an LKB Ultratome 1, sequentially stained with uranyl acetate and lead citrate, and examined in a Zeiss EM9S2B microscope. Five areas from four separate samples from each cornea were photographed at a magnification of ×8650, and on each of the 20 photographs the thickness of the endothelium was measured at 5 points. Under isotonic conditions, at this magnification, the thickness on the printed image was ~20 mm and was measured with a resolution of 0.5 mm. One-hundred such measurements were made on each cornea, and the average thickness of each endothelium was calculated.

Calculation of Cell Volumes

The dimensions of the endothelial cells were measured on micrographs prepared as described above. It was assumed that changes in relative volume calculated from these measurements were an accurate measure of changes in relative volume of the cells under the experimental changes. Thus, it was assumed that any changes in volume produced by processing either were negligible or occurred in equal proportion in all the samples. Endothelial cells were approximated as right prisms, the area of the end face being equal to the mean area of the cell surfaces measured in the ruthenium red preparations and the height being equal to the mean thickness of the endothelium measured in the electron micrographs. Most of the changes in volume in anisotonic media were related to changes in height (thickness) of the cells; the mean surface area increased by <15% in the hypotonic solutions and decreased by <12% in the hypertonic solutions. A Boyle van't Hoff plot was constructed using the mean volumes of the endothelial cells fixed at the osmolality of exposure and the quotient isotonic osmolality/osmolality of the bathing solution. Isotonic osmolality was taken to be 300 mosmol/kg. The volume at isotonic osmolality was calculated from the regression equation using the data from the four highest osmolalities (*see* below), and the absolute measured volumes were normalized to this volume; that is, the ordinate of the Boyle van't Hoff plot was converted to *relative* volume, where isotonic volume = 1.

EXPERIMENTAL RESULTS

Volume Response

Figure 3 shows the relationship between relative volume and relative inverse osmolality. It can be seen that when the osmolality of the bathing solution was $\geq 0.43 \times$ isotonic, the endothelial cell volume followed classical Boyle van't Hoff behavior in that there was a linear relationship between cell volume and inverse osmolality. A regression line drawn using the four highest osmolalities gave the following equation for the dependence of relative volume (V^{rel}) on relative osmolality (π^{rel}):

$$V^{rel} = 0.336 + \frac{0.664}{\pi^{rel}}$$

If the five highest osmolalities were used, the equation was:

$$V^{rel} = 0.314 + \frac{0.686}{\pi^{rel}}$$

The difference is small, and the line from four points is preferred since there was significant ultrastructural damage at the fifth point. The correlation coefficient was 0.9994. When the tonicity was $0.25 \times$ isotonic, the

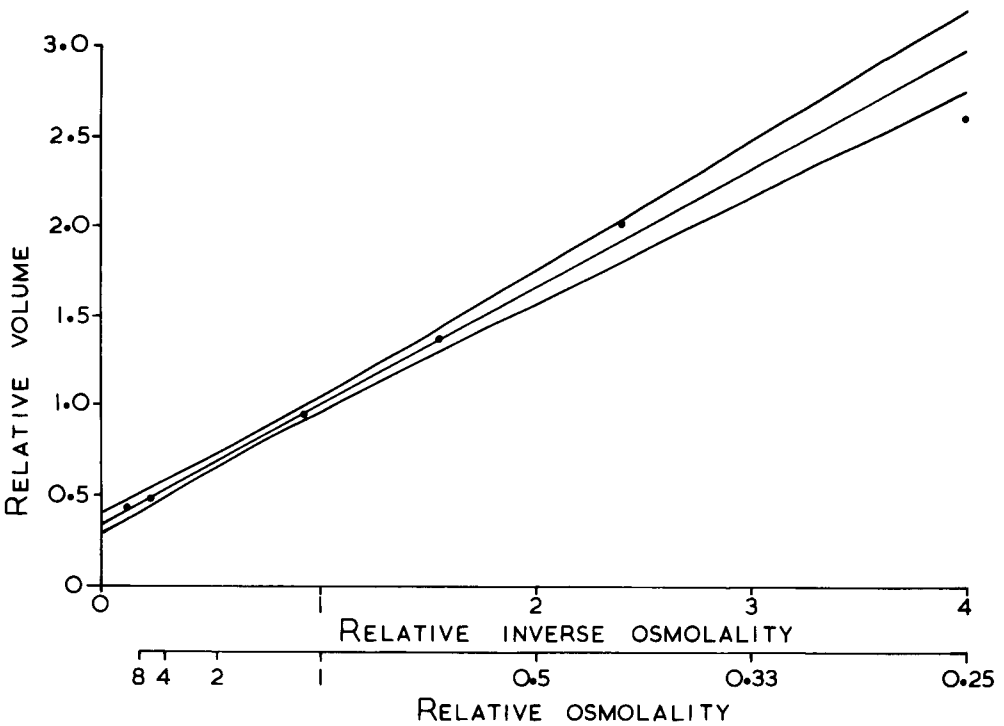

Fig. 3. Volume of endothelial cells relative to their volume in isotonic saline (300 mosmol) plotted against reciprocal relative osmolality (osmolality relative to 300 mosmol). At osmolalities $> 0.43 \times$ isotonic the relationship follows the classical Boyle van't Hoff law. The flanking lines show the 95% confidence limits: $R = 0.9994$. The ordinate intercept (0.336) is the so-called nonosmotic volume of the cells.

cell volume was less than predicted by the regression equation, and the hypotonic limit of reversible osmotic response probably lies between 0.43 and 0.25 × isotonic. However, shrinkage during processing could have caused an underestimation of the relative volume measured in the most hypotonic solution (*see* Discussion).

