STUDIES ON THE PHYSICAL STATE OF WATER IN LIVING CELLS AND MODEL SYSTEMS. IV. FREEZING AND THAWING POINT DEPRESSION OF WATER BY GELATIN, OXYGEN-CONTAINING POLYMERS AND UREA-DENATURED PROTEINS.

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Using a differential scanning calorimeter, we studied the freezing and thawing behavior of solutions of six globular proteins (hemoglobin, bovine serum albumin, y-globulin, \(\beta\)-lactoglobulin, egg albumin, and protamine sulfate); gelatin; and three synthetic polymers (polyvinylpyrrolidone (PVP), polyvinylmethylether (PVME), and poly(ethylene oxide) (PEO)). The native globular proteins in concentrations up to 50% produced no major change of the freezing temperature of the bulk phase water, or of the shape of the freezing peaks. In contrast, the synthetic polymers caused a lowering of the freezing temperature and a widening of the freezing peaks; the peaks disappeared at the highest macromolecular concentration and exothermic peaks appeared during subsequent warming (warming exothermic peak or WEX). Gelatin behaved like the three polymers and so did the globular proteins after denaturation with urea but not after denaturation with sodium dodecyl sulfate (SDS). These different patterns of freezing and thawing of solutions of native globular proteins and of SDS-denatured globular proteins, on the one hand, and of gelatin, PVP, PVME, PEO, and urea-denatured globular proteins, on the other, parallels perfectly the different abilities of these groups of substances to reduce the solvency of the water for solutes, reported earlier. The major new conclusion from this study is that the presence of macromolecules to a concentration as high as 50% does not necessarily inhibit or even delay to any appreciable extent the freezing of the bulk phase water. On the other hand, inhibition of ice-formation does occur in the presence of macromolecules (e.g., gelatin, PVP) that cause multilayer polarization of the bulk phase water. The findings allow new evidence to be derived that the bulk of water in living cells also exists in the state of polarized multilayers.

INTRODUCTION

The physical state of water in living cells has been the center of scientific dispute for a long time.\(^{1-4}\) According to the classical membrane-pump theory, the cell interior has essentially the properties of a dilute salt solution; the bulk of cell water is free.\(^{5-7}\) According to the association-induction (AI) hypothesis the cell represents an assembly of a cooperatively and closely associated system of proteins, water, and ions maintained at a high energy state; the bulk of cell water is adsorbed in multilayers.\(^{8,9,10}\) In the AI hypothesis, a matrix of more or less parallel chains containing alternating electronegative (N) and electropositive (P) sites with distances between nearest neighboring sites roughly equal to that of one water molecule is called an NP-NP-NP system. (Variants of this system may contain only N or P sites at distances equal to two water diameters and are called, respectively, NO-NO-NO systems or PO-PO-PO systems.) It was postulated that an NP-NP-NP system of extended matrix protein chains polarizes in multilayers virtually all the water in resting cells, and that water thus affected has reduced
solubility for Na⁺ salts, sugars, free amino acids, and other large and complex molecules found usually in low concentrations in living cells. Recent model studies have confirmed several basic predictions of this theory. Thus in in vitro experimental studies, proteins which for structural (e.g., gelatin) or other reasons (e.g., exposure to urea) exist in an extended form do indeed reduce the solubility of the water for Na⁺, sucrose, and glycine. Proteins which exist in native α-helical forms with NH-CO groups locked in intra- or intermacromolecular H bonds show little effect on water solvency. An important discovery from these early investigations was that certain linear oxygen polymers like poly(ethylene oxide) (PEO), polyvinylpyrrolidone (PVP), and polyvinylmethyl ether (PVME) satisfy the criterion of NO-NO-NO systems and are also quite effective in reducing water solvency for Na⁺, etc. much as gelatin and urea-denatured proteins do. Water in these model systems show other properties different from normal liquid water, including adsorption characteristics, NMR relaxation behavior, and osmotic activity. The association-induction hypothesis also suggests that the low temperature properties of multilayer water should be different from the normal water. This paper presents a study of the freezing and thawing properties of water in synthetic polymer solutions and in aqueous solutions of a number of native and denatured proteins by differential scanning calorimetry.

