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Biophysical chemical aspects of cellular cryobehavior

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Freezing tolerance and resistance in nature are among the most important and challenging aspects of biochemical adaptation to extreme environments. Some biochemical strategies are known but their mechanism is still poorly understood. Cryopreservation of cells and tissues of sensitive organisms is still generally based on physical chemistry rather than on biophysical chemical mechanisms. This paper describes the main aspects of these problems and features new trends in their study.

1. Introduction

Temperature plays one of the most important roles in living systems. For a given organism, each biological structure and function tolerates only a small range of temperature. In ecological systems, temperature can be controlled in different ways. Homeotherms use physiological strategies to obtain their body heat from their own metabolism. In contrast, poikilotherms cannot regulate their body temperature, but are the best equipped to avoid thermal stress through an array of biochemical strategies that can ultimately be traced to cellular activities, according to various and – in most cases – poorly understood mechanisms.

As temperature is lowered in any organism, the interactions and weak chemical bonds that are involved in all the degrees of structures become more stable and, below a certain temperature, ice forms in the extracellular space as well as in the cytoplasm [1]. This liquid-to-solid transition may be responsible for the cryosensitivity of organisms by altering the cellular ultrastructure or metabolism [2,3]. To overcome the often lethal effects due to extreme temperatures, living systems have 'invented' an array of behavioral, anatomical and physiological strategies [4–7].

In our opinion, extensive study of the strategies found in nature for overcoming damage caused by freezing along with a biophysical/chemical approach to the cryobehavior of cells is a prerequisite to the transformation of biological processes occurring at low temperature into a branch of biology, and to achieving the reliable cryopreservation of cells, tissues and organs of living systems.

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2. Freezing resistance / tolerance in nature

2.1. Prevention of ice formation by the use of intrinsic cryoprotectants

Poikilotherm organisms normally exposed to temperatures below those at which their body fluids freeze can prevent ice formation in the extracellular fluids (freezing resistance) [8–13]. Such behavior occurs only over a limited range of

temperatures but its full understanding might allow improvements to be made in the routine cryopreservation procedures for homeotherms, which is the goal of cryobiology. Larval and adult insects use glycerol, sugars [14,15] and sometimes proteins [16–20] to depress the freezing point of their body fluids over a varying but significant range of temperatures [13]. According to the literature, these metabolic compounds may permeate cells and invade the extracellular space, therefore being able to serve as integral antifreeze agents [21]. For instance, arctic insects withstand temperatures as low as -35 to $-40\,^{\circ}C$ and synthesize large amounts of glycerol from glycogen, and one finds a close correlation between glycerol content and freezing resistance [22]. The freezing temperature drops from late summer to late winter as the concentration of glycerol increases up to 20–30% in the body fluids. Glycerol is then transformed into glycogen [23], and during summer, the insects die at about -5 to $-6\,^{\circ}C$. However, although there is no doubt that glycerol can exert a cryoprotective influence on the organism, other biochemical strategies may be needed to achieve freezing resistance in some insect species where glycerol seems to be less effective [24].

Such behavior could make one consider adding antifreeze solvents or solutes to homeotherm organisms in order to prevent ice formation and possibly to favor cryopreservation. However, such tentative protocols are not yet successful, since cryoprotectants were shown to be toxic for the cell (for review, see ref. 25).

2.2. *Osmotic inactivation of cellular water*

Intertidal invertebrates also resist or tolerate freezing [26–29]. These organisms are often exposed, in temperate regions of the North, to air temperatures of -10 to $-20\,^{\circ}C$ during the colder winter months [26,28,29]. The best known example of such resistance is provided by the common mussel, *Mytilus edulis*, which is able to withstand temperatures down to approx. $-10\,^{\circ}C$ [27], and it seems that such behavior can be ascribed in part to the osmotic properties of its intracellular fluids. About 20% of cellular water is osmotically inactive, i.e., cannot be drawn from the cell during extracellular ice formation. The factors responsible for 'tying up' the cellular water of *Mytilus* are unknown, but Williams suggested that some cellular components might exert an influence out of proportion to their concentration and provoke dramatic changes within cellular ultrastructure.