Morphology

Corneas incubated in the standard GBR solution (322 mosmol/kg) and then fixed in cacodylate-buffered GA (320 mosmol/kg) exhibited no significant ultrastructural alterations to the endothelium when compared with corneas that had been fixed in GBR. Comparison with freshly isolated, immersion-fixed corneas again revealed no significant differences. In all cases the endothelium was firmly attached to Descemet's membrane, apical junctional zones were normal, and there was no widening of the intercellular space.

Corneas exposed to either 1317 or 2585 mosmol/kg and fixed at these osmolalities exhibited an endothelial layer that, although shrunken, was firmly adherent to Descemet's membrane (*see* Fig. 4). Cell organelles

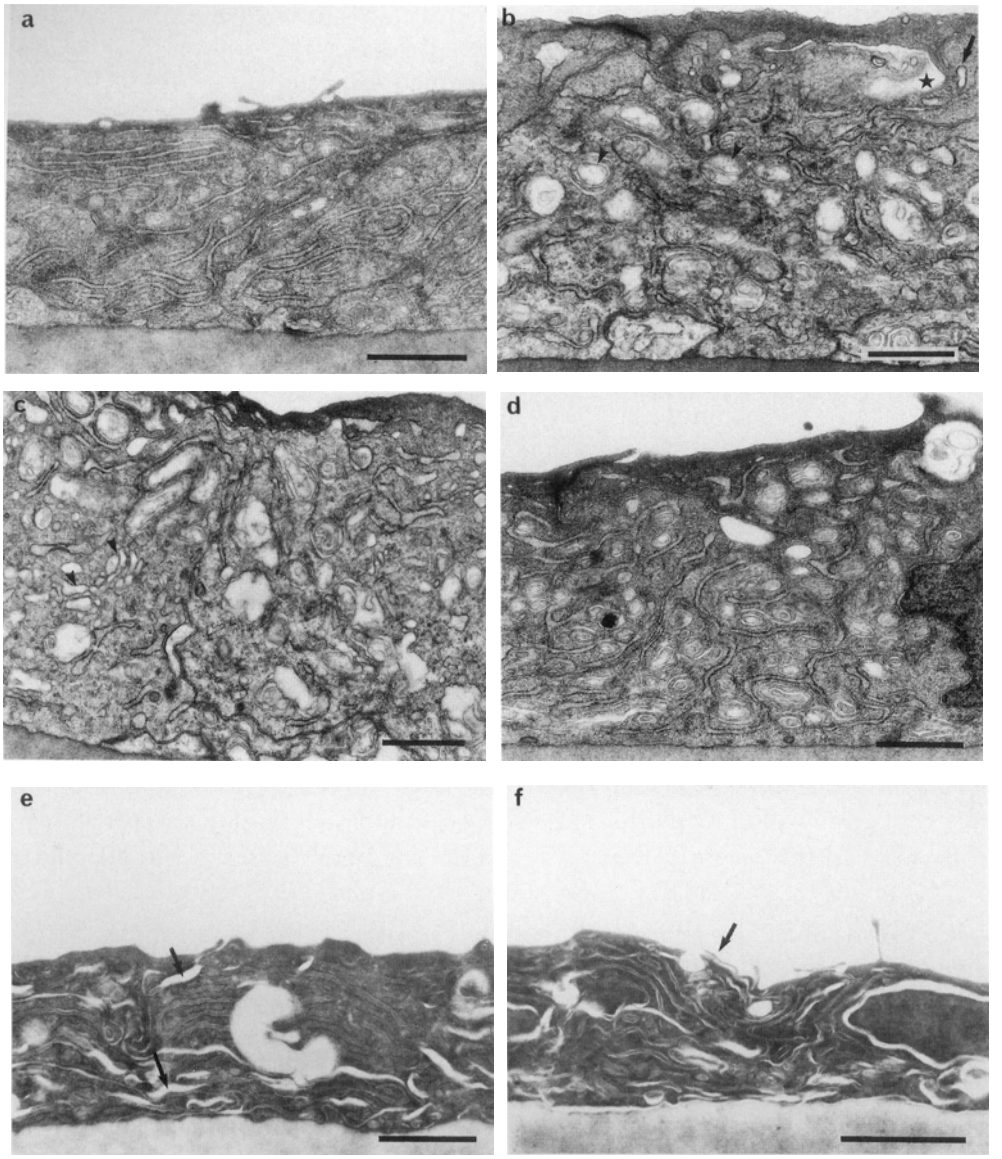

Fig. 4. Corneal endothelium exposed to hyper- and hypotonic GBR solutions and fixed in GBR/GA solutions of similar osmolality. (a) Cornea fixed in GBR/GA after exposure to a standard GBR solution of 322 mosmol/kg.

