Physiological Chemistry and Physics and Medical NMR, 15 (1983)

STUDIES ON THE PHYSICAL STATE OF WATER IN LIVING CELLS AND MODEL SYSTEMS. V. THE WARMING EXOTHERMIC REACTION OF FROZEN AQUEOUS SOLUTION OF POLYVINYLPYRROLIDONE, POLY(ETHYLENE OXIDE), AND UREA-DENATURED PROTEINS

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● *The warming exothermic peaks (WEX) first described by Luyet and coworkers in solutions of ethylene glycol, glycerol, and polyvinylpyrrolidone (PVP) was interpreted on the basis of a devitrification phenomenon, in which rapid cooling converts normal water to amorphous ice, which devitrifies on warming to the state of normal Ice I. Our present studies of the WEX phenomenon in aqueous solutions of urea-denatured proteins, PVP, PVME, and two types of poly(ethylene oxide) led us to a different conclusion: WEX is the result of a transformation of chilled water in the state of polarized multilayers to Ice I. Among the evidence for this is the fact that WEX of the polymer-water system is indifferent to the rate of cooling between 320°/min. and 1.25°/min. and that polymer-water systems cooled to −70° C remain viscous liquids and not (solid) vitreous ice. The transformation from water dominated by PVP, PVME, and urea-denatured proteins to Ice I can occur rapidly during warming or more slowly when the chilled polymer-water systems are kept at 223° K. To effect this transformation the polymers themselves must undergo movements which tend to be retarded most by the presence of bulky side chains, as in urea-denatured proteins, and least in polymers with no side chains (e.g., Polyox WSR-N750).*

INTRODUCTION

In the preceding paper, Ling and Zhang (1983) have shown that gelatin, urea-denatured globular proteins, polyvinylpyrrolidone (PVP), poly(ethylene oxide) (PEO), and polyvinylmethylether (PVME), all of which produce solvency reduction of water (Ling et al., 1980a, b; Ling and Ochsenfeld, 1983), also cause freezing-point and thawing-point depression of the water, a progressive widening of the freezing peak and a warming exothermic peak during thawing. In contrast, globular proteins and globular proteins denatured by sodium dodecyl sulfate (SDS), none of which produce solvency reduction of water, have only minimal or no observable effects on freezing and thawing temperatures of bulk phase water, or their freezing peak widths, nor do they produce warming exothermic peaks.

Warming exothermic peaks, to be referred to as WEX, were reported by Luyet, Rasmussen, and Kroener in 1966 in aqueous solutions of glycerol and ethylene glycol (Luyet et al., 1966) and later by Luyet and Rasmussen (1967) in aqueous solutions of PVP. Luyet and coworkers referred to the underlying mechanism as "devitrification," a change from amorphous ice to crystalline ice. Later Cooks et al. (1975) reported a similar observation during the thawing of "frozen" ternary systems of H_2O-NaCl-glycerol and of H_2O-NaCl-dimethylsulfoxide (DMSO). All of these studies were at least partly motivated by an interest in the mechanism by which glycerol, ethylene glycol, PVP, and DMSO exercise their well-known cryoprotective effects.

In the present paper we shall present results of a more detailed study of the WEX phenomenon in protein-water and polymer-water systems, using the technique of differential scanning calorimetry.

MATERIALS AND METHODS

The materials used in this investigation and their sources are essentially the same as described in the preceding paper (Ling and Zhang, 1983). Polyvinylpyrrolidone (PVP-360) was from Sigma Chemical Co., polyvinylmethylether (PVME) from GAF Corp., poly(ethylene glycol) (Polyox WSR-205, M.W. 600,000, Polyox WSR-N750, M.W. 300,000) from Union Carbide; urea from J. T. Baker; gelatin from Eastman; hemoglobin (Lot 98C-8040), bovine serum albumin (Lot 70F-9380), protamine sulfate (Lot 91C-0077), and pepsin (Lot 60F-8057) were all from Sigma Chemical Co.

The calorimetric measurements were made on a Perkin-Elmer DSC-2 differential scanning calorimeter. Temperature as low as 223° K was obtained by immersing the cooling block in a mixture of dry ice and 95% ethanol. As a rule, samples were cooled or heated at the cooling or heating rate of 10°/min. unless otherwise stated. Water and indium were used for calibrating the temperature.