3. Biophysical / chemical approach: the role of water

3.1. *Osmotic activity of water*

This analysis of freezing resistance encountered in nature shows that water is once again at the heart of the problems raised by cellular cryobehavior. There is some evidence that the properties of cellular water approach those of water in protein crystals more closely than in dilute solutions, since many crystals contain as little as 20% protein while dilute solutions comprise 0.1% or less [30]. For instance, actively growing cells contain between 17 and 26% protein by weight and red blood cells contain about 35% by weight [31]. Consequently, cells might contain two phases of water, as do protein crystals. This has been demonstrated in two different systems through quite different methods [32–35]. As described by Clegg [36], the first phase is osmotically inactive and of approximately constant volume, the other phase being osmotically active and behaving in its solvent properties, and presumably temperature behavior, like bulk water. However,there exists disagreement in the literature concerning the distance from the surface of proteins over which the structure and properties of water are changed. This factor varies, according to the investigators, from 6 to 500 Å [36–40]. This discrepancy is significant, since in the latter case practically all the cellular water would exhibit ordinary bulk properties. In fact, there is evidence that appreciable amounts of cell water display physical properties differing from those of the pure liquid [36,41], these amounts varying from none [42] to practically all [43] of the water in cells.

3.2. *Vitrification approach*

Under laboratory conditions, only the use of massive concentrations of cryoprotectant solvents

and mixtures, combined with cooling and thawing of preparations under precise conditions down to cryogenic temperatures, can prevent the lethal effect of intracellular ice formation in homeotherm cells [44].

Cryoprotectants depress the freezing point and extend the supercooling of body fluids by interacting with water molecules via hydrogen bonding [21,45–47]. These interactions reduce the degree of water-water bonding and, consequently, the formation of ice is delayed. Water may even form an amorphous solid (vitreous state) [48–51]. Such 'vitrification' is now considered as the best guarantee of achieving cryopreservation [52] and is being sought by most investigators multiplying and refining the 'recipes' based on the physics of the liquid-to-solid transition of aqueous systems.

Hydrophilic polymers of high molecular weight, such as polyvinylpyrrolidone (PVP) and hydroxyethylstarch (HES), give rise to the vitrification of water at high concentrations [53–56] and long-chain hydrophilic molecules at relatively low concentrations facilitate vitrification by cryoprotectants [56–59]. This result suggests that biopolymer networks of cellular structures and extracellular matrices might form submicroscopic amorphous phases in the presence of solvents. Some biopolymer networks could on their own form liquids of very high viscosity, and therefore might vitrify spontaneously after accurate treatment.

On the one hand, such an increase in viscosity followed by vitrification prohibits any damaging water motion. On the other hand, the absence of latent heat of melting might prevent harmful thermal events upon thawing.

3.3. Gel approach

The intricate meshwork of interacting proteins and polysaccharides in the extracellular space of tissues, as well as in regions of cells (ectoplasm and endoplasm), is known to form 'jelly-like' phases, and most macromolecular components extracted from these matrices and cells may gelate in the test tube under precise conditions [56]. Consequently, the liquid-to-solid transitions observed, sometimes by gentle cooling, are usually termed sol-to-gel transitions. Such gelated networks could confine water and thus prevent osmotic outflows and stresses over a range of cryogenic temperatures, while tied-up water would prevent their structure from collapsing into a compact mass. If this assumption is correct, specific physical/chemical properties can be expected which could be of extreme importance for the cryobehavior of cells and tissues. In particular, ice formation in the body fluids (extracellular matrices), or even in suspension media, might be without major consequences since a noticeable part of cellular water would remain osmotically inactive. This could be one of the 'secrets' of spontaneously freeze-tolerant organisms, and one way to obtain cryopreservation of cryosensitive cells and tissues.

Experimental distinction between highly viscous solutions and gels involving hydrophilic biopolymers is not an easy matter, but it could lead to a better understanding of the cryobehavior of cells and extracellular matrices.