(b), (c), and (d): Corneas fixed in GBR/GA after exposure to hypotonic GBR solutions of 75, 128, and 193 mosmol/kg, respectively. At osmolalities <193 mosmol, mitochondria are swollen [(b): arrow heads], rough endoplasmic reticulum is vesiculated [(b): arrows], the intercellular space is widened [(b): *], and the Golgi apparatus is swollen and disorganized [(c): arrow heads].

(e) and (f): Corneas fixed in GBR/GA after exposure to hypertonic GBR solutions of 1317 and 2585 mosmol/kg, respectively, show membranes in negative contrast. Numerous vacuoles and biconvex cavities [(e): arrows] are present, and the apical junctions sometimes appear open [(f): arrows]. Bar is 1 μm.

were indistinct, with mitochondrial membranes in negative contrast. Endoplasmic reticulum was not fragmented, but biconvex cavities were apparent within some profiles. There was also a significant widening of the perinuclear space. In the apical zone, some junctions appeared to be open, with the extra- and intercellular space continuous. Within the cytoplasm there were numerous cavities of unidentified origin. Corneal endothelium exposed to 193 mosmol/kg and fixed at this osmolality, though swollen, was substantially unaltered. Apical junctions were unaffected, the endothelium was firmly attached to Descemet's membrane, and cell organelles showed little alteration. Structural changes were confined to slight swellings of rough and smooth endoplasmic reticulum and the Golgi complex, together with some slight subcellular edema. Exposure to 128 mosmol/kg produced varying degrees of damage; some areas were markedly swollen and exhibited marked intracellular edema with pronounced mitochondrial swelling and vesiculation of mitochondrial membranes. Nuclear membranes appeared intact although there was a loss of heterochromatin. Other areas were less swollen, although the degree of structural alteration, especially to mitochondria, was no less severe. In some areas the intercellular space was enlarged and the apical junctions opened, and occasionally cells were found with ruptured apical membranes. These changes were constantly seen after exposure to 75 mosmol/kg, and some areas of the endothelium were found to be detached from Descemet's membrane and to exhibit severe intracellular damage.

The reversibility of the changes seen in anisosmolar solutions was assessed in the corneas that were fixed after being returned to standard GBR for 10 min (*see* Fig. 5). Corneas that had been exposed to 1317 mosmol/kg showed only minor damage to the endothelium: junctional complexes had reformed, there was no swelling of endoplasmic reticulum, and the perinuclear space had returned to normal. Structural alteration centered on the mitochondria, which showed a moderate degree of swelling. After exposure to 2585 mosmol/kg, the degree of cellular disorganization appeared more pronounced. Apical flaps were raised up from the endothelial surface, although apical junctional complexes had again reformed, and the intercellular space was of normal width. Some vesiculation of both the Golgi complex and smooth endoplasmic reticulum had occurred. The most pronounced change was again to the mitochondria, which were in many cases markedly swollen. However, the endothelium was still closely opposed to Descemet's membrane. Corneas examined after exposure to 193 mosmol/kg followed by return to the standard solution showed no significant ultrastructural changes. There were marked alterations after return from 128 mosmol/kg. Mitochondria remained hydropic, with disoriented and disintegrating cristae and a pallid matrix. Within the cytoplasm could be found large cavities , which appeared to be membrane-bound in some cases [*see* Fig. 5(c)]. Their origin was unclear, but they appeared to be associated with Golgi arrays. The inter-

