

## CHAPTER VI

# Structure and Properties of Water at Biological Interfaces\*

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## I. Introduction

The unusual properties of water are directly traceable to the complex structure of this remarkable liquid. Unfortunately, the structure of water has, up to now, escaped a precise description—in fact, several mutually contradictory theories are currently discussed in the literature. Even less is known about the structure of water in moderately concentrated aqueous solutions or about water near interfaces. Vicinal water containing a relatively high concentration of both electrolyte and nonelectrolyte solutes is exactly the type of water which is encountered in biological systems—"the witches brew," according to Professor Henry Frank. Up to now, biochemistry and biophysics, in attempts to describe biological systems, have been fairly successful merely by ignoring the problem of the detailed structure of water in the systems under consideration. The purpose of the present article is to call attention to several unique facets of vicinal water structure in the hope that the recognition of these features may assist in elucidating the role of water in biological systems. Essentially, the plan is first to delineate some ideas about water in general and vicinal water in particular and, next, to present evidence for unusual temperature dependencies of many types of aqueous phenomena: it will be demonstrated that a large number of these phenomena may be explained quite readily in terms of changes in the underlying structure of the vicinal water. With this information in hand, the likely structural characteristics of vicinal water in biological systems becomes more easily understandable, and this is illustrated through a review of some specific properties of a number of biologically interesting systems. The value of this approach is that, however qualitative it may be, it does afford the opportunity to make rather general (qualitative) predictions which can be tested against experience, and, thus, "the exercise is removed from the realm of pure speculation to that of empirical science." The present chapter is not intended to serve as a review—certainly not a critical, carefully annotated review. Instead, it is an attempt to speculate along a line of reasoning (based on water structure) that has not previously been exploited to any large degree. Hence, the chapter should be read for its "inspirational"

value" rather than as a final or definitive treatment of the subject of the role of water structure near the cell surface. Unfortunately, as will become apparent, the treatment will leave something to be desired, as our understanding of water is still highly fragmentary and incomplete. This, of course, ensures many generations of biochemists, biophysicists, and biologists a fertile field for future *Forschung*.

One can hardly overstress the incomplete understanding which presently prevails regarding the structure of water in general and in biological systems in particular. The essence of the present study is to advocate that future work in the fields of biophysics and biology should recognize from the onset the possibility that the vicinal water of living systems (and, indeed, possibly all the water of the cell) exists in a structurally modified form (compared to bulk water). Although basically not an unreasonable assumption, this approach is made exceedingly difficult by lack of the appropriate physical chemistry of aqueous systems in which the aqueous phase does not possess the structure and properties ordinarily discussed. On the other hand, it is undoubtedly better to seek in the darkness for that which is missing than to search under the light of oversimplified traditional conceptual (and mathematical) models and approaches where it is obvious that the answer is not to be found.

A thorough, general survey of the problem of water near the cell surface should start with a comprehensive review of water structure. However, the subject of water structure has been reviewed by many authors, and the reader is referred to the available survey articles and monographs; particular attention is called to the monographs by Kavanau (1964), Samoilov (1965), Luck (1964), and Eisenberg and Kauzmann (1969). See also the extensive writings by Frank (and co-workers) (1945, 1957, 1958, 1959a,b, 1963, 1965a,b, 1966, 1967). At this time a number of excellent general tools for water structure studies are rapidly becoming available, and these will also be useful for the study of water in biological systems—nuclear magnetic resonance (NMR) and neutron inelastic scattering are typical examples. Unfortunately, even these tools have their restrictions, ranging from instrumental to theoretical (for interpretation of data), or are otherwise limited, for instance, in their geometric resolution of the area or volume to be examined. They far surpass, however, many of the tools which have been available in the past, not to mention that even those tools were frequently not used; indeed, the entire problem has been rather neglected by most researchers. Furthermore, we often seem to encounter tremendous inhomogeneity in our overall approach to problems. Thus, our understanding of the structure of water is qualitative, fragmentary, and incomplete, whereas an eloquent mathematical treatment is developing for the description of irreversible

thermodynamics which is achieving great sophistication in its application to biological membrane phenomena. Yet, the advances are often made, not because of, but in spite of, our current understanding of the very medium in which the processes and phenomena of interest occur. In quantitative biology (as well as in a great number of other fields of research), numerical agreement between calculated and observed values for a single parameter (based on a simple, specific mechanism) is often taken as "proof" of a particular mechanism with little regard as to the ability of the underlying theory to explain—even qualitatively—one single additional parameter or set of phenomenological observations. To the present author, an attempt to achieve great theoretical sophistication in, for instance, the analysis of membrane functioning or enzyme reactions seems currently an inordinately misdirected effort because of the uncertainty regarding the influence of the very structure of the phases in which the processes take place.

## II. Overview of Structure of Water

### A. STRUCTURE OF BULK WATER

#### 1. *Safford's Survey of Water Structure Models*

As indicated in the introduction to this chapter, the problem of the structure of water (in bulk) remains unsolved. In fact, several contradictory and mutually exclusive theories are currently discussed in the literature. A number of these theories are outlined briefly in Section II,A,2. At this point we present, in tabular form (Table I), an overview of current water structure theories, as prepared by Safford and Leung (1971). (The original report by Safford is recommended for more detailed information, and, specifically, for a careful analysis of the use of neutron inelastic scattering as a tool for determining water structure.)

#### 2. *Comments on Specific Models*

The question of the possible existence, in liquid water, of discrete structural entities of geometric, identifiable characteristics is one of the most fundamental current problems regarding water structure. The existence of such structural entities has often been proposed, but the alternative view (i.e., that, on the average, there is no order in water, neither long-range nor short-range order) has been maintained for a number of years, particularly by some spectroscopists. The notion that structurally identi-