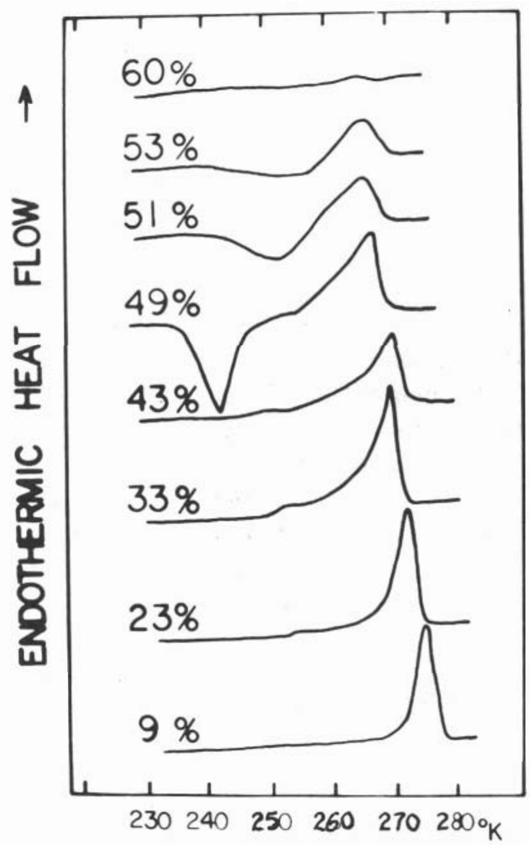

FIGURE 1. DSC thermograms of various concentrations of PVP-360 solutions during thawing.

RESULTS

Figure 1 shows the thawing DSC thermogram of polyvinylpyrrolidone (PVP) solution in concentrations ranging from 9% to 60%. The 51% PVP thermogram is virtually the same as that reported for 50% PVP by Luyet and Rasmussen (1967). The more complete

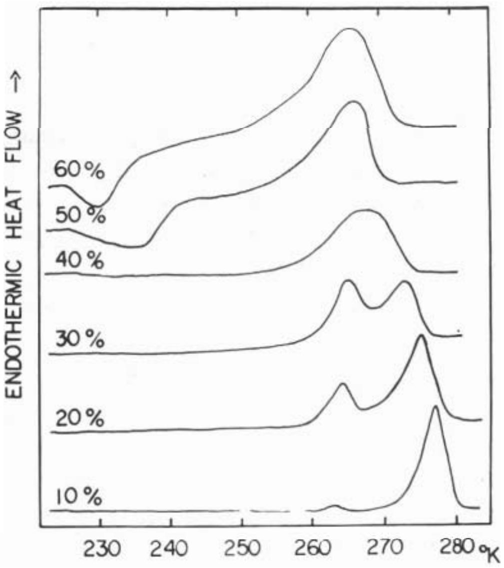

FIGURE 2. DSC thermograms of various concentrations of PEO (Polyox SWR-205) (MW 600,000) solutions during thawing.

data presented here covering a variety of PVP concentrations show variability of the thawing behavior with even minor changes of the polymer concentration. Note the absence of the warming exothermic peak (WEX) at 43% PVP concentration in contrast to the pronounced WEX in a solution only 6% stronger. From 49% to 51% there was another marked change in the size of the WEX and temperature at which it occurs. At 60%, neither WEX, nor an observable endothermic thawing peak could be seen. Note also that in concentrations at and below 43%, and before the onset of WEX, there were small endothermic peaks which moved to lower temperatures as the concentration of PVP increased.

Figures 2 and 3 are thawing DSC thermograms of two types of poly (ethylene oxide) (PEO). Only in solutions of Polyox WSR-205 was the WEX observed; in solutions of Polyox WSR-N750, no WEX was visible even at a PEO concentration of 69.4%. In the thawing scans of 9 or 10% solution of PEO there were secondary endothermic peaks at temperatures below those at which the main thawing peaks occurred. These secondary peaks resemble those seen in 23% and 33% PVP though they were prominent and grew more so until at still higher concentration, they merged with the main endothermic peak as the main endothermic peak moved progressively to lower temperatures.