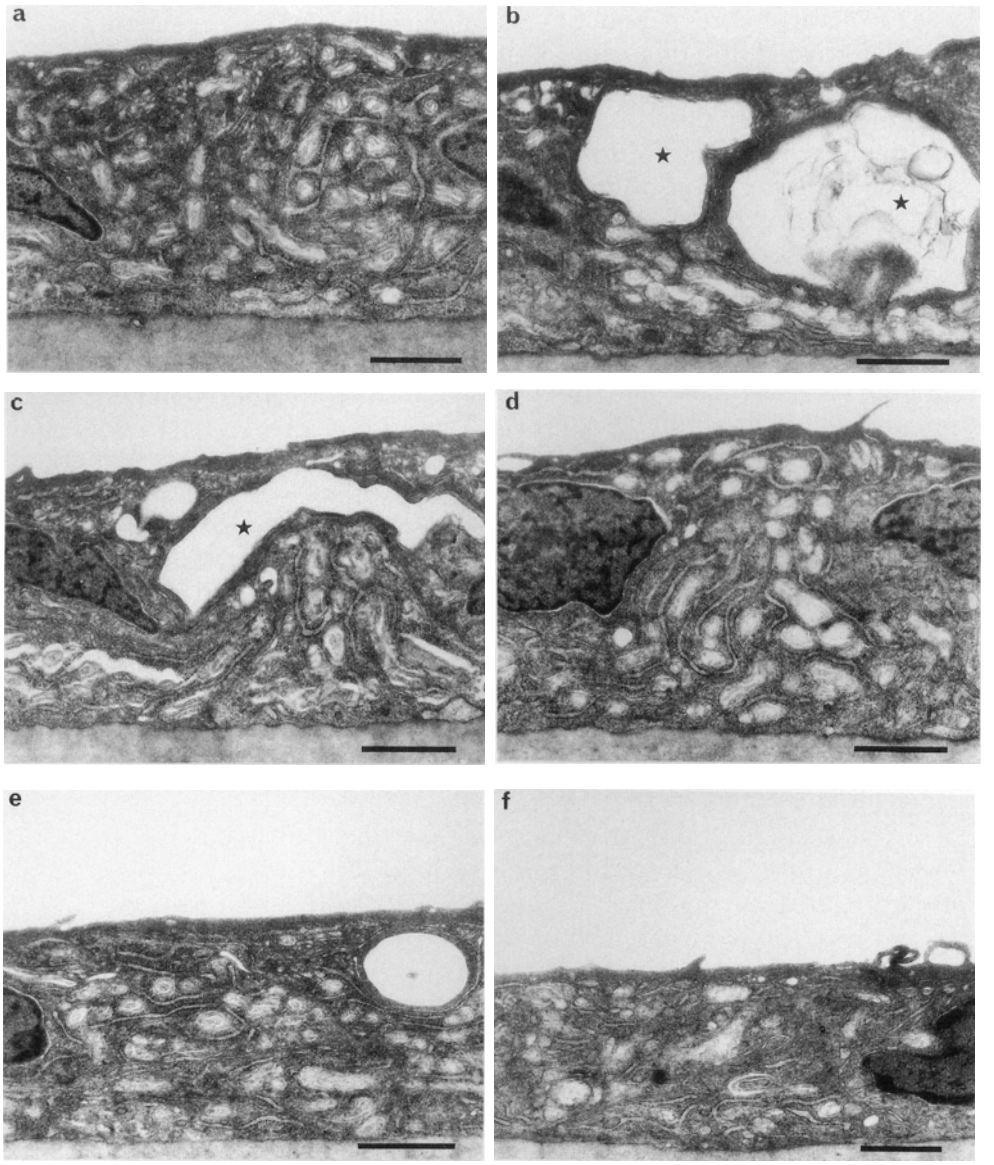

Fig. 5. Corneal endothelium exposed to hyper- and hypotonic GBR solutions and fixed in GA after return to standard GBR. (a): Freshly isolated cornea. (b), (c), and (d): Corneas fixed after return to standard GBR from hypotonic GBR solutions of 75, 128, and 193 mosmol/kg, respectively. At osmolalities <193 mosmol the endothelium produced large vesicles on return to standard GBR [(b), (c): *]. Mitochondria were swollen with a pallid matrix. (e) and (f): Corneas fixed after return to standard GBR from hypertonic GBR solutions of 1317 and 2585 mosmol/kg, respectively. Bar is 1 μm.

cellular space had apparently returned to normal width, and apical junctions had reformed. There was only slight swelling of the endoplasmic reticulum, but an increase in the number of small intracellular vesicles. The endothelium remained firmly bound to Descemet's membrane. Cor-

neas fixed after return from 75 mosmol/kg exhibited a range of structural profiles from that similar to endothelium returned from 128 mosmol/kg to extremely damaged endothelium detached from Descemet's membrane.

Under the conditions used in these experiments, it would appear that the cornea will tolerate hypertonic solutions up to 4.4 × isotonic and hypotonic solutions of 0.64 × isotonic and can then be returned to isotonicity without marked structural damage to the endothelium.

DISCUSSION

Validity of the Volume Measurements

Changes in cell dimensions can occur during processing for electron microscopy and may be osmotic or chemical in origin (*24*), but our technique was designed to minimize any such effects. Samples were fixed in GA solutions containing salts in a concentration that was isosmolar with the test solution, that is, it was assumed that GA made no contribution to the effective osmotic pressure of the solution; its reflection coefficient was zero. This assumption is supported by a number of studies (*25–27*). Other workers have suggested that GA may contribute significantly to effective osmotic pressure during fixation, but even then an effect was detectable only when the concentration exceeded 5% (*26,28,29*). At low concentrations and when the system was well-buffered, the effect was negligible (*30*) or undetectable (*31*). We used 3% GA. In one published study specifically on rabbit corneal endothelium, using 2.5% GA, no shrinkage was detected with respect to fresh tissue (*32*), nor was there any evidence of a selective osmotic effect of GA on damaged cells (*33*). Not only are cell membranes freely permeable to GA, but exposure to GA also alters the semipermeable properties of some cells; for example, ascites tumor cells (*26*) and erythrocytes (*34*) were found to be freely permeable to ions and small molecules after GA fixation, although studies with smooth muscle (*29*) and lung cells (*35*) suggest that some osmotic reactivity may persist. There is no doubt, however, that exposure to osmium tetroxide does completely destroy osmotic responsiveness (*25,29,35*). In our study, cells were postfixed in osmium tetroxide in salt solutions that were isosmolar with the test solutions.

Changes in cell dimensions can also be caused by nonosmotic mechanisms, particularly during dehydration with ethanol (*24*). However, these changes are minimal when dehydration is started with an intermediate concentration (*36*), as in our study, or when tissue has been processed with GA and osmium tetroxide and is embedded in epoxy resin (*24,37–39*). Nonosmotic volume changes during osmium treatment have been shown to depend on pH and divalent cations (*40,41*). In our study the pH and concentration of such cations remained constant throughout the test exposure and processing. Thus, we feel confident in our assump-

tion that any changes in dimensions during processing were negligible. Moreover, any changes that might occur would affect all samples; if changes were to affect all samples in equal proportion, then they would have no affect on the relative changes in volume. If, on the other hand, changes were to affect all samples by an equal amount, there would be a small effect on volume ratios. Thus, if all cells shrank by an amount equal to 5% of the normal cell volume (far more than we consider likely), then the apparent relative volumes of 2.61 and 0.43 (the highest and lowest values recorded) would actually represent real relative volumes of 2.70 and 0.40. Reference to Fig. 3 shows that such an error would have a negligible effect on the regression line, in fact, it would improve the fit at low relative osmolalities.