fiable units are present in liquid water has been advocated for a long time; thus, mixture models have been envisioned, employing (in older papers), for instance, the existence of dimers, tetramers, hexamers, octomers, and other "polymers" of water (conceptually, the notion of large polymers resembles the more recent idea of clusters). In the 1930s and 1940s, "cybotactic swarms" were envisioned by Stewart (1931) and by Frenkel (1955). In each swarm, the molecules were all presumed to possess one or more physical properties in common (such as dipole orientation) and were considered to contain as many as 10,000 water molecules. Presently, liquid crystals (nonaqueous systems) are probably the closest known to cybotactic swarms. However, even before the turn of the century, it was suggested that other types of structured elements might exist in water; as an example, in one of these mixture models the presence of "microcrystalline chunks" of Ice-Ih\* lattices was assumed. The presence of ice-like elements, in this view of the liquid structure, were seen merely as the result of the (incomplete) thermal breakdown (upon melting) of the ice lattice. In view of the enormous amount of information available regarding water and aqueous solutions (often data of extreme precision), it is truly remarkable that the problem of a continuum versus a mixture model continues to be the subject of active discussion in the literature. Currently, the majority of investigators apparently favor the mixture model (to which the present author also subscribes). In connection with the mixture models, we call attention in particular to some aspects which have been stressed by Frank (see, for instance, Frank, 1970) and co-workers. The first of these is in the fact that regardless of the specific nature of the structural units, they probably have the attributes of "flickering clusters." This means that the lifetimes (stabilities) of the structured units are quite short in terms of the times studied by most ordinary analytical tools, but are still long compared to individual, molecular vibrations. A reasonable estimate for the lifetime of any structural arrangement in liquid bulk water might be  $10^{-11}$  second (within a factor of  $10^{\pm 1}$ ). Thus, in approximately  $10^{-11}$  second the structure entity may break down due to an unfavorable thermal fluctuation which disrupts the local order. However, the unit elements, in a similar interval of time, will regroup or reform in part (or in connection with molecules from other, disrupted structures) to form new, short-lived, structured elements. Another feature of some, but not all, mixture models is the possibility that a certain degree of cooperativeness may occur due to the nature of hydrogen bonding. This subject is also one of active discussion in the literature; Del Bene and Pople (1969) in particular are studying this problem through purely quantum

\* Ordinary, hexagonal ice is referred to here as Ice-Ih.

TABLE I  
MODELS OF WATER STRUCTURE<sup>a</sup>

Description and principal features of the model	References	Comments
I. Continuum Models		
Tetrahedral—four-coordinated hydrogen-bonded local ordering. No local domains having structural differences. Bonds become bent at melting point	J. D. Bernal and R. H. Fowler, <i>J. Chem. Phys.</i> , <b>1</b> , 515 (1933)	Based on X-ray data
Upon melting, bonds become relatively flexible and may bend continuously, resulting in rotational distortions	J. Leonard-Jones and J. A. Pople, <i>Proc. Roy. Soc., Ser. A</i> , <b>295</b> , 155 (1951)	Based on measurements of dipole moments
No separate vapor-like and structured regions, but rather a continuum without definitive structure	T. F. Wall and D. F. Hornig, <i>J. Chem. Phys.</i> , <b>43</b> , 2079 (1965)	Based upon the widths and shapes of observed Raman lines. The observed linewidths and the width of maxima in the X-ray radial distribution function are correlated with continuous variations in the O—O distances of bonded molecules
II. Specific Structure Models		
Water has distorted or expanded Ice-I structure in which defects may occur and in which a definite number of H <sub>2</sub> O molecules may pass through faces of surrounding tetrahedra and take up interstitial positions. A molecule which has relaxed from the framework occupies a shallow potential in a void and is partially hydrophobized by the high symmetry of the field in the void. The interaction between framework and void must be weak in order that the framework itself does not collapse	O. Ya. Samoilov, "Structure of Aqueous Electrolyte Solutions and the Hydration of Ions," Consultants Bureau, New York, 1965; E. Forslind, <i>Acta Polytech.</i> , <b>115</b> , 9 (1962); Yu. V. Gurikov, <i>J. Struct. Chem. (USSR)</i> , <b>4</b> , 763 (1965)	Based in part upon X-ray radial distribution functions. Fisher and Andrianova, and Gurikov have calculated such quantities as mean coordination number, fluctuation in the coordination number, isothermal compressibility, entropy, and free energy for this model. They find agreement with experiments on mean coordination number and a low percentage of molecules in the voids. The agreement with entropy and fluctuation in the coordination number is found to be poor unless an excess of about 3% of the molecules can exist in the framework. In addition, they point out that the lack of knowledge on the long-range order of the system and its influence on the entropy can give rise to significant error
It has been shown that water has a quasi-tetrahedral short-range structure and that the radial distribution curve is not compatible with an octahedral water structure. The short-range ordering in water is described in terms of a blurred-out Ice-I structure in which molecules have been displaced from the structure by thermal excitation and have occupied interstitial positions. Each framework oxygen atom has three neighbors at 2.94 Å and one at 2.77 Å, and each interstitial has neighbors at 2.94, 3.30, 3.40, and 3.92 Å. The interstitials show a larger temperature coefficient associated with their longer neighbor distances. At 25°C, 50% of the framework cavities are filled	J. Morgan and B. E. Warren, <i>J. Chem. Phys.</i> , <b>6</b> , 666 (1938); G. W. Brady and W. J. Ramshaw, <i>ibid.</i> , <b>32</b> , 306 (1960); A. H. Norton, M. D. Danford, and H. A. Levy, <i>Discuss. Faraday Soc.</i> , <b>43</b> , 97 (1967)	This model was based upon fits to observed X-ray radial distribution functions. Such a fit is necessary, but alone does not constitute a sufficient test for the model's validity