MATERIAL AND METHODS

Material. The following polymers were used: polyvinylpyrrolidone (PVP-360, 94C-0049) from Sigma Chemical Co.; polyvinylmethyl ether (Gantrez M154 PVME) from GAF Corp.; poly(ethylene oxide) (Polyox WSR-205 and Polyox WSR-N-750) from

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FIGURE 1. Cooling thermograms of various concentrations of native bovine hemoglobin solutions without AgI crystals.

FIGURE 2. Cooling thermograms of various concentrations of native bovine hemoglobin solution in the presence of seeding crystals, AgI.
UNION CARBIDE. Approximate mol. wt. of Polyox WSR-205 is 600,000; that of Polyox WSR-N-750 is 300,000. Gelatin was obtained from Eastman (Lot B4B, 1EP 47). The following proteins were purchased from Sigma Chemical Co.: bovine serum albumin (BSA) (A4378, Lot 70F-9380); hemoglobin (H2500, Lot 98C-8040; H2625, Lot 112F-9300); β-lactoglobulin (L6879, Lot 11F-8025); α-chymotrypsinogen (C4879, Lot 11F-8055); γ-globulin (G5009, Lot 100F-9390); pepsin (P7012, Lot 60F-8057); protamine sulfate (P4505, Lot 91C-0077). Egg albumin was obtained from Nutritional Biochemicals Corp. Urea was obtained from J. T. Baker Chemical Co. (5-4204). Sodium dodecyl sulfate (SDS) was from Fisher Scientific Corp.

Preparation of Samples. The synthetic polymers and natural proteins were dissolved in distilled water at concentrations varying from 5% to 70% (weight/volume). The solutions were shaken in a constant temperature room (25°C) for about 3 days until fully dissolved and homogeneous. Denatured proteins were prepared by dissolving dry proteins in aqueous solutions of 3, 5, 7, and 9M urea or of 0.05, 0.1, and 0.3 M sodium dodecyl sulfate (SDS). The final protein concentration in the denatured protein was held as a rule at 28.5%. Samples weighing between 5 to 30 mg were placed in tared Perkin-Elmer sample pans (Type 219-0062) for volatile solvents, which were then hermetically sealed with the aid of a special sealer provided by the Perkin Elmer Company. Silver iodide crystals were prepared in the dark from reagent grade AgNO3 and NaI and dried.

Recording thermograms and measuring the temperature and enthalpy of fusion. The calorimetric measurements were made with a Perkin-Elmer DSC-2 differential scanning calorimeter. Temperature as low as 223°C was achieved by immersing the cooling block in mixtures of dry ice and 95% ethanol. To produce still lower temperature (i.e., 123°C) liquid nitrogen was used. As a rule, the sample was cooled or heated to the desired temperature at a cooling rate of 10°C/min controlled by a temperature programmer unless otherwise indicated. The thermogram was recorded in both cases. Nitrogen was used as a purge gas.

The intersection of the slope of the leading edge of the thermogram with the scanning base line was used as an indication of melting point. The area of the thermogram was integrated with a planimeter to calculate the phase-transition enthalpy. Water and indium were used for calibrating the temperature and enthalpy.

RESULTS

Figure 1 shows the cooling thermograms of solutions of bovine hemoglobin. The temperature at which freezing occurred was not materially changed when the hemoglobin concentration increased from 5% to 50%. All samples froze at a temperature some 13 degrees below 0°C. Even lower freezing temperatures were obtained in other human hemoglobin preparations. Inclusion of seed crystals of silver iodide, well known for its power to induce ice crystal formation, raised the freezing temperature of the hemo-
TABLE I. The freezing temperature of several native globular proteins in the presence of AgI crystals.