Osmotic Response

Endothelial cells show a classical Boyle van't Hoff osmotic response down to the osmolality at which obvious, severe ultrastructural damage is seen. We have been unable to find a value for the normal total water content of these cells in the literature, but by analogy with other cells, it seems unlikely to be as low as the 66.4% that our present results would require if there were no nonosmotic fraction of water. It is probable, therefore, that corneal endothelial cells would exhibit a positive ordinate intercept on a plot of cell water (V_i^{rel}) vs reciprocal osmolality ($1/M_c^{rel}$).

Osmotic Tolerance

Our results indicate that corneal endothelial cells are more tolerant of a reduction in volume than expansion. The tolerated limits, as defined by a normal cell volume after return to isotonic conditions, and minimal or nonexistent ultrastructural evidence of damage can be calculated from the Boyle van't Hoff regression equation as 50–140% of the isotonic cell volume. If it is assumed that the intercept in that equation represents a nonosmotic volume, then the limits of intracellular osmotic water volume ($V_{i.osm}^{rel}$) are found to be 23–160% of isotonic volume, since:

$$V_{i.osm}^{rel} = \frac{1}{\pi^{rel}}$$

The hypotonic limit is in agreement with published data for both rabbit and human cornea (*42*) and is comparable to that for early mouse embryos (*13*). Armitage (*43*) has recently published a study of osmotic tolerance of the rabbit cornea, using its ability to control corneal hydration on the specular microscope as a criterion of damage. He reported that function was maintained after exposure to osmolalities between 0.12 and 2.7 osmol/kg. We, however found significant ultrastructural changes at the extremes of this range and, consequently, have been somewhat more conservative in suggesting tolerable osmotic limits.

Implications for Cryopreservation

In the following analysis it is assumed that endothelial cells have a nonosmotic volume that includes both cell solids and abnormal water and is equal to 0.336 of the normal volume, that Me_2SO penetrates only the normal water, and that there are no changes in osmotic coefficient or nonosmotic volume. First we develop equations that describe the volume of endothelial cells in equilibrium with solutions of Me_2SO and NaCl; a similar analysis has been presented for human red blood cells and glycerol (*44*). Then we describe the changes in solution composition that occur during the freezing of such a system, and, finally, we calculate the volume of endothelial cells during freezing, thawing, and return to isotonic saline.

Volume of Endothelial Cells Exposed to the Ternary System NaCl/Me_2SO/H_2O

Consider 1 L of endothelial cells, under isotonic conditions, having a nonosmotic volume of V_n L: The physiological intracellular normal water vol $= 1 - V_n$ L $= 1 - V_n$ kg. If normal intracellular osmolality $= 0.3$ osmol/kg water, then, if the volume of normal water in the cell, V_w, changes, but the solutes remain inside the cell:

$$\text{intracellular osmolality} = \frac{0.3(1 - V_n)}{V_w} \text{ osmol/kg} \qquad [7]$$

Consider now the solution that bathes the cells, and consider first the impermeant solute, NaCl: Assume that NaCl dissociates into two ions and has an osmotic coefficient of 0.925 and a mol wt of 58.45, and that S is the concentration of NaCl in g/100 g solution, and M is the concentration of Me_2SO in g/100 g solution.

Then, on an osmolal scale: External salt concentration =

$$\frac{10 \times S \times 2 \times 0.925}{58.45} \times \frac{100}{100 - S - M} \text{ osmol/kg} \qquad [8]$$

Since the cells are assumed to be in equilibrium with the surrounding fluid, and Me_2SO is equally distributed across the cell membrane, osmotic balance is determined solely by extracellular NaCl. Hence, equating the right-hand sides of Eqs. [7] and [8]:

$$0.3 \frac{(1 - V_n)}{V_w} = \frac{31.65S}{100 - S - M}$$

$$V_w = \frac{0.3\,(100 - S - M)\,(1 - V_n)}{31.65S}$$

$$= \frac{0.009479\,(100 - S - M)(1 - V_n)}{S} \text{ L} \qquad [9]$$

(The constant 0.009479 results from combining the assumed values for

the normal intracellular osmolality, the number of species into which NaCl dissociates, an osmotic coefficient for NaCl, and the mol wt of NaCl.)