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| The associated clusters in water are clathrate structures similar to those of the gas hydrates. Twenty of the water molecules lie at the corners of a labile pentagonal dodecahedron with an unbonded molecule at the center of the dodecahedron.  | L. Pauling, <i>Science</i> <b>134</b> , 15 (1964)  | The radial distribution function calculated for this model is inconsistent with that measured for water [see M. D. Danford and H. A. Levy, <i>J. Amer. Chem. Soc.</i> <b>84</b> , 3965 (1962)].  |
| A hydrogen-bonded framework or Pauling clathrate structure is assumed with interstitial molecules to calculate the statistical, mechanical and thermodynamic properties of water. The framework may relax or "flicker" yielding a third state of $\text{H}_2\text{O}$ 's which are unbonded and have not yet re-formed a cluster.  | H. S. Frank and A. S. Quist, <i>J. Chem. Phys.</i> <b>34</b> , 604 (1961)                  | Yields a satisfactory representation of the PVT properties of water over a limited range of pressure and temperature. It is suggested that the inclusion of the third state would yield a better fit of the heat capacity of water and partial molar properties of nonpolar solutes.   |
| An equilibrium is assumed to exist between species having densities similar to Ice-I and Ice-III. Both fluidized vacancies and monomers exist on melting, and the monomers may pack into voids in the Ice-I-like units.  | M. S. Jhon, J. Gross, T. Ree, and H. Eyring, <i>J. Chem. Phys.</i> <b>44</b> , 1469 (1966) | Good agreement is obtained for molar volumes, the vapor pressure below the boiling point, the specific heat, and the pressure dependence of the viscosity.   |
| Open- and close-packed structures formed of "puckered hexagonal rings" coexist in equilibrium. In particular, the rings are not viewed as static structures at a given temperature, but as representative of localized short-time interactions. A given water molecule is not always in a ring and is unbonded or monomer-like only when changing from one state to another. Thus, this model characterizes the monomers in terms of a residence or relaxation time for the structure, and rearrangement takes place by jumps of individual molecules. | C. M. Davis, Jr. and A. T. Litovitz, <i>J. Chem. Phys.</i> <b>42</b> , 2563 (1965)         | Accounts for the radial distribution curve out to 4 Å, the thermal expansion of water between 0° and 100°C, the relaxation portion of the isothermal compressibility, and the specific heat. The fraction of hydrogen bonds was estimated by Raman spectroscopy.   |
| III. Cluster Models  | G. W. Stewart, <i>Phys. Rev.</i> <b>37</b> , 9 (1931)                                      | Based on X-ray diffraction only  |
| The existence of clusters containing 10,000 water molecules was postulated.  | H. S. Frank and W. Y. Wen, <i>Discuss. Faraday Soc.</i> <b>24</b> , 133 (1957)             | These arguments were based upon the partially covalent cluster of the hydrogen-bonded molecules and supported by data on densities, relaxation times, structural changes in solution of nonpolar solutes, and thermodynamic parameters.  |
| Nemethy and Scheraga have done a statistical thermodynamical calculation for the flickering cluster model. The compact clusters are made up of four distinct species corresponding to molecules with one, two, three, and four hydrogen bonds and a sharp energy level is assigned to each species. A fifth species is the unbonded monomers which is in equilibrium with the clusters. No long-range ordering is specified and irregular arrangements in the clusters are allowed.  | G. Nemethy and H. A. Scheraga, <i>J. Chem. Phys.</i> <b>36</b> , 3382 (1962)               | Agreement is obtained with calculated values of free energy enthalpy, and entropy, but poorer agreement with the heat capacity. This theory was also able to account for first and second nearest-neighbor maxima in the X-ray radial distribution curves. Nemethy and Scheraga have pointed out that variations as large as 50 cm <sup>-1</sup> in the frequencies used for the partition functions produce almost negligible changes in the calculated thermodynamic functions. The frequencies considered correspond to torsional oscillation and hindered translation of $\text{H}_2\text{O}$ molecules bonded to structured units in the liquid and occur below 500 cm <sup>-1</sup> . However, shifts of 50 cm <sup>-1</sup> in these frequencies are not negligible and can correspond to significant changes in the bonding and the lattice geometry for $\text{H}_2\text{O}$ molecules. |

TABLE I (Continued)

Description and principal features of the model	References	Comments
Buijs and Choppin obtained near infrared results that, in general, were in agreement with the theory of Nemethy and Scheraga. However, they considered only three species with zero, one, or two of the OH groups of an H <sub>2</sub> O molecule bonded. Best agreement on the relative numbers of hydrogen-bonded water molecules was obtained at low temperature	D. Buijs and G. R. Choppin, <i>J. Chem. Phys.</i> , <b>39</b> , 2035 (1963)	Both the theory and the infrared results have been subject to recent questions [see D. F. Hornig, <i>J. Chem. Phys.</i> , <b>40</b> , 3119 (1964); D. D. Boettger, D. D. Harders, and D. D. Luck [ <i>J. Phys. Chem.</i> , <b>71</b> , 459 (1967)] have pointed out that the populations obtained by Buijs and Choppin for the species are not unique. Further, Stevenson [D. P. Stevenson, <i>J. Phys. Chem.</i> , <b>69</b> , 2145 (1965)] has argued that the concentration of non-hydrogen-bonded water molecules between 0° and 100°C, as predicted by models of Nemethy and Scheraga and by Buijs and Choppin, are two orders of magnitude too high. He concludes that the number of monomers in liquid water between 0° and 100°C is less than 1% of the molecules in the liquid. More recent studies by Griffith and Scheraga [J. H. Griffith and H. A. Scheraga, <i>16th Meet. Amer. Chem. Soc.</i> , 1966, <i>Abstract I-45</i> ] give lower concentrations of monomers than in previous mixture models, but still well above those estimated by Stevenson. This model yields good agreement with the Helmholtz free energy, the internal energy, and the specific heats
Vand and Senior argued that better agreement is obtained between the results of Buijs and Choppin and theory if the sharp energy levels for each species, as assumed by Buijs and Choppin, are replaced by broad energy bands. As these bands overlap, a continuous distribution of molecular states is approached. Thus, the energy state of a species varies with the coordination. Branched chains of molecules exist and can be either free or attached to a cluster. In the limit of this picture, water would be viewed as a loosely bound solid. Vand and Senior have shown, furthermore, that the particular model they chose is not unique in being able to explain the thermodynamic data, but a model based upon one species with an energy band distribution that also fits the thermodynamic data	V. Vand and W. A. Senior, <i>J. Chem. Phys.</i> , <b>45</b> , 1869 (1966); W. A. Senior and V. Vand, <i>ibid.</i> p. 1873; V. Vand and W. A. Senior, <i>ibid.</i> p. 1878	The data of Walrafen appears in contradiction to that of Wall and Hornig. The broad Raman lines the widths of which Wall and Hornig ascribed to a continuous variation in O—O distances, appear partially resolved into components in Walrafen's spectra. From the changes in the line intensities with temperature, he obtains reasonable estimates of enthalpy, entropy, and fair agreement with the heat capacity of water of coordinations
Walrafen, from Raman measurements, has proposed a model of water similar in certain features to those of Nemethy and Scheraga and of Vand and Senior. Intermolecular vibrational modes corresponding to tetrahedral five molecule units having C <sub>1v</sub> symmetry are observed and are in equilibrium with unbonded species which contribute little, if at all, to the spectra. With increasing temperature, these tetrahedral species may distort and break down, giving an increase in species of a lower degree of coordinations	G. E. Walrafen, <i>J. Chem. Phys.</i> , <b>40</b> , 3249 (1964); <b>44</b> , 1546 (1966)	The data of Walrafen appears in contradiction to that of Wall and Hornig. The broad Raman lines the widths of which Wall and Hornig ascribed to a continuous variation in O—O distances, appear partially resolved into components in Walrafen's spectra. From the changes in the line intensities with temperature, he obtains reasonable estimates of enthalpy, entropy, and fair agreement with the heat capacity of water of coordinations