The moderate decrease of the thawing temperature in solutions of increasing urea concentration (Figure 4) contrasts with much larger changes of their freezing temperatures (Ling and Zhang, 1983). Figures 5, 6, and 7 are the thawing thermograms of urea-treated hemoglobin (28.5%), bovine serum albumin (BSA) (28.5%), and protamine sulfate (28.5%) respectively. Exposure to 3 M urea produced a lowering of the melting temperature and a

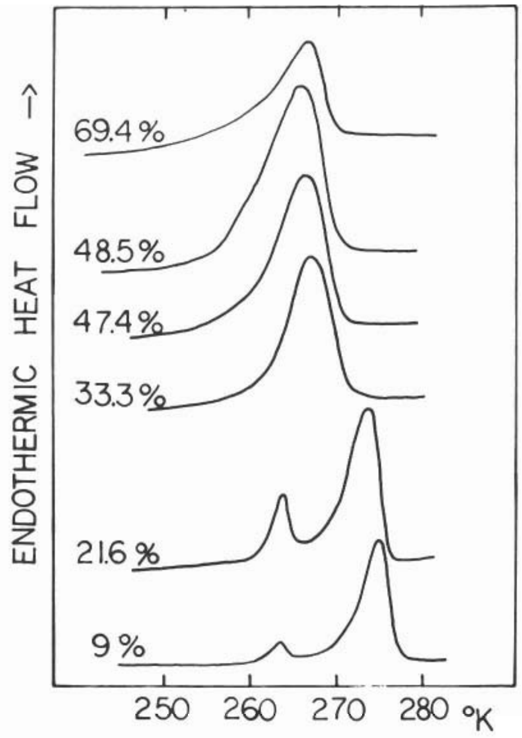

FIGURE 3. DSC thermograms of various concentrations of PEO (Polyox WSR-N750) (MW 300,000) solutions during thawing.

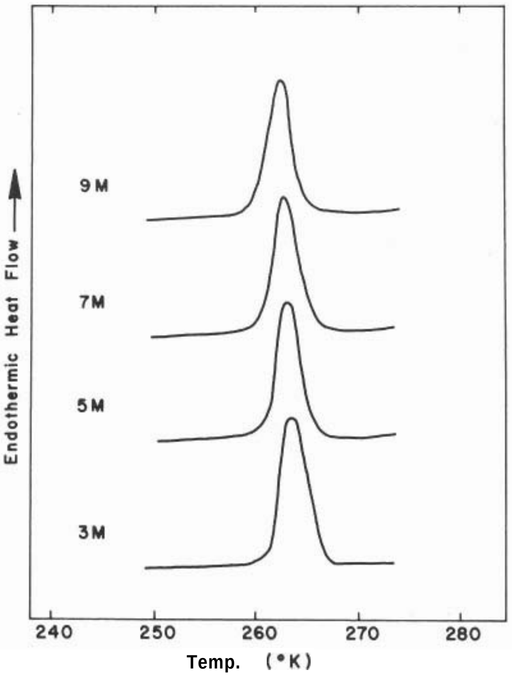

FIGURE 4. DSC thawing thermograms of solutions of urea of various concentrations.

broadening of the peak for all these proteins. A **WEX** appeared in 28.5% hemoglobin treated with 5 M urea; the **WEX** became sharper at higher urea concentrations (Figure 5). The appearance of **WEX** began at 7 M for BSA (Figure 6) and at 9 **M** for protamine sulfate (Figure 7). Thus exposure to 9 M urea, converted the globular proteins with sharp melting peaks to the complex pattern of exothermic peaks followed by the endothermic melting peak, very similar to that seen in 49% **PVP** and in 40% **PVME** described in the preceding paper (Ling and Zhang, 1983).

Figure 8 shows the effect of pepsin concentration on the **WEX** phenomenon. An aqueous solution of 28.5% pepsin and 9 M urea did not exhibit a **WEX;** the thawing peak was quite similar to that of 28.5% pepsin except its shift to a lower temperature presumably on account of activity of urea. It should be mentioned that resistance of pepsin to urea denaturation has long been known (Lineweaver and Schwimmer, 1941). However a 9 M urea solution containing 50% pepsin showed a fully developed **WEX.** Since 28.5% hemoglobin, 28.5% bovine serum albumin as well as 28.5% protamine sulfate all exhibited a **WEX** in the presence of 9 **M** urea, clearly the concentration of proteins needed in the presence of 9 **M** urea to create a **WEX** varies with the nature of the proteins.