Consider now the Me_2SO in the external solution. Assuming a mol wt of 78.13, the molal concentration is:

$$\frac{10 \times M}{78.13} \times \frac{100}{100 - S - M} \text{ mol/kg water}$$

The density of Me_2SO is 1.1014 kg/L:

$\therefore$ 1 L Me_2SO contains 14.1 mol
$\therefore$ The molar volume of Me_2SO = 0.0709 L/mol

Assuming that the partial molar volume of Me_2SO is also 0.0709 L/mol in the solutions under consideration, the volume concentration of Me_2SO in the bathing solution is given by:

$$\frac{1000 \times 0.0709}{78.13} \times \frac{M}{100 - S - M} = \frac{0.9075 \times M}{100 - S - M} \text{ L } Me_2SO/\text{L water} \quad [10]$$

Assuming the same ratio of Me_2SO to normal water inside the cells, the intracellular volume of Me_2SO, V_m, is given by:

$$V_m = V_w \times \frac{0.9075 \times M}{100 - S - M} \text{ L}$$

Substituting Eq. [9] for V_w:

$$V_m = 0.008602\ (1 - V_n) \times \frac{M}{S} \quad [11]$$

(The constant 0.008602 results from combining the partial molar volume of Me_2SO, the mol wt of Me_2SO, and the constant 0.009479 from Eq. [9].)

Hence, the total cell volume, V, is given by:

$$V = V_n + V_w + V_m$$

$$= V_n + \frac{0.009479\ (100 - S - M)\ (1 - V_n)}{S} + \frac{0.008602 \times (1 - V_n) \times M}{S} \quad [12]$$

$$= V_n + \frac{(1 - V_n)}{S}\,[0.9478 - 0.009478S - 0.000876\ M]\text{L} \quad [13]$$

Note particularly that the volume of Me_2SO within the cell is not determined solely by M (Eq. [11]), but by the ratio M/S, which is the R value of the solution. The R value does not change during freezing, at least until the pseudobinary eutectic temperature is reached, since only water is being removed from the system as freezing proceeds. Thus, the equilibrium amount of Me_2SO inside the cell does not increase as freezing progresses, only its concentration changes as a result of water movement. Hence, it is not necessary to postulate a particular Me_2SO permeability in any kinetic analysis, and in the following analysis (which is not kinetic) it is necessary to assume only that water movement remains in equilib-

rium. This is a reasonable assumption since the cooling rates used in practice are slow (~1°C/min).

Solute Concentrations During Freezing of the Ternary System NaCl/Me_2SO/H_2O

The fact that the R value of a ternary solution, such as the one under consideration, does not, in general, change during freezing, has the useful result that the two solutes can be treated as a single solute, giving rise to a single liquidus curve. Equations have been developed that describe the family of liquidus curves for all R values of the systems NaCl/glycerol/H_2O and NaCl/Me_2SO/H_2O (*45,46*). The equation for the Me_2SO system is:

$$\Delta t = AC + BC^2 - 0.00045C^3 \quad [14]$$

where Δt is the melting point in °C, C is the total solute concentration in g/100 g solution, and R is the weight ratio of Me_2SO/NaCl in the following equations for A and B:

$$A = -0.6 + 0.17 \tan^{-1}(R) \quad [15]$$

$$B = \frac{\tan^{-1}\left(\frac{R}{2}\right)}{132} - 0.001 \quad [16]$$

(The inverse trigonometrical functions have no analytical significance, but merely provide a convenient transformation to model the dependence of the coefficients in Eq. [14] on R).

Using Eq. [14] it is possible to determine C, the total solute concentration in equilibrium with ice, for all temperatures down to the pseudo-binary eutectic temperature (t_{pb}) during freezing and thawing. The value of t_{pb} in °C is given by:

$$t_{pb} = -21.2 - 6.3C + 0.16C^2 \quad [17]$$

and this sets the lower limit to the validity of Eq. [14]. The concentration of NaCl (S) and of Me_2SO (M), both in g/100 g of solution, are readily calculated from C and R by the equations:

$$S = \frac{C}{R + 1} \quad [18]$$

and:

$$M = R \times S \quad [19]$$

Volume of Endothelial Cells During Freezing, Thawing, and Dilution

Assuming initial equilibration of Me_2SO, instantaneous water movement, and a constant, nonosmotic volume, Eqs. [13] and [14] were used to calculate relative cell volumes during freezing and thawing. The data for isotonic saline and 0.5, 1, and 2M Me_2SO in 300 mosmol saline are

shown in Fig. 6. The ability of Me_2SO to delay the onset of cell shrinkage and to reduce its extent at any given subzero temperature is clearly shown. The relevance of this to theories of freezing injury is apparent. The minimum concentration of Me_2SO to prevent shrinkage beyond the lower limit established in this study is about 2*M*.

In the absence of permeability parameters for Me_2SO and corneal endothelial cells it is not possible to model the time-course of volume changes during the removal of Me_2SO, but Table 1 shows the maximum volumes that would be produced if osmotic equilibrium were attained solely by water movement with the indicated single-step reductions in concentration of Me_2SO. In kinetic terms, this is equivalent to assuming infinitely rapid water movement and infinitely slow movement of Me_2SO, which is not altogether unreasonable as a first approximation. Cell volume was calculated as follows: First, the volume of water and Me_2SO in cells equilibrated in the initial solution was calculated by Eqs. [9] and [10] and added to the nonosmotic volume to obtain the initial cell volume. The osmolality of the initial suspending solution was then calculated by obtaining its freezing point from Eq. [14] and multiplying by the molal freezing point depression constant, −1.86. This involves approximations that have been discussed in detail by Mazur and Schneider (*13*), but are considered acceptable in the present context. The internal osmolality was assumed equal to the external osmolality. Next, the osmolality of the second suspending solution (that is, the diluting solution) was calculated by the