\* Table prepared by Safford and Leung (1971) and reprinted here by permission of the authors.

mechanical calculations (see also Hankins *et al.*, 1970). The results to date suggest strongly that stabilization of hydrogen bonds may occur where cyclic arrangements of chains of water molecules are possible. We finally stress that some mixture models allow for the existence of discrete sites or voids in the structure. Later in this chapter we return to specific possible structured elements in water, and particularly in water near interfaces. The structured elements include ice (that is, the structural elements similar to ordinary hexagonal ice, i.e., Ice-Ih), high-pressure ice polymorphs, clathrate hydrates, and clusters (in the Nemethy-Scheraga sense). For a review of ice polymorphism, see the excellent report by Kamb (1968) and the report by von Hippel and Farrell (1971); the general field of clathrates has been surveyed by van der Waals and Platteau (1959) and by Gawalek (1969).

We can summarize a number of significant features. The results of certain theoretical calculations notwithstanding, at present, it appears that the evidence for a mixture model for water structure is quite substantial—water is probably best described in terms of a mixture of structured elements and, possibly, monomers (or other relatively low molecular weight associated species). The major likely candidates for the structured elements are Ice-Ih (somewhat unlikely), the high-pressure ice polymorphs, clathrate cage structures, or various types of clusters.

## B. WATER IN ELECTROLYTE SOLUTIONS

### 1. *Safford's Survey of Water-Ion Interactions and Electrolyte Solution Structure*

The reader is referred to Tables II and III (from Safford and Leung, 1971) for a short survey of recent work on the subject. Further references can be found in the books by Kavanau (1964), Samoilov (1965), and review articles by the present author (Drost-Hansen 1967a) and by Hertz (1970). For an excellent review of hydration of ions, see the article by Desnoyers and Jolicoeur (1969). Very readable accounts of molal volume aspects of electrolyte solutions are presented by Millero (1970, 1971).

### 2. *Solute-Solvent Interactions*

It appears that most solutes in water affect the structure in the vicinity of the solute molecule (or ion) and several different types of structural arrangements may result. Some solutes (especially electrolytes) are known as "structure breakers," whereas others are "structure makers." One of the difficulties in this connection is that of semantics. Thus, a structure maker

TABLE II  
MODELS OF WATER-ION INTERACTIONS<sup>a</sup>

Description and principal features of the model	References	Comments
Water molecules in primary hydration layer subject to strong centric-symmetric forces and are ordered. Beyond this region is a zone of disordered water structure which, at larger distances from the ion, blends into the undisturbed water structure	H. S. Frank and W. T. Wen, <i>Discuss. Faraday Soc.</i> <b>24</b> , 133 (1957)	This model was based upon corroborative evidence from measurements on heat capacities, dielectric relaxation, diffusion of H <sub>2</sub> O salt solutions, ionic mobility, entropies of solution, and viscosity
Water forms frozen patches or microscopic icebergs about solute molecules. The hydration of ions—their ability to break the water structure and the sizes and bonding of their hydration layers—depends both on size and on the charge of the solute molecules	H. S. Frank and M. W. Evans, <i>J. Chem. Phys.</i> <b>13</b> , 507 (1945)	Based on observed entropy measurements and the large negative partial molal heat capacities of ions—ions such as Al <sup>3+</sup> , Mg <sup>2+</sup> , Li <sup>+</sup> , and F <sup>-</sup> —can build icebergs about them and decrease the fluidity. In particular, ions of high charge, such as La <sup>3+</sup> and Eu <sup>3+</sup> , may give rise to a “superlattice” ordering of H <sub>2</sub> O molecules with sufficient quasi-solid-like ordering to support lattice vibrations
Ions may either increase or decrease the activation energies of H <sub>2</sub> O molecules in their vicinity and act, respectively, as positive or negative hydrators. Ions such as Mg <sup>2+</sup> , Ca <sup>2+</sup> , Li <sup>+</sup> , and Na <sup>+</sup> show positive hydration, whereas ions such as K <sup>+</sup> , Cs <sup>+</sup> , Cl <sup>-</sup> , Br <sup>-</sup> , and I <sup>-</sup> show negative hydration. This hydration is assumed to occur in the region of the ion and largely determines the kinetics of the solution. There is also a more distant region where the water is mainly influenced by the ionic field	O. Ya. Samoilov and T. A. Novanova, <i>J. Struct. Chem. (USSR)</i> <b>6</b> , 767 (1965); O. Ya. Samoilov, <i>[Structure of Aqueous Electrolyte Solutions and the Hydration of Ions.]</i> Consultants Bureau, New York, 1965	Samoilov argues that, in dilute aqueous solutions, ions interact with H <sub>2</sub> O molecules so as to yield the minimum modification of the solvent structure. X-Ray data are cited to indicate that regions having structures similar to pure water can coexist with hydrated ions. At higher concentrations, water molecules coordinate to ions so as to yield a similar local ordering to that of solid salt hydrates
Ions are shown to fall into two classes depending on the parameter $\sqrt{Z/r_f}$ , where Z is the charge and $r_f$ is the distance from the ion to the dipole center of the first layer of H <sub>2</sub> O molecules about the ions. Ions with $\sqrt{Z/r_f}$ greater than about 0.3 (i.e., Li <sup>+</sup> , Mg <sup>2+</sup> , La <sup>3+</sup> ) are surrounded by an ice structure varying from 3 to 10 water molecules	E. Glueckauf, <i>Trans. Faraday Soc.</i> <b>61</b> , 914 (1965)	Data on molar volumes have been analyzed to draw conclusions on the structure of water about ions and the iceberg-building tendency of ions
Small ions (i.e., Li <sup>+</sup> and Na <sup>+</sup> ) may fit into ‘holes’ in the water structure without breaking, but only slightly stretching and bending bonds. Larger ions, such as K <sup>+</sup> , would not fit into the structure and would break it down	F. Yaslov, <i>J. Phys. Chem.</i> <b>70</b> , 2286 (1966)	Electrostatic energies of alkali ions in the field of a water calculated as a function of the angle of the ions with the dipole axis of the water molecule. A series expansion was used for the potential due to the ionic charge, together with quadrupole moments of the water molecule
Dissolved electrolytes produce distortions in open structure of water lattice. The solute-solvent interaction in general does not give rise to the formation of a distinct coordination sphere of H <sub>2</sub> O about the ion. Rather, the ion is integrated into the water lattice by ordinary chemical bonds of variable strength and duration	E. Bergqvist and E. Forslund, <i>Acta Chem. Scand.</i> <b>16</b> , 2069 (1962)	Based on concentration dependence of proton magnetic resonance chemical shifts