The Cooling Rates and the Appearance of WEX. Luyet and Rasmussen (1967) believed that the **WEX** phenomenon represents a devitrification phenomenon. In this view, **WEX** can only occur if the water in an aqueous solution is caught in an amorphous state by very rapid cooling and becomes

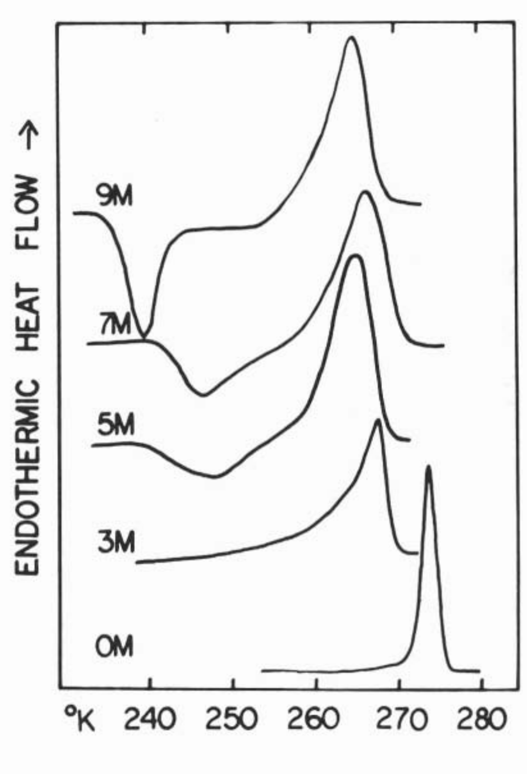

FIGURE 5. DSC thawing thermograms of urea-denatured hemoglobin (28.5%). Concentration of urea indicated in molarity.

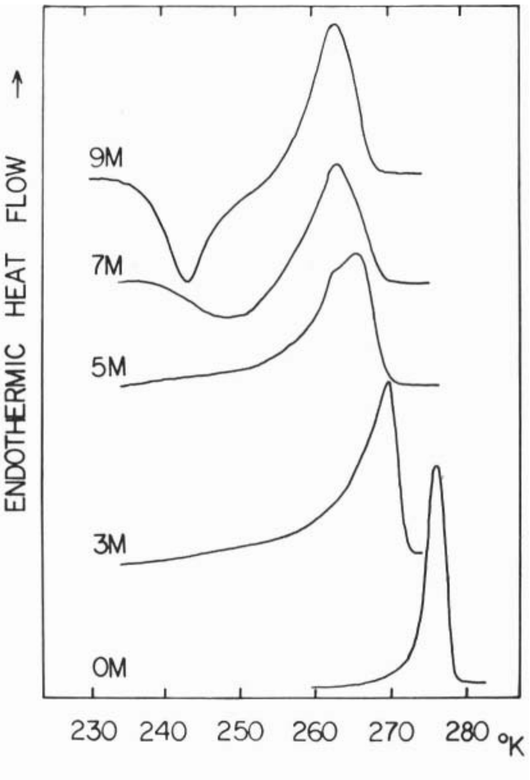

FIGURE 6. DSC thawing thermograms of urea-denatured BSA (28.5%). The concentration of urea indicated in molarity.

vitrified ice. We tested their hypothesis by altering the cooling rate of 50% PVME from 320 degrees per minute to 256 times slower (I.E. 1.25°/min). The same WEX was observed in all cases as shown in Figure 9, in contradiction to the expectation based on the devitrification theory.

Effect of the Duration of Prolonged Exposure to 223° K on the WEX. Although the WEX does not depend on the rate of cooling in the range studied, it did vary with the time that the "frozen" solution is kept at 223 K (−50°C).

Thus as shown in Figure 10, 1 min. after cooling to 223 K, 49% PVP-360 showed a pronounced WEX, which, however, became progressively less prominent following further stay at this' temperature until after one hour the WEX disappeared altogether. A small secondary endothermic peak can then be discerned which may well be the same kind of secondary endothermic peaks seen in Figure 1 in PVP-360 at concentrations up to 43%.

In the case of 50% PVME (Figure 11) it took about 2 hours at 223° K for the WEX to disappear. After that a double-headed endothermic peak remained.

In contrast to the two polymers, urea denatured hemoglobin was the slowest to lose its WEX (Figure 12). The rates of disappearance of WEX, expressed as a ratio of the area of WEX over that of the main endothermic peak is shown in Figure 13. One recalls that urea by itself does not produce a WEX (Figure 4).