4. Gels

4.1. Physical gels

Physical gelation is a complex process which has been extensively studied in recent years on a macroscopic scale [60–67]. Physical gelation is thermoreversible and requires some mechanism of cross-linking in order to form a three-dimensional network. One possibility is the intertwining of two or more strands of helical structures. Another is the formation of microcrystallites which are incapable of excessive growth. A third involves the association of like segments of a copolymer dissolved in a solvent that is good for one segment and poor for the other.

Recent works carried out on isotactic polystyrene have shown its mechanism of gelation. When the system is cooled below its θ temperature, a phase separation occurs, which forms regions of high or low polymer concentration. The strands in the polymer-rich phases then become organized into microcrystallites. An additional phase separation in the diluted phase can take place in some solvents, which manifests itself by

the appearance of turbidity. In other solvents, the appearance of turbidity is related to different or at least accompanying phenomena; conformational change of the polymer strands from the extended helix present in the gels in the first place to a folded helix, or growth of chain-folded microcrystals. The latter phenomena are irreversible until the melting temperature of the folded helices has been reached, while the first one is fully reversible and reproducible. For agarose gels formed in water, gelation was also attributed to a phase separation prior to crystallization of polymer strands in the concentrated regions [61–64]. Gelation of many natural polymers could thus possibly arise from a quite general mechanism.

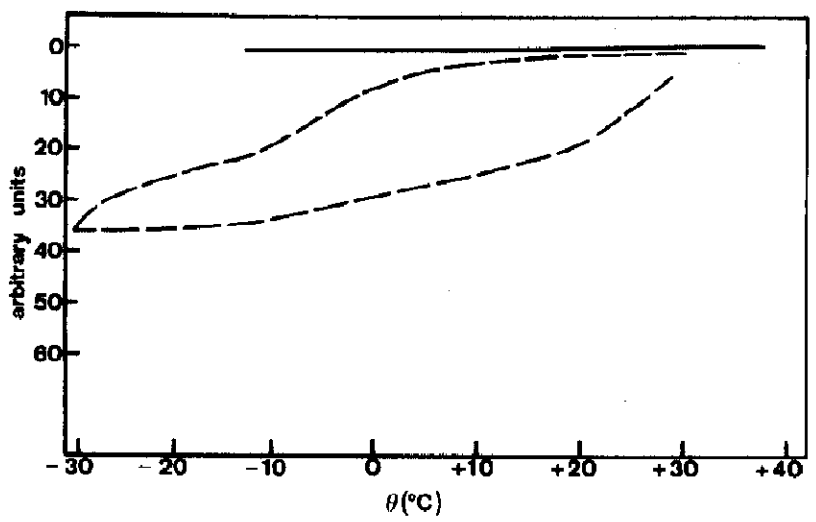

Fig. 1. Light scattering intensity evolution during thermal cycles (≈15 °C/min) for 0.1% (w/v) collagen solutions in 0.1 M Tris-HCl buffer (pH 7.2) alone (———) or containing 30% methanol (— — —).

4.2. *Biopolymer gels*

Physical gelation is a common phenomenon in biopolymers. The gels exhibit sharply defined melting behavior. Upon heating, the gel suddenly becomes a liquid, and such a solid-to-liquid transition is perfectly reversible on cooling with a temperature hysteresis. The gelation and melting temperatures can be measured by a ball-drop method [68] and through differential scanning calorimetry. Gels are usually soft, resilient and clear just below the gelation temperature, but can become turbid upon further cooling.

Agarose and collagen, accepted as being typical of extracellular polysaccharides and proteins, have been investigated in aqueous and mixed solvents through thermal cycles ranging between normal, subzero and cryogenic temperatures. Using light scattering [69], the slope, magnitude, and hysteresis of clear-to-turbid transitions have been recorded in different solvents at various rates of cooling and warming.

Dilute aqueous solutions of thermally denatured collagen (0.1%, w/v) show a constant light scattering intensity over the range of ambient and subzero temperatures studied (fig. 1). More concentrated solutions (1–3% collagen) in the presence of 10–30% ethylene glycol or glycerol show supercooling over a broad range of subzero temperatures without any clear-to-turbid transition.