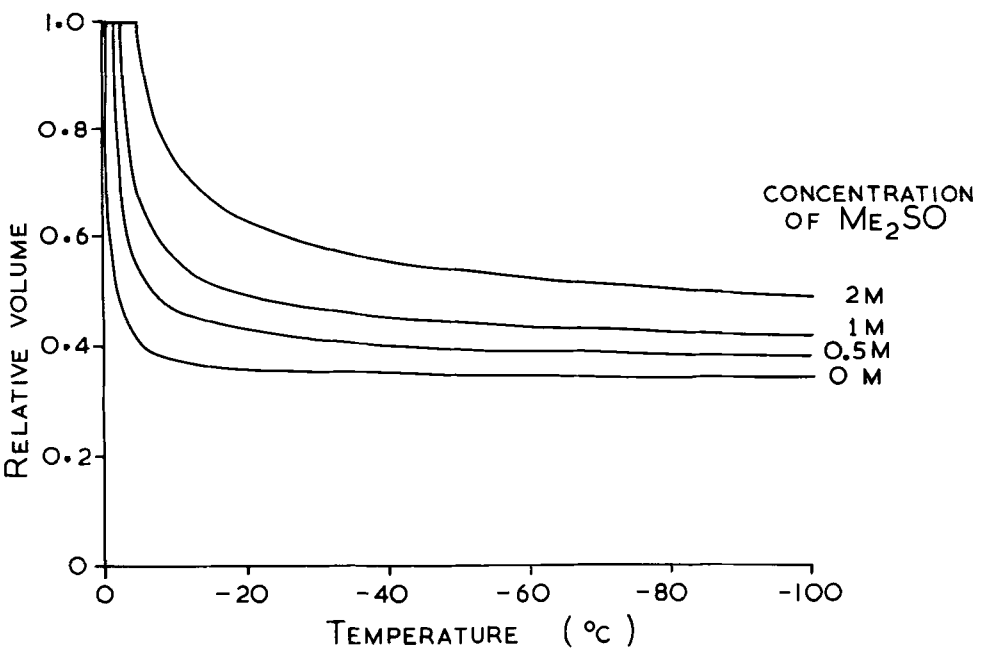

Fig. 6. Calculated relative volume of endothelial cells during freezing to −100°C in a range of solutions. 2*M* = 2 molar Me_2SO in 300 mosmol saline. 1*M* = 1 molar Me_2SO in 300 mosmol saline. 0.5*M* = 0.5 molar Me_2SO in 300 mosmol saline. 0*M* = 300 mosmol saline.

TABLE 1
Calculated Maximum Volumes Produced when Endothelial Cells are Transferred from Solutions of Me_2SO in Saline to more Dilute Solutions

300 mosmol saline plus indicated molarity of Me_2SO,		Maximum volume,
Initial solution	Diluting solution	Proportion of original volume
2.0*M*	1.0*M*	1.47
1.0*M*	0.5*M*	1.37
0.5*M*	0.25*M*	1.29
0.25*M*	0.0*M*	1.69
0.5*M*	0.0*M*	2.27
1.0*M*	0.0*M*	3.32

same procedure, and the volume of intracellular water was assumed to increase in inverse proportion to the ratio of the two osmolalities. The final volume was then calculated assuming that the nonosmotic volume and the volume of Me_2SO remained unchanged. The proportional increase in volume was the final volume/initial volume. Dilution procedures in current use commonly involve steps in which the concentration of Me_2SO is halved until a concentration of 0.5 or 0.25*M* Me_2SO is reached, with a final transfer to isotonic saline or a similar solution. The present calculations suggest that such a process may cause unacceptable excursions in volume, particularly in the final dilution.

ACKNOWLEDGMENTS

The authors acknowledge their indebtedness to Dr. Peter Mazur of Oak Ridge National Laboratory. It was his work that stimulated us to carry out this study, and he has given generously of his time in discussion with us. We also thank Dr. M. J. Taylor of this group and Dr. W. J. Armitage of Bristol University for their critical reading of the manuscript.

REFERENCES

1. Lovelock, J. E. (1953), *Biochim. Biophys. Acta* **10,** 414.
2. Mazur, P. (1977), Slow-freezing injury in mammalian cells, in *The Freezing of Mammalian Embryos* (Elliott, K., and Whelan, J., eds.), Elsevier, Amsterdam, pp. 19–42.
3. Lovelock, J. E. (1953), *Biochim. Biophys. Acta,* **11,** 28.
4. Mazur, P., Leibo, S. P., Farrant, J., Chu, E. H. Y., Hanna, M. G., Jr., and Smith, L. H. (1970), Interactions of cooling rate, warming rate, and protective additive on the survival of frozen mammalian cells, in *The Frozen Cell. Ciba Symposium* (Wolstenholme, G. E. W., and O'Connor, M., eds.), J. and A. Churchill, London, pp. 69–85.
5. Meryman, H. T. (1970), The exceeding of a minimum tolerable cell volume in hypertonic suspension as a cause of freezing injury, in *The Frozen Cell*