DISCUSSION

The Question of Vitreous Ice. Luyet, Rasmussen and their coworkers (Luyet et al.,

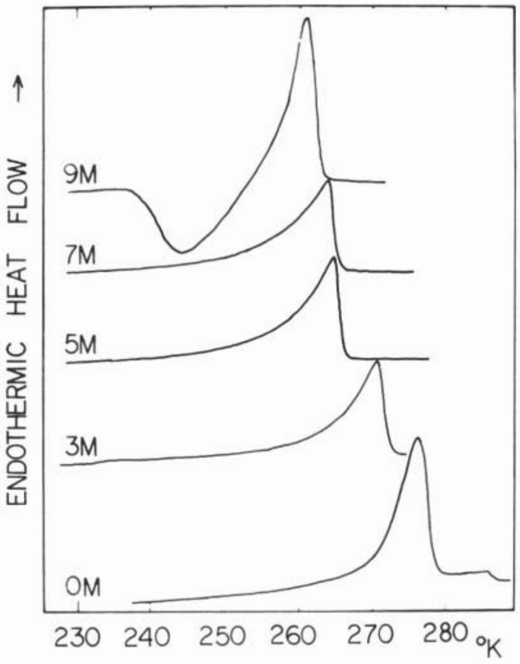

FIGURE 7. DSC thawing therrnograms of urea-denatured aqueous protamine sulfate (28.5%). The concentration of urea indicated in molarity.

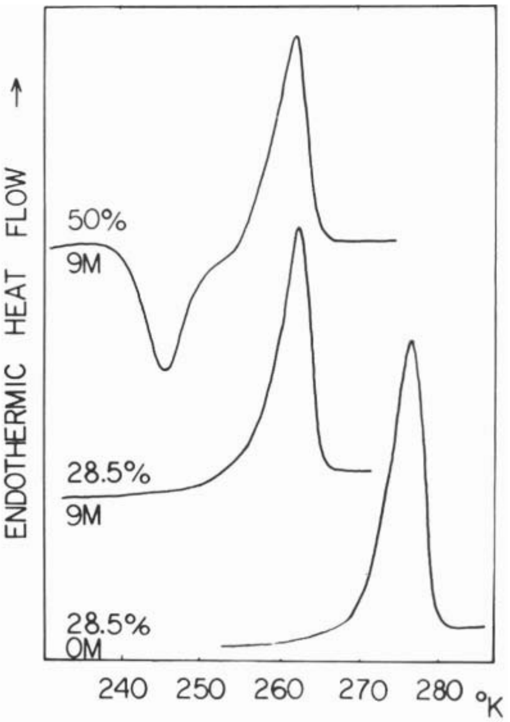

FIGURE 8. DSC thawing thermograms of urea-denatured pepsin. The concentration of pepsin is given in percentage and those of urea in rnolarity.

1966; Luyet and Rasmussen, 1967) first reported what we now refer to as the WEX phenomenon in aqueous solutions of glycerol, ethylene glycol, and PVP. They explained the phenomenon as due to "devitrification," contending that it was a prior rapid cooling that created the vitreous ice from normal liquid water in the sample and that it was the transformation of this vitreous ice into normal Ice I during rewarming that gives rise to the heat-releasing WEX.

There are several reasons that argue against this interpretation:

(1) Supercooling cannot create vitreous ice: Kamb (1972, p. 11) wrote: "The vitreous form of ice has been made by condensation of water vapor below −150° C, but it is impossible, as far as we know, to convert liquid water to a vitreous form by rapid cooling."

(2) The coexistence of water with a large quantity of macromolecules (e.g., 50% globular protein solutions) does not necessarily impede normal ice formation (Ling and Zhang, 1983).

(3) WEX does not depend on rapid cooling: A 250 fold slow-down of the cooling rate did not alter the WEX of a PVME solution (Figure 9) and left little doubt that WEX did not originate from very rapid cooling.

(4) Water in a chilled PVME solution is not in the state of ice: Vitreous ice like all other ices by definition is a ***solid*** and rigid. Yet a drop of 50% PVME remains as a viscous liquid and yields readily to manipulation when held between a pair of glass rods and immersed in a mixture of ethanol and dry ice kept at about −70° C.