These results can be compared to previous observations indicating that relatively low concentrations of synthetic or natural macromolecules amplify the supercooling due to cryoprotectants [56–59]. The viscosity plays an important role in such supercooling, which may lead to vitrification. From another standpoint, the solvent may be confined within the pores of a three-dimensional network formed by the polymer strands. Anyway, denatured collagen in polyol/water mixtures might be used as a suspension medium favoring either the formation of a glass or the trapping of the solvating medium, any of which would prevent the massive outflow of cellular water during freezing of extracellular matrices.

From our present point of view, it is certain that native collagen fibers, which are known to resist stretching when considered in the extracellular matrix, should not play a significant role in the fate of water during freeze-thaw operations. Only denatured collagen could constitute a three-dimensional protein network and be effective.

Dilute aqueous solutions of agarose (0.1%, w/v) present a significant clear-to-turbid transition at temperatures depending on the cooling rate (between 12 and 20 °C at about 15 °C/min, between 30 and 35 °C at 0.1 °C/min). The reverse turbid-to-clear transition occurs with a hysteresis of at least 50 °C (fig. 2).

Agarose solutions in polyol/water mixtures also demonstrate reversible clear-to-turbid transitions in spite of their high viscosity, a result which, compared to that obtained with collagen, clearly

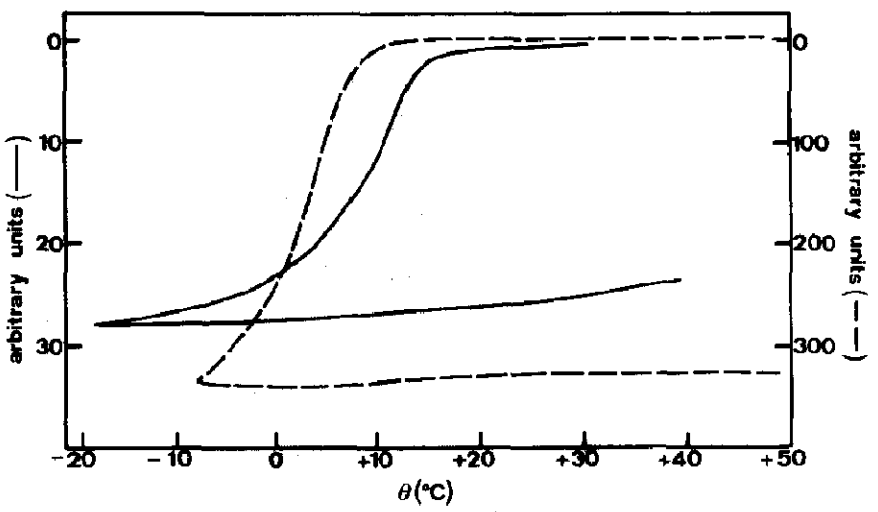

Fig. 2. Light scattering intensity evolution during thermal cycles (≈15 °C/min) for 0.5% (w/v) agarose solutions in 0.1 M phosphate buffer (pH 7.0) alone (———) or containing 30% methanol (— — —).

indicates the lability of the agarose networks.

In the presence of methanol (30%, v/v) both agarose and denatured collagen present reversible and reproducible clear-to-turbid transitions, a surprising result for collagen (figs. 1 and 2). The moderate viscosity of these methanol/water mixtures compared to pure water may be partly involved in the behavior of collagen. The intensity of the transition as well as the extent of hysteresis during thermal cycles (about 50 °C) suggests that the initially random network of flexible agarose chains cross-linked by noncovalent bonds may undergo significant phase separation upon cooling. Regions of low or high polymer density would result from such phase separations and be responsible for turbidity, while the pore size of the network would increase dramatically. These assumptions are a reminder of the large fluctuations in pore size of polyacrylamide gels (and more generally chemical gels) both upon cooling or addition of poor solvents to the solutions [70–73]. As for collagen, its significant clear-to-turbid transition could be related to a loss of solubility of the strands and formation of aggregates upon cooling.