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>Freezing Temperature (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bovine hemoglobin</td>
<td></td>
</tr>
<tr>
<td>5%</td>
<td>265.8</td>
</tr>
<tr>
<td>50%</td>
<td>265.5</td>
</tr>
<tr>
<td>BSA</td>
<td></td>
</tr>
<tr>
<td>5%</td>
<td>265.7</td>
</tr>
<tr>
<td>50%</td>
<td>265.7</td>
</tr>
<tr>
<td>γ-globulin</td>
<td></td>
</tr>
<tr>
<td>5%</td>
<td>266.8</td>
</tr>
<tr>
<td>50%</td>
<td>266.0</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td></td>
</tr>
<tr>
<td>5%</td>
<td>265.5</td>
</tr>
<tr>
<td>50%</td>
<td>265.0</td>
</tr>
<tr>
<td>egg albumin</td>
<td></td>
</tr>
<tr>
<td>5%</td>
<td>266.0</td>
</tr>
<tr>
<td>50%</td>
<td>265.8</td>
</tr>
<tr>
<td>protamine sulfate</td>
<td></td>
</tr>
<tr>
<td>5%</td>
<td>262.2</td>
</tr>
<tr>
<td>50%</td>
<td>262.8</td>
</tr>
</tbody>
</table>

globin solutions by several degrees. As in other cases without the AgI crystals, no or very little change in the freezing temperature could be detected accompanying a large increase of hemoglobin concentration (Figures 2, 3). Table I shows that the freezing behavior of hemoglobin is shared by other native globular proteins studied. Like native bovine hemoglobin 5% and 50% solutions of BSA, γ-globulin, egg albumin, and β-lactoglobulin in the presence of AgI crystals have similar freezing temperatures. (The lower freezing temperature of protamine sulfate was most likely due to the high concentration of SO\(_4\)\(^{2-}\) present.) The fact that the freezing temperature of water with AgI (Table I) is still lower than its melting temperature (Figure 3B) indicates that AgI did not completely overcome the supercooling effect.

The cooling thermograms of solutions of the polymer PVME (Figure 4) are quite different from those of solutions of native globular proteins (Figures 1, 2). At 30% or lower concentration of polyvinylmethylether (PVME) there was a conspicuous exothermic peak but neither the width of the peak nor the freezing-temperature remained the same with changing polymer concentration. Rather, the peak widened and moved to lower and lower temperatures as the concentration of the polymer increased. From 40% to still higher concentrations of PVME, the sharp peaks disappeared altogether. Only very weak humps are seen; the total heat given off at these humps are much less than at the lower concentrations as a visual inspection of the total areas of the peak clearly shows. At 65%, there was virtually no observable exothermic reaction at all. The cooling thermograms of two other polymers studied,

FIGURE 4. Cooling thermograrns of various concentrations of PVME solutions without AgI crystals.
PVP-360 and PEO (Polyox WSR-205) were quite similar (Figure 5). However, another PEO (Polyox WSR-N-750) showed a somewhat different behavior (Figure 6). Here even at the concentration of 69.4%, an exothermic hump remained visible. Otherwise, the behaviors were quite similar to those of PVME, PVP-360, and PEO WSR-205.

Figures 7 and 8 show the cooling thermograms of varying concentrations of PVME and PEO-205 in the presence of AgI crystals. Comparing Figure 7 with Figure 4 and Figure 8 with Figure 5, one notices that the seeding AgI crystals have caused the bulk of the large peaks (at lower polymer concentrations) to move to higher temperatures, indicating once more the effectiveness of AgI in countering the supercooling effect. However, the up-

temperature migrations of part of the freezing water did not involve all the water present. It left behind another peak, the freezing temperature of which remained more or less the same as before AgI addition. Clearly multilayer polarization of water by PVME and PEO, as evidenced, for example, by their solute exclusion properties,\textsuperscript{13} enhanced the supercooling of the water to a temperature far below the lower limit observed in normal liquid water (i.e.,$-40^\circ$C)\textsuperscript{19,20} and the AgI was effective in overcoming some of this enhanced supercooling effect though not all of it.

Figure 9 shows a thawing thermogram of solutions of hemoglobin. Thawing thermograms as a rule are more easily reproducible. These thermograms are counterparts of the cooling thermogram of hemoglobin solutions and exhibit very similar characteristics: uniform peak width and nearly the same peak temperature at all concentrations studied (5% to 50%). But the melting temperature is uniformly $15^\circ$C above the freezing temperature.
Figure 10 is a thawing thermogram of solutions of bovine serum albumin. Except for a shift of about 3°C of the peak temperature toward a lower temperature at the highest concentration and a somewhat wider peak width, the thermograms are very similar to these of hemoglobin. Indeed Figures 9 and 10 are typical examples of the thawing thermograms of all eight globular proteins studied.