<sup>a</sup> Table prepared by Safford and Leung (1971) and reprinted here by permission of the authors.

does not necessarily mean a solute that enhances the intrinsic (or latently present) structural characteristics of pure water, but, rather, merely indicates the enhancement of some type of "lattice rigidity" over that present in the bulk solution. On the other hand, a structure breaker is a solute which disorders the (local) intrinsic water structure. It should be mentioned that there is some evidence that, even in rather concentrated solutions, there appear to persist structural elements characteristic of pure bulk water (see, for instance, Safford, 1966; Drost-Hansen, 1967b). The suggestion of continued existence of undisturbed water structure elements in strong electrolyte solutions (say, 0.5 M) is, indeed, surprising. In such solutions of a strong 1-1 electrolyte, the ratio of water molecules to individual ions is approximately 1:50. The distance between ions, measured in terms of diameters of the solvent molecules, is only 3 or 4. Thus, it would be expected that all of the water molecules would be under the strong centrosymmetric force fields of the ions, and that no undisturbed elements of the original water structure would remain (see, however, Vaslow, 1963).

### C. WATER IN NONELECTROLYTE SOLUTIONS

#### 1. General Comments

The physical chemistry of aqueous solutions of low molecular weight nonelectrolytes has been studied far less frequently than that of aqueous electrolyte solutions. With regards to biological systems, it is even more unfortunate that practically no studies have been made on electrolytes in aqueous nonelectrolyte solutions. We return briefly to that specific problem later in this chapter. Much of the available information on aqueous non-electrolyte solutions has come from the Russian school of authors (see, for instance, Samoilov, 1965; Mikhailov, 1968; Krestov, 1969, and Yastrem-skii, 1963), from England (Franks, 1967; Symons and Blandamer, 1968; Blandamer *et al.*, 1969a,b), and from the studies by Ben-Naim (1969). Discussions of the water–nonelectrolyte mixed solvent systems can be found in the proceedings from two recent Symposia (Franks, 1967; Covington and Jones, 1968). We summarize below a few of the more important facets of these studies. Again, no general agreement as to interpretation of experimental data exists regarding structural features of an aqueous nonelectrolyte solution. However, a number of pertinent generalizations can be made.

#### 2. Aqueous Alcohol Solutions

Franks and Ives (1966) have presented a lucid review of the structural properties of alcohol–water mixtures. Aqueous solutions of the lower

TABLE III  
PARTIAL SUMMARY OF EXPERIMENTAL RESULTS ON THE STRUCTURE OF IONIC SOLUTIONS\*

Technique—solute	Ref.*	Information obtained
X-Ray $\text{Ag}(\text{NO}_3)_2$ , $\text{Pb}(\text{NO}_3)_2$	(a)	$\text{Th}^{4+}$ and $\text{UO}_2^{2+}$ have a regular or "super" arrangement in the liquid. The ions are surrounded only by $\text{H}_2\text{O}$ molecules. $\text{Ag}^+$ , $\text{Pb}^+$ , and $\text{Ba}$ nitrates show a large fraction of dissociated (gas-like) molecules. $\text{I}^-$ , $\text{Br}^-$ , and $\text{Rb}^+$ show no arrangement, but only a broad interference between heavy ion and surrounding molecule.
KCl, $\text{NaCl}$ , $\text{LiCl}$	(b)	At low temperatures these solutions showed the second maximum in the radial distribution curve of pure water which is interpreted to show a nonhomogeneous structure at low temperatures, with water-rich regions.
$\text{LiCl}$ , $\text{LiBr}$ , $\text{RbCl}$	(c)	The 4.0 and 2.58 M $\text{LiBr}$ and $\text{RbBr}$ retained the principal diffraction maximum of water. In more concentrated solution (i.e., 13 M $\text{LiCl}$ ), the structure resembles that of the correspondingly hydrated salt.
KOH, KCl	(d)	For KOH, the $\text{K}^+$ substitutes an $\text{H}_2\text{O}$ in the quasi-tetrahedral water structure, whereas the $\text{OH}^-$ occupies an interstitial position. For KCl, the $\text{Cl}^-$ breaks down $\text{H}_2\text{O}$ structure by distorting the tetrahedral coordination and squeezing out $\text{H}_2\text{O}$ molecules.
LiCl	(e)	The water structure is broken by $\text{Li}^+$ ions. Hydrated $\text{Li}^+$ ions then pack around $\text{Cl}^-$ ions giving a hydration number of 8 or 9. The hydrated $\text{Li}^+$ ions have four water molecules in a tetrahedral configuration about the ion.
$\text{EtCl}_2$ and $\text{ErI}_4$	(f)	The $\text{H}_2\text{O}$ molecules are firmly held in octahedral arrangement around the $\text{Er}^{4+}$ ions. There is evidence for an ice-like ordering of $\text{H}_2\text{O}$ molecules resulting from the higher degree of orientation about the cation.
	(g)	For lithium and sodium halide solutions, 7-9 water molecules occupy the first hydration layer of the halide ions. Their number increases slightly with ion size. Second and third hydration layers are correlated with the anions. The region of influence of $\text{Li}^+$ and $\text{Na}^+$ is in general smaller than that of the anions and corresponds to first and second hydration layers. The halide ions lie along the OH axes, whereas cations lie on the dipolar axes of the primary waters. Considerable ion-ion contact was detected in the cesium salt solutions, but not in the others.
Raman	(h)	From a correspondence of frequencies in the region 700-1600 $\text{cm}^{-1}$ between the solutions and the solid hydrates, it was concluded that the relative placement of the ions in the concentrated solutions is characteristic of the solid hydrate
Concentrated ( $>N$ ) solutions of $\text{Li}$ , $\text{Ca}$ , $\text{Al}$ , $\text{Cr}$ , and $\text{Th}$ nitrates were compared to their corresponding solid salt hydrates	(i)	Lines in the 360-400 $\text{cm}^{-1}$ region are assigned to metal-oxygen stretching frequency of cation-water complexes. The increased binding of the hydration sheath is correlated to increased ionic charge. There is considerable electron sharing in the metal-oxygen bond.
Solutions of the nitrates, sulfates and perchlorates of $\text{Cu}$ , $\text{Zn}$ , $\text{Hg}$ , $\text{In}$ , $\text{Mg}$ , $\text{Tl}$ and $\text{Ga}$	(j) (k)	A similar conclusion was reached from IR studies for metal-oxygen bonding in solid hydrates. Strongly hydrated units exist in electrolyte solutions. Vibrational frequencies of $\text{H}_2\text{O}$ molecules in the primary hydration layer of the ions are observed between 900 and 400 $\text{cm}^{-1}$ . Reasonable agreement is obtained for $\text{O}-\text{HOH} \cdots \text{Cl}$ and $\text{O}-\text{HOH} \cdots \text{I}$ vibrations in solutions and those reported for solid hydrates.
Concentrated solutions of $\text{In}_2(\text{SO}_4)_3$ , $\text{In}(\text{ClO}_4)_3$ , $\text{In}(\text{NO}_3)_3$ ,	(l)	Changes in the region below 500 $\text{cm}^{-1}$ indicated formation of large, highly ordered clusters of $\text{H}_2\text{O}$ molecules centered on $\text{In}^{3+}$ ions and extending several water molecules in depth. Solutions become more ordered with decreasing temperature. $\text{NO}_3^-$ and $\text{SO}_4^{2-}$ tended to displace $\text{H}_2\text{O}$ molecules from the hydration sphere of the cations at +25°C.