The main results obtained on agarose solutions clearly suggest that polysaccharides, including those present in the ground substance of extracellular matrices, might undergo phase separations upon cooling. In addition, temperature and solvent composition are not the only parameters capable of inducing phase transitions. These can also be brought about at constant temperature and solvent composition by changes in pH or ionic strength altering the effective ionization of the polymer network [74]. Glycosaminoglycans and proteoglycans may also form a highly hydrated, jelly-like 'ground substance' capable of giving rise to phase transitions upon cooling and thus to large fluctuations in biopolymer density, with contracted and swollen regions. As a critical temperature is approached, these fluctuations may become infinitely slow and eventually remain fixed in a particular configuration before the freezing point. The coagulated and swollen regions would then represent the separated phases of the gel.

Although we are still far from a detailed understanding of the physical/chemical basis of such an imaginary picture, the properties of synthetic gels provide models for the essential elements of these phenomena.

5. Physical properties of the cytomatrix and cytoskeleton

Cryotolerance and cryopreservation could also be achieved at the intracellular level.

The cytoplasm of a cell is now described, organelles excepted, as an aqueous phase of polymers, small molecules and ions. In spite of its low viscosity, which is of the order of that of aqueous solutions of highly asymmetric proteins at comparable concentrations, the whole framework is highly organized, of good mechanical coherence and minimal rigidity (fig. 3). Under the action of specific agents, changes in the mechanical and contractile properties of the cytomatrix occur that can be regarded as sol ⇄ gel transitions. Thus, it has been shown for decades that changes in the cytoplasm or regions of the cytoplasm from a solution to a quasi-thyxotropic gel [75] and vice versa are essential for the cell's vital processes. Investigations carried out on the cytoplasm of certain types of cells, and of egg cells in particular, have demonstrated the complex behavior (solation, gelation) of the cytoplasmic substance which can be influenced by many factors, such as temperature [76–79].