(Wolstenholme, G. E. W., and O'Connor, M., eds.), J. and A. Churchill, London, pp. 51–64.
6. Steponkus, P. L. (1984), *Ann. Rev. Plant. Physiol.* **35,** 543.
7. Fong, L. P., Hunt, C. J., Taylor, M. J., and Pegg, D. E. (1986), *Br. J. Ophthalmol.* **70,** 751.
8. Taylor, M. J. (1986), *Cryobiology* **23,** 323.
9. Maurice, D. M. (1984), The cornea and sclera, in *The Eye. Vegetative Physiology and Biochemistry* (Davson, H., ed.), Academic, New York, NY, Third Ed., vol. 1b.
10. Taylor, M. J., Hunt, C. J., and Sherrard, E. S. (1981), Assessment of corneal endothelial integrity by specular microscopy after cryopreservation, in *The Cornea in Health and Disease,* Academic, and RSM, London, pp. 437–444.
11. Madden, P. W. and Easty, D. L. (1982), *Cryobiology,* **19,** 666.
12. Taylor, M. J., and Hunt, C. J. (1985), *Curr. Eye Res.* **4,** 963.
13. Mazur, P., and Schneider, U. (1986), *Cell Biophys.*
14. Dick, D. A. T. (1966), *Cell Water,* Butterworths, Oxford.
15. Hazelwood, C. F. (1979), A view of the significance and understanding of the physical properties of cell-associated water, in *Cell-Associated Water,* (Drost-Hansen, W., and Clegg, J., eds.), Academic, New York, NY, pp. 165–259.
16. Ling, G. N., and Ochsenfeld, M. M. (1973), *Ann. NY Acad. Sci.* **204,** 325.
17. Garlid, K. D. (1979), Aqueous phase structure in cells and organelles, in *Cell-Associated Water* (Drost-Hansen, W., and Clegg, J., eds.), Academic, New York, NY, pp. 293–361.
18. Dick, D. A. T. (1959), *Int. Rev. Cytol.* **8,** 387.
19. Armitage, W. J. (1986), *J. Physiol.* **374,** 375.
20. McConaghey, P. D., and Maizels, M. (1961), *J. Physiol.* **155,** 28.
21. Fong, L. P., Hunt, C. J., and Pegg, D. E. (1987), *Current Eye Research* Vol. 6, pp. 569.
22. Hayes, A. R. (1978), *J. Clin. Pathol.* **31,** 696.
23. Luft, J. H. (1971), *Anat. Rec.* **171,** 369.
24. Lee, R. M. K. W. (1984), A critical appraisal of the effects of fixation, dehydration, and embedding on cell volume, in *Science of Biological Specimen Preparation* (Revel, J. P., Barnard, T., and Haggis, G. H., eds.), SEM Inc., AMF O'Hare, IL, pp. 61–70.
25. Bone, Q., and Denton, E. J., (1971), *J. Cell. Biol.* **49,** 571.
26. Pentilla, A., Kalimo, H., and Trump, B. F. (1974), *J. Cell Biol.* **63,** 197.
27. Arborgh, B., Bell, P., Brunk, V., and Collins, V. P. (1976), *J. Ultrastruct. Res.* **56,** 339.
28. Barnard, T. (1976), *J. Ultrastruc. Res.* **54,** 478.
29. Lee, R. M. K. W., McKenzie, R., Kobayashi K., Garfield, R. E., Forrest, J. B., and Daniel, E. E. (1982), *J. Microsc.* **125,** 77.
30. Mathieu, O., Claassen, H., and Weibel, E. R. (1978), *J. Ultrastruc. Res.* **63,** 20.
31. Hillman, H., and Deutsch, K. (1978), *J. Microsc.* **114,** 77.
32. Jensen, O. A., Prause, J. U., and Laursen, H. (1981), *Albrecht Graefes. Arch. Klin. Ophthalmol.* **215,** 233.
33. Prickaerts, J. P., Wilson, G. J., Bayliss, C. E., and Baffour, R. (1984), *J. Microsc.* **135,** 169.
34. Carstensen, E. L., Aldridge, W. G., Child, S. Z., Sullivan, P., and Brown, H. H. (1971), *J. Cell Biol.* **50,** 529.
35. Wangensteen, D., Bachofen, H., and Weibel, E. R. (1981), *J. Microsc.* **124,** 189.

36. Weibel, E. R., and Knight, B. W. (1964), *J. Cell Biol.* **21,** 367.
37. Gusnard, D., and Kirschner, R. H. (1977), *J. Microsc.* **110,** 51.
38. Boyde, A., and Boyde, S. (1980), Further studies of specimen volume changes during processing for SEM: Including some plant tissue, in *Scanning Electron Microscopy* II, SEM, AMF O'Hare, IL, pp. 117–124.
39. Eins, S., and Wilhelms, E. (1976), *Microscopie* **24,** 29.
40. Tooze, J. (1964), *J. Cell Biol.* **22,** 551.
41. Elbers, P. F. (1966), *Biochim. Biophys. Acta* **112,** 318.
42. Edelhauser, H. F., Hanneken, A. M., Pederson, H. J., and Van Horn, D. L. (1981), *Arch. Ophthalmol.* **99,** 1281.
43. Armitage, W. J. (1986), *Cryobiology* **23,** 566.
44. Pegg, D. E. (1984), *Cryobiology* **21,** 234.
45. Pegg, D. E. (1983), *Cryo Lett.* **4,** 259.
46. Pegg, D. E. (1986), *Cryo Lett.* **7,** 387.