An Alternative Interpretation of the WEX in Terms of Water Polarized in Multilayers. Luyet, Rasmussen and their coworkers believed that water in the aqueous solutions

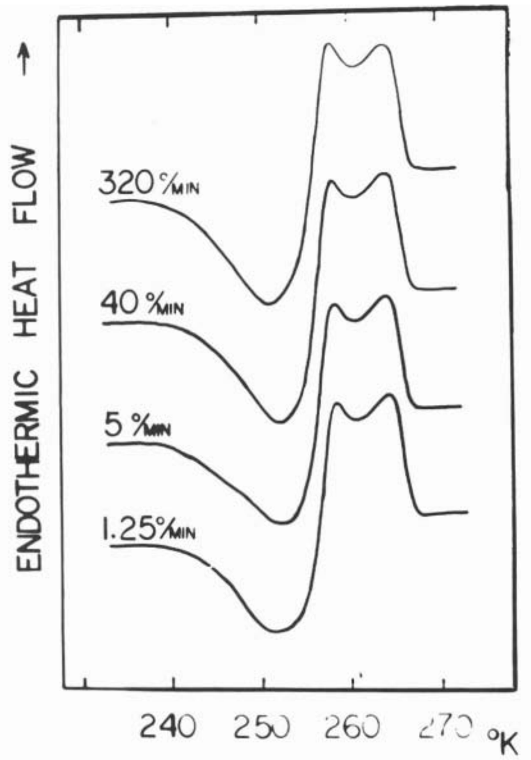

FIGURE 9. DSC thawing thermograms of 50% PVME after cooling down to 223° K at various cooling rates as indicated.

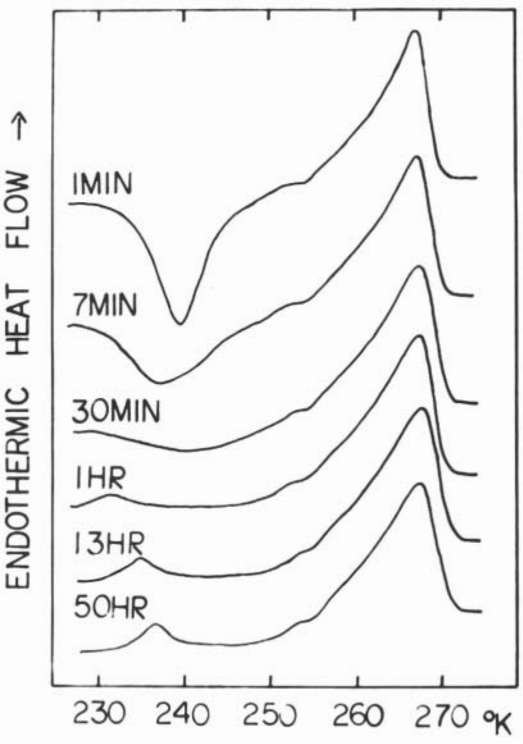

FIGURE 10. WEX decay. 49% PVP-360 solutions were held at 223°K for various lengths of time as indicated before warming began.

of PVP, gelatin, glycerol, etc., is normal liquid water. However, normal liquid water is not known to have been supercooled to beyond –40°C (Dorsey, 1940; Hallet, 1965). That is to say, in cooling solutions of PVP and gelatin to −70°C (i.e., the temperature produced by the dry-ice-alcohol mixture), Ice I should have been formed instantly, contrary to the facts. This then led Luyet and coworkers to the postulation that rapid cooling turned the water in these systems into vitreous ice. Clearly at that time they shared the common belief, now disproven (Ling and Zhang, 1983), that the presence of macromolecules invariably slows down the rate of ice-formation.

The purpose in postulating the existence of vitreous ice is to produce, at a temperature far below the freezing point of normal water, a condition which keeps the water molecules from the rotational and diffusional motions necessary to transform them into Ice I. The explanation we would like to offer is that in the presence of gelatin, PVP, PVME, etc. of suitable concentration, the bulk of water does not exist as normal liquid water. Rather it exists in the state of polarized multilayers, and cooling to –50°C of water in this state does not promptly turn this water into Ice I because there is a substantial energy barrier between these two states. To overcome this energy barrier, one can increase the temperature and hence the average kinetic energy, kT, of the water molecules, as one does during warming. This is why during a WEX heat is given off during warming in apparent contradiction to the Le Chatelier principle. Another way to achieve the transformation from the chilled multilayer state to the Ice I state is by lengthening of the time of exposure to –50°C so that in spite of the lower average kinetic energy, represented by kT, eventually all the water molecules come across the energy barrier, albeit slowly. As mentioned above, this expectation too, has been confirmed (Figures 10, 11). An implicit assumption of the present explanation is that the