Electronic Spectra CoCl <sub>2</sub> , CoBr <sub>2</sub> , CoI <sub>3</sub> and NiCl <sub>2</sub> , NiSO <sub>4</sub> , Ni(NO <sub>3</sub> ) <sub>2</sub> ErCl <sub>3</sub> , Er(NO <sub>3</sub> ) <sub>3</sub>	(m) (n, o)	The observed frequencies below 500 cm <sup>-1</sup> , characteristic of a cation complex, appear within 30 cm <sup>-1</sup> of the corresponding solid hydrate in each case. In the solutions thermally activated lattice vibrations were seen which closely paralleled those of the solid hydrate. The structures in the spectra of the solution were more diffuse than in the solid spectra. They became more diffuse with decreasing concentration. The cation appears surrounded by a quasi-solidlike patch which can support lattice vibrations. Frank and Evans have argued that a similar superlattice may be associated with La <sup>+</sup> ions in solution
Isotopic Mobility Concentrated LiNO <sub>3</sub>	(p)	The solution contains aggregates with molecular orientations similar to the crystal lattice of the solid hydrate
Solubility KSCN	(q)	The nearly linear SCN <sup>-</sup> to an extent occupies channels in the water structure
Nuclear Magnetic Resonance (NMR) Indium halides General review of data for many salts	(r) (s)	Evidence exists for In(H <sub>2</sub> O) <sub>4</sub> <sup>+</sup> complexes in solution. In general, the hydrated ion destroys the structure of water and forms complexes of type Me(H <sub>2</sub> O) <sub>6</sub> . However, ions may enhance strength of hydrogen bonds of H <sub>2</sub> O molecules beyond the first hydration layer due to polarization.
1:1 Electrolytes	(t)	Vibrations of the complex are in general not harmonic The ion-water complex is treated as a molecular species and effective hydration numbers are calculated. A decrease in the effective hydration number occurs with increasing ionic radius. Among halide ions, it is suggested that only the F <sup>-</sup> forms a hydrate structure. The larger halide ions break down the water structure. A structure-making effect is suggested for Li <sup>+</sup>
NMR On Absorption Aqueous solutions of H <sup>+</sup> , Li <sup>+</sup> , Be <sup>2+</sup> , Mg <sup>2+</sup> , Ba <sup>2+</sup> , Sr <sup>2+</sup> , Hg <sup>2+</sup> , Ga <sup>3+</sup> , Bi <sup>3+</sup> Quadrupole Relaxation KCl, CsCl, NaCl, LiCl, MgCl <sub>2</sub> , AlCl <sub>3</sub>	(u) (v)	Exchange times for H <sub>2</sub> O molecules between the hydration shell of an ion and the solvent were obtained. For Al <sup>3+</sup> , Be <sup>2+</sup> and Ga <sup>3+</sup> , Be <sup>2+</sup> , and Ga <sup>3+</sup> , the time exceeds 10 <sup>-4</sup> second, whereas for all others it is less than 10 <sup>-4</sup> second K <sup>+</sup> and Cs <sup>+</sup> increase the rotational freedom of H <sub>2</sub> O molecules in the hydration sphere of the ion. In contrast, Na <sup>+</sup> , Li <sup>+</sup> , Mg <sup>2+</sup> , and Al <sup>3+</sup> reduce it
Proton Relaxation Alkali halide solutions	(w)	The configuration of H <sub>2</sub> O molecules is more stable about Li <sup>+</sup> , Na <sup>+</sup> , or F <sup>-</sup> ions than for pure water. For other ions, it is less stable. The degree of stability decreases with increasing ionic radius
Theory Alkali-metal cations and halide anions	(x)	Estimates of interaction energies of ions with their nearest H <sub>2</sub> O molecules have been obtained using LCAO-MO theory. The change in the energy of electrons on hydration in the sequence Li <sup>+</sup> > Na <sup>+</sup> > K <sup>+</sup> > Rb <sup>+</sup> > Cs <sup>+</sup> . The energy changes are smaller for anions than for cations. The translational mobility of H <sub>2</sub> O molecules close to the ion should increase in going from Li <sup>+</sup> to Cs <sup>+</sup> . The Raman studies summarized above also indicate metal-oxygen electron sharing in aqueous solutions. Infrared studies yield similar results for the solid salt hydrates

<sup>a</sup> Table prepared by Sanford and Leung (1971) and reprinted here by permission of the authors.

aliphatic alcohols appear to be the only systems for which reasonable estimates can be made of the free energy, enthalpy, and entropy of solutions at infinite dilution. Practically no systematic information is available which covers a wide range of concentrations, and most of the available data were obtained only at one (or at best, a few) temperatures. The study of aqueous alcohol solutions involves the separation of solute-solvent effects from solute-solute and solvent-solvent effects and attempts to understand the alcohols in terms of the interaction with the solvent of the functional group (the hydroxyl group) and the (nonpolar) hydrocarbon part of the molecule. In the studies of solutes at infinite dilution, it is, of course, the solvent-solute interaction in which we are interested. This interaction will naturally depend on the water structure, that is, the solvent-solvent interaction. However, the available experimental data often pertain to a wide concentration range of the solute, but not necessarily at very high dilutions. This necessitates considering also the solute-solute interactions, and, because the measurements are frequently not carried out to sufficiently high dilutions, the extrapolation to infinite dilution often becomes tenuous and sometimes impossible. The studies by Franks (1966, 1968) are especially interesting in connection with the alcohols; he has also reviewed more generally the state of organic non-electrolytes in water. The reader is referred specifically to the recent article on effects of solutes on the hydrogen bonding in water (Franks, 1968).