The state of the cytoplasm is influenced by the

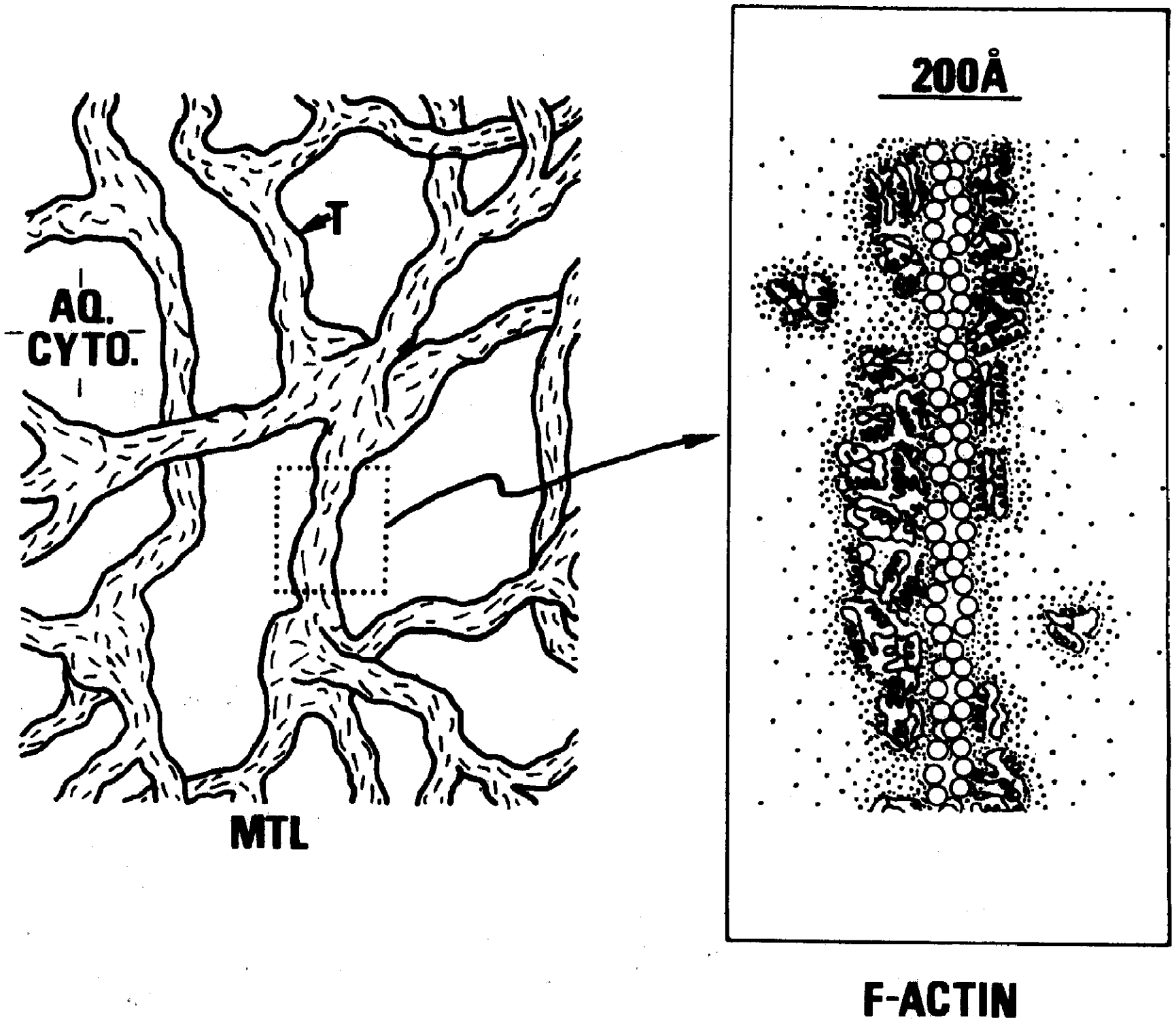

Fig. 3. The cytomatrix is a meshwork of actin filaments. Thus a microtrabecular lattice (MTL) is surrounded by aqueous cytoplasm (AQ. CYTO.) (left). One possible organization of a trabecula (T), postulated by Clegg [36], is shown on the right. It can be seen that a central helical F-actin filament is associated with various proteins, some tightly, some loosely bound (see text). Redrawn from ref. 36 by copyright permission of The Rockefeller University Press.

behavior of the cytoskeleton. Asymmetric biopolymers participating in the cytoskeleton (microfilaments, microtubules) can assemble or disperse and these dynamics play an important role in the cryobehavior of cells. There exists a dynamic equilibrium between the monomeric and polymeric forms of actin [80–87] and tubulin [88–93]. Actin filaments may undergo structural transformations to form bundles or jelly-like networks as a result of the effect of associated proteins [94–100]. Actin bundles may serve the same purpose as the microcrystallites found in physical gels. Any parameter able to influence the conformational equilibrium of the cytoskeleton may be of importance in the physiological properties and cryobehavior of the cell. For instance, it has been reported that microtubules are cold sensitive [101–105] and that amoebae which contain a greater proportion of monomeric actin are more tolerant to freezing [106].

Thus, a biophysical/chemical approach was adopted. The above processes as well as gel ⇄ sol transitions may occur in situ as part of the usual chain of events in the cell. They might involve changes in the cytomatrix surface, and in the amount of water that has 'bulk-like' properties [33]. Up to now, cryopreservation has been mostly considered via a physical approach involving water as a solvent rather than as a versatile component of biological systems. Our approach is based on this last consideration and on the fact that, due to the high level of organization and degree of hydration of biological macromolecules in most living systems, the physical state of soft living tissues is intermediate between solid (macromolecules) and liquid (solvation). These two components present a complex interplay which is still poorly understood and which is probably the key factor involved in cryobehavior.

Recent work in this laboratory has shown that treating early rabbit embryos with propanediol determines the depolymerization of cortical actin filaments [107], as can be visualized with the aid of fluorescence microscopy (fig. 4). Direct evidence of this depolymerization has been obtained in vitro. Actin depolymerization might be one of the reasons for the efficiency of the cryoprotection offered by propanediol for early stage rabbit embryos.

6. Conclusion

Thus, much remains to be done, both in situ and in vitro to answer such essential questions as a possible correlation between the behavior of the cytoskeleton and the fate of frozen cells. However, present highly preliminary observations clearly suggest that gelated biopolymer networks 'frozen in' at low temperature may be not beneficial to cryopreservation. Disassembly and depolymerization prior to or during cooling may improve cryotolerance, both situations being closely related to the fate of intracellular water as a versatile component of systems, and possibly involving a cascade of physical and chemical events which are currently under investigation.