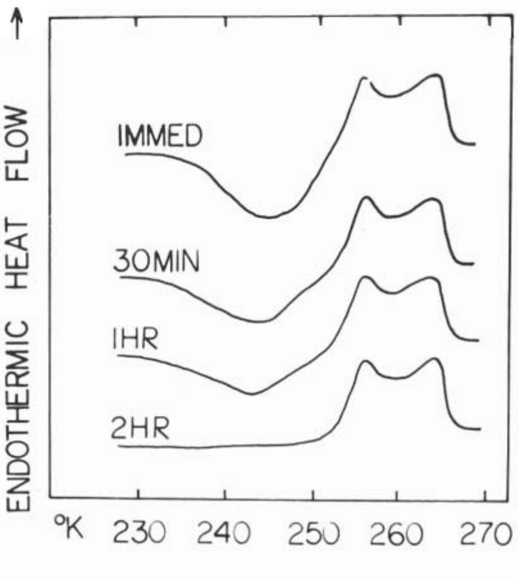

FIGURE 11. WEX decay. 50% PVME solutions were held at 223° K for various lengths of time as indicated before warming began.

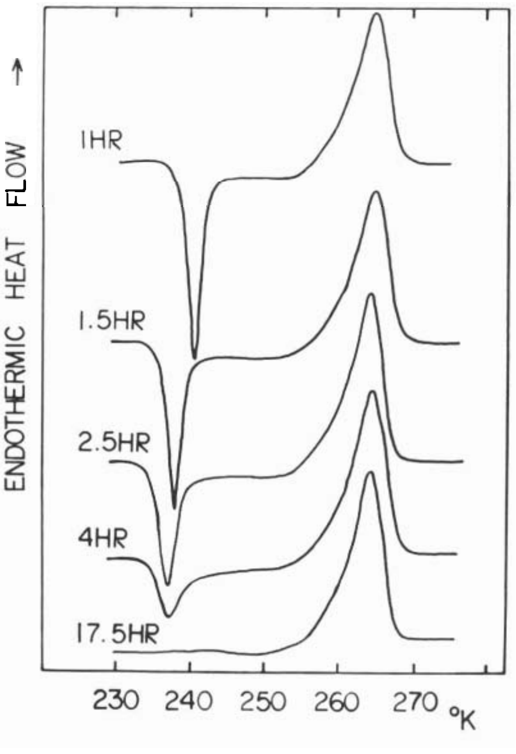

FIGURE 12. WEX decay. Hemoglobin in 9 M urea was held at 223° K for various lengths of time before warming began. Concentration of hemoglobin was 28.5%

state of Ice I, though not immediately reached, nevertheless does represent a lower energy and hence more stable state. However, in very concentrated solutions of water-polarizing polymers (e.g., 65% PVME, see Figure 11 of preceding paper), this may not be the case. Here, neither a freezing peak, nor a WEX, nor a thawing peak was observed. Apparently water is an energetically more favorable state in the polarized multilayer state in the presence of 65% PVME than is the Ice I state at the low temperatures studied.

The Involvement of Polymer Chain Translation and Rotation in the WEX Phenomenon. 60% PVP (Figure 1), and 65% PVME (Figures 4 and 11 of Ling and Zhang, 1983) do not form ice under all the conditions studied. Nor do they freeze or demonstrate a WEX. Yet 53% PVP does exhibit a WEX, though a weak one. The only difference between 53% PVP and 60% PVP is 7% additional PVP and 7% less water. Clearly the presence of PVP has greatly reduced the tendency to form ice either during cooling or warming. Since the physical state of water in 53% PVP and 60% PVP can hardly be fundamentally different (Ling and Ochsenfeld, 1983), it is more likely that the difference lies in the greater degree of entanglement or other stabilizing influences the few additional percentage of PVP adds to the already dense PVP-chain assembly. If this explanation is correct, it would suggest that due to the stronger interaction between the water multilayers and the polymer chains, for the water in the chilled polarized multilayer state to transform into Ice I, it is not just water molecules that have to undergo the proper movements but the polymers must also move in unison. When this polymer movement is sufficiently hindered, as it seems to be in the 60% PVP or 65% PVME, the water will remain in the polarized multilayer state during "freezing"and will not change to Ice I.