A great deal of information has been learned about the behavior of non-electrolytes in water from studies of molal volumes. Figure 1 shows a characteristic set of curves of partial molal volumes for ethanol, dioxane, and hydrogen peroxide. The pronounced minimum for alcohol near 0.1 mole fraction is practically interesting, and Fig. 2 shows some additional data for ethanol and *t*-butanol at various temperatures. The behavior is obviously highly complex and no doubt reflects properties intrinsic not only to the water structure but also to the solute which, in turn, interacts with the water through the functional group as well as through the non-polar hydrocarbon part. Indeed, (a) the water structure may contract, (b) the solute may fit into already existing voids in the water, (c) the solute may induce voids in the water structure, or (d) the solute may experience a confining effect due to the water, reducing possible (larger-volume) conformations of the nonpolar group. Franks and Ives (1966) and, more recently, Franks (1968) have discussed the possibilities in great detail. We quote Franks and Ives:

It is therefore proposed, for purposes of present discussion, that the effect need not depend exclusively on use by solute molecules of the pre-existing cavities natural to pure water (although this may be preferred), nor to the formation

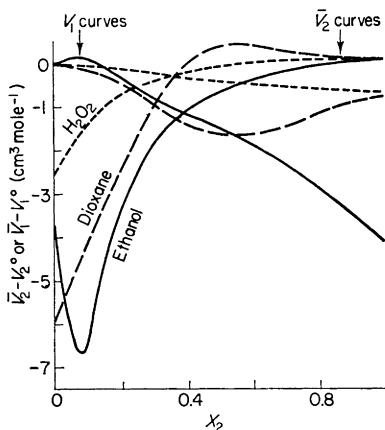


FIG. 1. Partial molar volumes for the ethanol-, dioxane-, and hydrogen peroxide-water systems, respectively, at 0°, 25°, and 0°C. (Franks and Ives, 1966, reproduced with permission from the Chemical Society, London.)

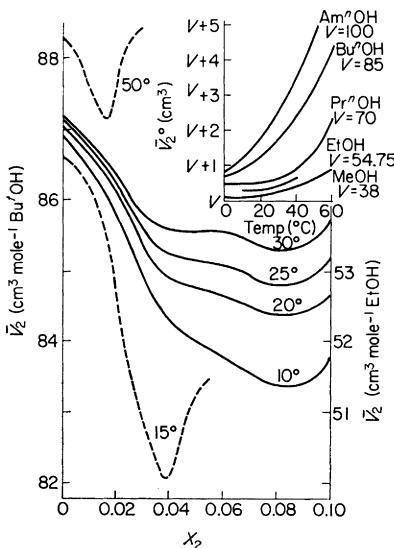


FIG. 2. Partial molar volume of ethanol (solid lines) and *t*-butanol (dotted lines) in aqueous solution. Top diagram: partial molar volumes at infinite dilution for various alcohols, as function of temperatures. (Franks and Ives, 1966, with permission from the Chemical Society, London.)

of cavities peculiar to the structures of a limited number of stable gas-hydrates. Instead, it is envisaged that, in virtue of its versatility in three-dimensional hydrogen bonding, water may have an intrinsic cavity-stabilising function that meets, in greater or lesser degree, the stearic requirements of any solute mole-

cule, perhaps better for a spherical molecule than for one of another shape. In effect, a solute molecule will control and protect the hydrogen-bonded packing of water molecules in its vicinity, replacing by its own volume one or more of the natural cavities that would otherwise have been present, with appropriate overall economy of space.

In passing, it should be observed that anomalies have been reported by numerous investigators for properties of aqueous alcohol solutions in the concentration range of about 0.05 to 0.1 mole fraction. Volumetric, ultrasonic absorption, and spectral data are available to demonstrate such anomalies. However, recently Lang and Zana (1970) have studied the ultrasonic absorption of nonaqueous alcohol mixtures. In all cases studied, the excess absorption, when plotted against mole fraction, displayed sharp maxima (for concentrations less than 0.1 mole fraction). This undoubtedly is related to dimerization (or higher degrees of association) of hydrogen-bonded aggregates of the alcohol in the organic solvent. Although the interpretation which is usually placed on the explanation of similar experiments in aqueous solutions is most likely essentially correct (i.e., the water structure is, indeed, profoundly changed), future work must also more carefully allow for the self-association of the alcohols in the aqueous systems.

Robertson and Sugamori (1969) have studied the kinetics and mechanisms of hydrolysis reactions and, in particular, the energy and heat capacity of activation for solvolysis of *t*-butyl chloride in alcohol-water mixtures. As would be expected from previous studies by Arnett and co-workers and from the studies by Symons, Blandamer, and co-workers, dramatic changes were found to occur in the structure of the aqueous solvent as a function of the alcohol concentration and this is reflected in the kinetic parameters. The paper by Robertson and Sugamori (1969) is of interest because of the light it throws on the question of enhanced structuring in aqueous nonelectrolyte solutions for low concentrations of the nonelectrolyte (0–0.15 mole fraction). However, it is perhaps equally significant that Robertson and Sugamori recognized the tremendous advantage of extremely careful measurements which permit evaluation not only of the apparent enthalpies of activation and apparent entropies of activation but, more importantly, of the temperature dependencies of the apparent enthalpy of activation—in other words, the apparent specific heat capacity. That this approach is presently possible is largely due to Robertson and co-workers who have long demonstrated an impressive ability to perform exceedingly precise measurements. A similar accomplishment (to which we return below) has been made by Goring and co-workers (see Ramiah and Goring, 1965) in their studies (for instance, volumetric measurements) of water-polyhydric alcohol, including cellulose interactions.

### 3. Classifications of Solutes

Table IV is an attempt by Franks (1968) to classify solutes depending on the relative magnitude of the excess mixing functions. From this table, it is seen that various solutes of interest to biochemical systems may have opposite effects on the water structure.