Figure 11 displays the thawing DSC thermograms of PVME solutions. It is evident that the thawing process of polymer solutions is considerably different from that of solutions of globular proteins. The samples were cooled from room temperature to 223°C at the cooling rate of 10°C/min. When the temperature equilibrium was reached, an upward heating scan was started at the rate of 10°C/min. The endothermic peak seen represents the heat for melting. With the increase of the polymer concentration, the melting point decreased to lower and lower temperatures. However, when the concentration reached 40%, an unusual phenomenon occurred. Here, an exothermic peak made its appearance during heating in the range of temperature from 235°C to 255°C. This exothermic peak was followed by a broad endothermic peak at a higher temperature. With increase of PVME concentration to 55% the exothermic peak was diminished in size and occurred at a higher temperature. Both the warming exothermic peak and the warming endothermic peak disappeared at 65% concentration. We named this exothermic peak “warming exothermic peak” and an acronym, WEX. This WEX phenomenon will be discussed at length in a following paper. A similar phenomenon was observed by Luyet, Rasmussen, and Kroener in aqueous solutions of glycerol and ethylene glycol, and by Luyet and Rasmussen in solutions of PVP.

**FIGURE 7.** Cooling thermograms of various concentrations of PVME with AgI crystals.

**FIGURE 8.** Cooling thermograms of various concentrations of PEO WSR-205 with AgI crystals.
So far we have seen in both cooling and thawing processes, the behavior of (native) protein solutions profoundly differs from that of PEO, PVP, and PVME. This difference parallels the separation of their solvency-reduction effects. The next question is, "What would the thermal properties be like in the case of gelatin, which in solvency-reducing properties resembles PVP, PEO, and PVME?"

What we found was that gelatin resembles decisively the polymers rather than the (native) proteins both in its cooling thermogram (Figure 12) and in its thawing thermogram (Figure 13). Thus virtually no exothermic responses occurred in 51.0% gelatin as shown in the top curve in Figure 12. There was a conspicuous shift of the freezing temperature toward lower temperature with increasing gelatin concentration, accompanied by a somewhat irregular but nevertheless observable widening of peak width. In thawing, the gelatin solution at 47% also showed a weak WEX. With further increase of gelatin concentration to 51%, the WEX became more pronounced though still less prominent than the WEXs of synthetic polymer solutions.

Figure 14 shows the cooling thermograms of different concentrations of gelatin in the presence of AgI crystals. Note that as in the case of PEO (WSR-205) (Figure 8), the seeding crystals separated the freezing peaks at the lower gelatin concentration into two peaks, one remaining more or less as before, and the other moving to a higher temperature. Since the larger and sharper peak remained at a substantially lower temperature, one suspects that the gelatin-dominated water had not only a reduced freezing temperature, but effectively blocked the propagation of ice (I) formation in the supercooled water.

Ling et al have shown that in so far as the water-solvency-reducing effect is concerned, the inactive native globular proteins can be made active by exposure to denaturants like
FIGURE 11. Warming thermograms of various concentrations of PVME.

FIGURE 12. Cooling thermograms of various concentrations of gelatin solutions without Ag\text{I} crystals.

FIGURE 13. Warming thermograms of various concentrations of gelatin solution without Ag\text{I} crystals.

FIGURE 14. Cooling thermograms of various concentrations of gelatin solutions with Ag\text{I} crystals.
FIGURE 15. Cooling thermograms of urea-denatured bovine hemoglobin 9M, 3M, etc., refer to urea concentrations without AgI.

FIGURE 16. Cooling thermograms of different concentrations of pure urea solutions without AgI.

FIGURE 17. Cooling thermograms of various concentrations of bovine hemoglobin in 9M urea without AgI crystals.

FIGURE 18. Cooling thermograms of various concentrations of bovine hemoglobin in 9M urea with AgI crystals.
urea that liberate the NHCO groups from their alpha-helical and other inter- and intra-macromolecular H-bonds and expose these NHCO groups directly to the bulk phase water. We next seek to find out if exposure to urea will change the thermal behavior of native globular proteins as well.