The concept of a mandatory requirement of coordinated movements of the polarizing polymers with water in the WEX phenom-

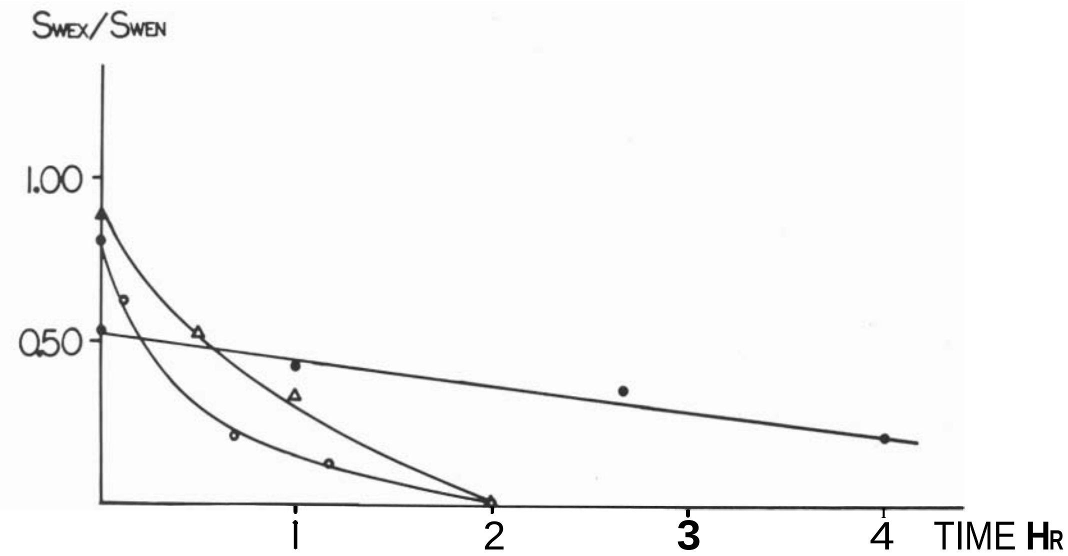

FIGURE 13. The change in the size of the warming exothermic peak of solutions of polymers and urea-denatured protein after having been kept at 223° K for varying length of time. The ordinate represents the urea of the integrated area of the warming exothermic peak (SWEX) expressed as a ratio to the integrated area of melting peak (SWEN) for PVP-360 (O)PVME (A), and urea-denatured hemoglobin. (O).

enon is significant in that this idea can help to understand the unusual behavior of PEO (especially Polyox N750).

PEO has the same solvency reduction effect as PVP, PVME, and gelatin. Indeed, PEO is one of the most effective among these polymers and proteins. It differs from PVP, PVME, and gelatin in several characteristics:

(1) Unlike PVP, PVME, and gelatin, which at high concentrations do not freeze at al, PEO doesfreeze at a concentration as high as 69.4% (Ling and Zhang, 1983, Figure 6). The explanation of this apparent anomaly is that in contrast to the other polymers, gelatin, and urea-denatured proteins, all of which possess side chains that can impede rotational motional freedom, PEO, a polymer containing only the repeating units of ($—CH_2$-O-$CH_2—$) has no side chains. It is therefore logical to expect the PEO chains to rotate or otherwise move more readily than polymers with side chains to follow the movements of water molecules toward the Ice I state.

(2) Polyox WSR-205 (MW 600,000) which is on the average, twice as long as Polyox WSR-N750 (MW 300,000) demonstrated a WEX, whereas none could be seen in the case of Polyox WSR-N750 at all. Clearly with the easy rotation and other motions of Polyox WSR-N750, all the polarized water has become transformed to Ice I during the freezing process and in the subsequent brief period of time of incubation at 223° K. The longer Polyox WSR-205 molecules do impose some restriction on freezing due to the greater energy barrier for the chain movement. As a result, Polyox WSR-N750, even at as high a concentration as 69.4% could rapidly form ice during the freezing at 10°/min whereas a 60% Polyox WSR-205 would require a higher kT provided by the warming to execute the same motional adjustments required for ice formation.

This investigation was supported by NIH Grants 2-R01-CA16301-3 and 2-R01-GM11422-13; Office of Naval Research Contract N00014-79-0126; and Pennsylvania Hospital General Research Support.

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(Received October 19, 1983; revised October 31, 1983)