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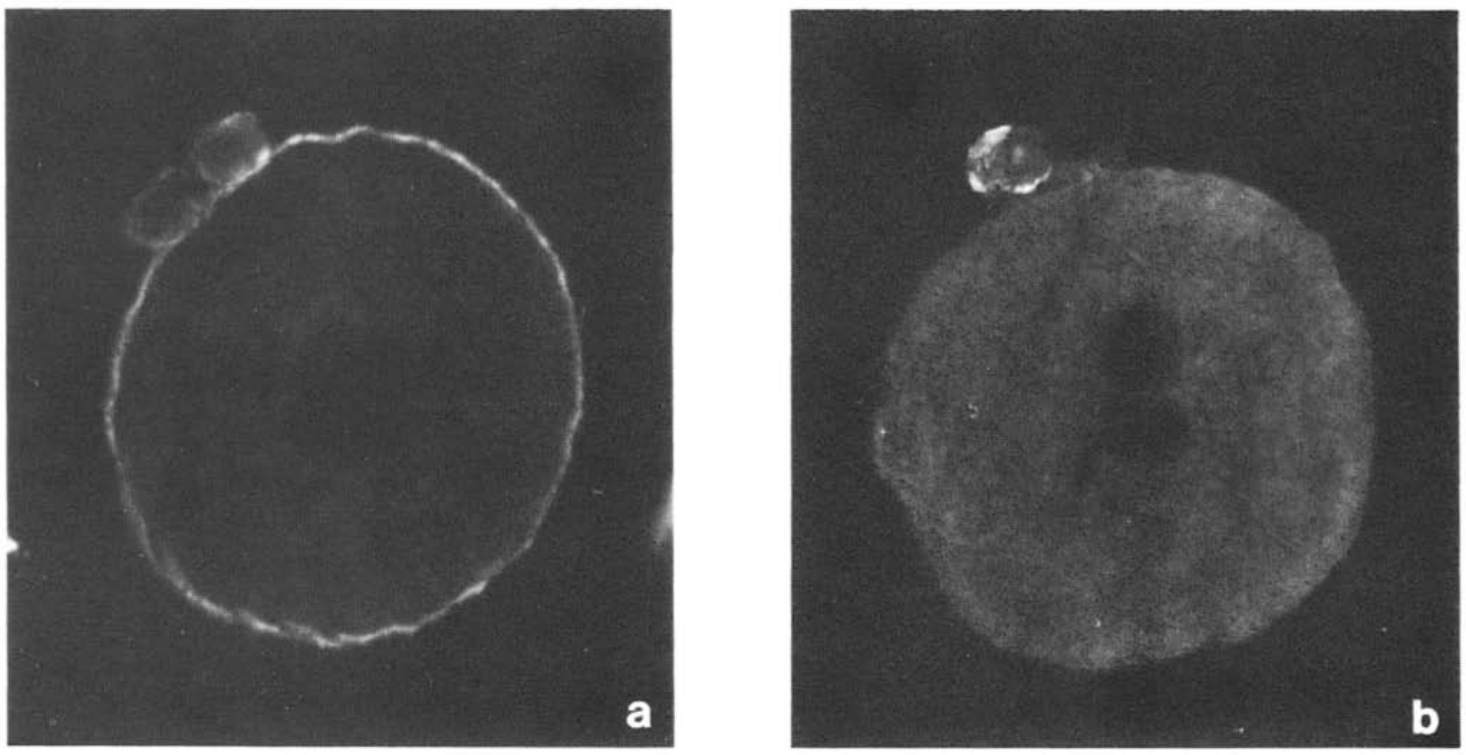

Fig. 4. Fluorescence microscopy of embryo cryosections 10 μm thick stained with NBD-phallacidin to reveal the polymerized actin: control embryo (a), and embryo exposed to propanediol (30 min in 1.5 M cryoprotectant, then 5 min in 2 M cryoprotectant with 0.5 M sucrose) (b).

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