Figure 15 shows the effect of exposure of a 28.5% bovine hemoglobin solution to increasing concentrations of urea on the cooling thermogram. Note that as the urea concentration increased, there was a gradual decrease of the freezing temperature and a progressive widening of the peak width. Thus urea has changed the cooling thermogram of a 28.5% BSA from the type typical of solutions of native globular proteins (Figures 1 to 3) to one more like 30% PVME (Figure 4). However, increasing concentrations of urea itself also caused a progressive lowering of freezing temperature (Figure 16).

Figure 17 shows that increase of hemoglobin concentration in the presence of a constant concentration of urea (9 M) produced concentration-dependent shifts of freezing temperature and that AgI crystals added to the lower concentration of urea-denatured hemoglobin caused a part of the freezing water to move to a higher temperature (Figure 18), as seen in the PVME, PEO (Figures 7 and 8) and gelatin solutions (Figure 14).

It is known that denaturants like sodium dodecyl sulfate (SDS) disrupt only the tertiary structure and do not unravel the secondary structure of proteins. According to the A1 hypothesis only proteins with the backbone NHCO groups directly exposed to the bulk phase water have the water solvency reducing effect. SDS, confirming the prediction of the A1 hypothesis, does not increase the solvency-reducing effect while urea does."

**FIGURE 19.** Thawing thermograms of 28.5% BSA solution denatured with 0.05 M, 0.1 M, and 0.3 M SDS.

**FIGURE 20.** Depression of melting point of polymer solutions in relation to their concentrations.

- Hemoglobin
- BSA
- Gelatin
- PVP-360
- PVME-MIS4
- PEO WSR-205
Figure 19 shows the thermograms of 28.5% BSA solution dissolved in 0.05 M, 0.1 M, and 0.3 M sodium dodecyl sulfate (SDS) separately. The thermogram did not show pronounced changes, nor was the WEX phenomenon observable. Essentially the same results were obtained for all the other seven globular proteins treated with SDS.

Figure 20 summarizes the melting-point depression of various solutions. The two native globular proteins, hemoglobin and BSA, show a very small change of the melting temperature ($T_M$) over the entire concentration range studied. In contrast, gelatin, which depresses the freezing point more markedly, shows a more pronounced melting point depression. However, it is the three polymers, PVP, PVME, and PEO-205, that exert the most pronounced effect on the melting point of water.

As mentioned earlier, not all water in a protein or polymer solution freezes at 223°K. One way of measuring the non-freezing water is by measuring the total amount of heat observed during thawing. With the aid of a planimeter the total area of the endothermic melting peak was measured. The integral enthalpy of each sample thus obtained was plotted against its water content in grams per gram solid. The non-freezable water was obtained by extrapolating the curve to zero enthalpy. Table II gives the data so obtained. To be noted is that the amount of non-freezing water found in bovine serum albumin agrees with values cited in the literature. Much larger quantities of non-freezing water are found in gelatin, also in agreement with values obtained by earlier workers. Why should there be less non-freezing water in PEO than in gelatin will be discussed in a following paper.

DISCUSSION

In this paper we described three notable phenomena. They are: freezing and thawing point depression; the widening and disappearance of freezing and thawing peaks; and the warming exothermic peak (WEX). In each case, the effects of the macromolecules studied parallel their effect on the solvency of water previously reported by Ling et al. All the native globular proteins and the SDS-denatured globular proteins studied which are known to have no effect on the solvency of the water in these solutions did not produce a concentration-dependent progressive freezing and thawing point depression, did not materially widen the freezing and thawing peaks, nor did they show the WEX phenomenon. In contrast, gelatin, urea-denatured globular proteins, and all the synthetic polymers studied (e.g., PVP, PEO, and PVME) depress the freezing and thawing points, cause a widening of the freezing and thawing peaks, and, at very high concentrations, cause the disappearance of the freezing and thawing peaks. Moreover, all of them cause a reduction of the solvency of the water for Na salts, free amino acid, and sucrose.

Table III summarizes these comparisons. In the present discussion we shall concentrate on the freezing and thawing point depression and the peak-width changes, leaving further discussion of the WEX phenomena for a following paper.

**The indifference of freezing and thawing of the bulk-phase water to the concentration of native globular proteins.** The near constancy of both the freezing (and thawing) temperature.

<table>
<thead>
<tr>
<th>TABLE II. Non-freezable water of protein and polymer solutions.</th>
<th>(g·H₂O/g·dry solid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>0.35</td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.47</td>
</tr>
<tr>
<td>PVP-360</td>
<td>0.85</td>
</tr>
<tr>
<td>PVME-M154</td>
<td>0.65</td>
</tr>
<tr>
<td>PEO WSR-205</td>
<td>0.35</td>
</tr>
</tbody>
</table>
tures and the widths of the freezing (and thawing) peaks in solutions of hemoglobin and other globular proteins varying in concentration from 5% to 50% is of critical significance. It shows clearly that in the phenomenon of the freezing-point depression in biological systems, the mere coexistence of water with a high concentration of macromolecules does not necessarily delay or otherwise interfere with ice formation and propagation in the bulk-phase water. Aside from a rather small but finite amount of non-freezing water (Table 11), the bulk of the water in the water in these systems all froze and thawed at more or less the same temperature and more or less the same rate. This conclusion is of considerable importance in reevaluating some past conclusions.

One may recall that in the 20's and early 30's, a considerable amount of evidence for the existence of bound water in cells was collected by Rubner, Jones and Gortner, and many others on the basis of the freezing-point depression of water in biological materials. The near total rejection of the then "bound water" concept was to a considerable extent based on the argument that all the observed freezing-point depression was an artifact due to the presence of proteins and other macromolecules which blocked ice-crystal formation and propagation, thereby preventing the reaching of equilibrium in ice formation (see 3). The present findings showed that as much as 50% native globular proteins had little if any effect on either the freezing or thawing of the bulk-phase water. Clearly, simple mechanical blockage cannot explain the freezing point depression of the bulk-phase water observed in biological materials.

The profound effect of gelatin, PEO, PVME, and urea-denatured proteins on freezing and thawing of water. Having eliminated simple mechanical blockage as the cause of freezing point depression of bulk-phase water, we ask the question, "What is then the true cause of the dramatically different behaviors of solutions of the protein gelatin and of polymers like PVME?" The new interpretation we offer can be readily derived from the polarized multilayer theory of cell water and its model systems.

<table>
<thead>
<tr>
<th>TABLE III. Comparison of freezing properties and solvency reduction of water in various protein and polymer solutions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native globular proteins</td>
</tr>
<tr>
<td>Gelatin</td>
</tr>
<tr>
<td>Denatured globular proteins</td>
</tr>
<tr>
<td>urea SDS</td>
</tr>
<tr>
<td>Synthetic polymers (PEO, PVME, PVP)</td>
</tr>
</tbody>
</table>
Water freezes at a low temperature because the average kinetic energy of the water molecules has fallen to the critical point so that they no longer can effectively combat the gain of enthalpy on the assumption of the ordered ice structure. As a result, it undergoes a cooperative transition to become ice. The reason that a fall of the average kinetic energy, $kT$, favors ice formation is because in the liquid state there are many more quantum mechanically allowed energy levels for each water molecule than for ice. A decrease in temperature and hence the average kinetic energy $kT$ (where $T$ is the adsorption temperature, $k$ the Boltzmann constant) partially nullifies this advantage of being in the liquid state. Now according to the polarized multilayer theory of cell water and of water in its model systems, the bulk of water in living cells and in the more concentrated solutions of PVME and other models, exist in the state of polarized multilayers. That is to say, as a result of the propagated electrical polarization emanating from the charged sites (NHCO groups in certain proteins and O in PVME), the average water molecule in the state of polarized multilayers is held to its neighbors more tightly. The rotational as well as translational motional freedoms are thus more restricted than in normal liquid water. Consequently, the activity of water in the state of polarized multilayers is reduced. Now a quantitative measure of the activity of water is the partial vapor pressure. Ling\textsuperscript{17} has recently shown that indeed the vapor pressure of aqueous solutions of PEO, PVP, and gelatin are lowered by these polymers and proteins far beyond that predicted from their molar concentration.

FIGURE 21. Diagrammatic illustration of a possible reason for the freezing and thawing point depression of gelatin, polymers, and urea-denatured proteins.
As temperature decreases, both the vapor pressure of water and of ice decreases but per degree of temperature lowering, the vapor pressure of ice falls faster than that of liquid water. The freezing point is the temperature at which liquid water and ice have the same vapor pressure (see Glasstone\textsuperscript{36}). Therefore, PEO, PVP, and gelatin, which lower the vapor pressure according to their concentration, will also depress the freezing point (and thawing temperature) of water. This phenomenon is illustrated in the diagram shown as Figure 21.

**The relation between melting point depression and the solvency reduction.** In Figure 20 we have shown the relative effectiveness in depressing the melting point by the proteins and polymers. Note that at the same concentration of proteins or polymers, their effectiveness in depressing the melting point goes hand-in-hand with their abilities to reduce

FIGURE 23. Ice crystal formation in A. glycerol (6 M), B. sucrose (50%), C. 50% gelatin, and D. 35% bovine serum albumin (~2°C). Note hexagonal ice formation pattern in A, B, and D. Ice formation pattern became much distorted and blunted in C (from Luyet and Rapatz, 1956, by permission of Dr. Rapatz).
the solvency of the water for Na' salts: "PEO, PVME > PVP > Gelatin > BSA, Hb."

What do the present findings tell us about the physical state of water in living cells? Chambers and Hale\(^{31}\) long ago showed that if the cut end of a supercooled frog muscle cell was touched with an ice crystal, one or more thin long spikes with no branches would rapidly form and increase in length in the longitudinal direction. They also showed that the direction of the ice spike propagation followed the orientation of muscle protein filaments. Thus if the muscle fibers were twisted, the spikes also followed a twisted pattern. Chambers and Hale’s work was confirmed by Miller and Ling\(^{22}\) who further extended their earlier findings. Thus Miller and Ling showed that if the opposite end of a cut muscle fiber was first brought into contracture by local exposure to a Ringer solution containing 5 mM caffeine, the rapidly propagating ice spikes came to a halt. With time, the single spike grew in width (Figure 22) in a bulbous, irregular manner in the contracted region.

It was suggested that the long ice spikes first described by Chambers and Hale are in fact artifacts due to the local perturbing effect at the tip of the advancing ice spike which splits and disrupts water in the state of polarized multilayers much as in the splitting of bamboo poles. As the myofilaments are split apart by the tip of the advancing spike more normal liquid water is liberated from its polarized multilayered state and it freezes, adding further length to the spike. A persuasive evidence for this interpretation is another observation reported by Chambers and Hale: if the frozen muscles were thawed and the muscle cooled again to below freezing temperature, the ice spikes would reform following precisely the tracks of the previous ice spikes.

The most significant question is, "Why do these ice spikes in the frog muscle cells not branch as ice crystals invariably do in more normal environments?" The answer offered was that the "structured" multilayer polarization prevents water from forming the normal hexagonal ice. However it is the series of new calorimetric observations reported in this paper that offer definitive confirmation of this view, because what we have demonstrated here is that only in water polarized in multilayers (by the NP-NP-NP or NO-NO-NO systems, as in gelatin, urea-denatured protein, PVME, and PEO) is there a profound inhibition of the formation and propagation of normal ice at a specified sub-zero temperature. In aqueous solutions of native globular proteins ice forms at the regular freezing temperature and with branches (see below).

Normal ice forms six radiating arms; it has a hexagonal symmetry. Luyet and Rapatz\(^{33}\) showed that this hexagonal ice formation is characteristic not only of normal water but also of aqueous solutions of glycerol (6M), sucrose (50%), and of a 35% solution of the native globular protein, bovine serum albumin (Figure 23). Of a variety of solutions whose freezing properties they studied, only two were exceptional: aqueous solutions of PVP and gelatin. As discussed earlier, these two also showed pronounced freezing and thawing point depression effects. Furthermore, ice formed in PVP and gelatin solutions showed lobed, club-like shapes of uniform width ("irregular dentrites") which no longer exhibit the regular hexagonal structures (Figure 23). These irregular dentrites resemble the kind of ice formed in muscle cells after prolonged exposure to below freezing temperature (~3°C) as shown in Figure 22. The similarity between "irregular dentrites" seen in gelatin and PVP solutions on the one hand and those in frog muscle cells after prolonged cooling once more confirms the view that in these cells in the normal resting condition, and even during caffeine contracture, the bulk of the water exists in the state of polarized multilayers.
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