The typical aqueous mixtures are those which predominantly seem to enhance water structure (act as structure makers). A tempting possibility for the interpretation of such structure stabilization is a model of water that allows for the discrete existence of sites or voids into which the nonpolar moieties may fit or in which the nonpolar part of the solutes

TABLE IV  
CLASSIFICATION OF SOLUTES ACCORDING TO THE RELATIVE  
MAGNITUDE OF EXCESS MIXING FUNCTIONS

Typical nonaqueous	Typical aqueous
$ \Delta H^E  > T \Delta S^E $	$ \Delta H^E  < T \Delta S^E $
Hydrogen peroxide	(Hydrocarbons)
Nitriles	Alcohols
Dimethyl sulfoxide	Amines
Amides	Ketones
Urea	Glycols
Glycerol	Ethylene oxide
Glycerides (?)	Polyoxyethylene derivatives
Polyhydroxy compounds	Pyridine bases
Carbohydrates	Hexamethylene tetramine (?)
Polyamino compounds	Ethers
	Dioxane (?)

may be locally stabilized or enhanced. A particularly useful parameter in this connection is obviously the partial molal volume. It is, for instance, well known (see the preceding section) that the molal volume of alcohols in water shows a pronounced minimum at some low mole fraction, corresponding approximately to 1 solute molecule per 20 water molecules.

The typical nonaqueous solutes will, in general, tend to disrupt the water structure, and an inspection of Table IV will show that this may well be due to the presence of multiple functional groups per molecular moiety, which may interact destructively with the intrinsic water structure. The problem of the structure of such solutions is extremely difficult, and, in fact, rather contradictory conclusions have been reached on the basis of different experimental techniques. This is important particularly to the biologist in connection with solutes such as urea and the glycerides. Table V shows the current status of interpretation of the effects on water struc-

ture of dioxane in dilute solutions. This table clearly illustrates the diversity of conclusions reached by employing different techniques.

Part of the Russian school of physical chemists appears to favor primarily an interpretation of nonpolar solutes in water as promoting structure through clathrate cage formation. On the other hand, the school, led by Samoilov (1965), is built essentially on an interstitial, ice-like model of water. The reader is referred to the papers by the Russian authors for de-

TABLE V  
INTERACTIONS BETWEEN DIOXANE (D) AND WATER IN DILUTE  
AQUEOUS SOLUTIONS

Experimental method	Type of interactions proposed	Reference
Raman scattering	Hydrogen bonds between D and H <sub>2</sub> O	Rezeav and Shchepanyak (1965)
Dielectric relaxation	D Promotes water structure	Haggis <i>et al.</i> (1952)
Dielectric relaxation	D Breaks water structure	Clemmott <i>et al.</i> (1964)
X-Ray diffraction	D Breaks H <sub>2</sub> O structure	Cennamo and Tartaglione (1959)
<sup>1</sup> H Chemical shifts	D·H <sub>2</sub> O and D·2H <sub>2</sub> O	Muller and Simon (1967)
Ultrasound absorption	D·2H <sub>2</sub> O and (D·H <sub>2</sub> O) <sub>2</sub>	Hammes and Knoche (1966)
Density, viscosity	D·4H <sub>2</sub> O and D·2H <sub>2</sub> O	Tsyplin and Trifonov (1958)
Density	D·3H <sub>2</sub> O and D·2H <sub>2</sub> O	Schott (1966)
Conductance	D·2H <sub>2</sub> O only	Trifonov and Tsyplin (1959)
Freezing points and enthalpy of mixing	D·6H <sub>2</sub> O, D·3H <sub>2</sub> O, D·2H <sub>2</sub> O	Goates and Sullivan (1958)
<sup>1</sup> H Chemical shifts	D·2H <sub>2</sub> O	Hall and Frost (1966)
<sup>1</sup> H Nuclear magnetic resonance relaxation	D Promotes structure in water	Clemmott (1967)

tails. It should be pointed out that the Russian school of physical chemists has probably provided more data than any other school on the properties of electrolytes in aqueous nonelectrolyte mixtures. The reader is also referred to the writings of Ben-Naim (1965, 1967, 1968, 1969), Arnett and McKelvey (1965, 1966), Arnett (1967), Arnett and co-workers (1969), Wetlauffer *et al.* (1964b), and Frank and Franks (1968). Finally, attention is called to the extensive studies by Symons and Blandamer (1968), Blandamer *et al.* (1969a,b, 1970) and especially Blandamer and Fox (1970). These authors have contributed greatly to the elucidation of aqueous nonelectrolyte studies in water (for instance, through very extensive studies on aqueous solutions of tertiary butanol).

#### 4. Discrete Size Effects

Before leaving the subject of nonelectrolytes in water, attention is called to possible discrete size effects of organic nonelectrolytes on the structure of water. Figure 3 shows the mole fraction solubility and the free energy of solution of the normal paraffins as a function of carbon number. This illustration is taken from a note by Franks (1966) and clearly suggests that the size of the solute affects the thermodynamic properties. This is undoubtedly of signal importance in understanding aqueous solutions of biologically important materials such as proteins and enzymes with non-polar side chains of the sizes illustrated in Fig. 3.

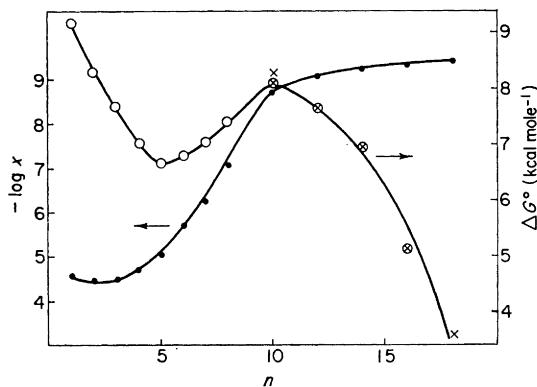


FIG. 3. Solubility (mole fraction) and free energy of solution for the normal paraffins in water as function of carbon number. (Franks, 1966, with permission from the publisher of *Nature*.)

An interesting study of the discrete effects of carbon chain length was reported by Clifford *et al.* (1965) in an NMR and Raman study of water in colloidal systems. These authors measured the initial slopes of the intensity of absorption at  $3450\text{ cm}^{-1}$  Raman band against concentration as a function of chain length. The "hydrocarbon" was a sodium alkyl sulfate. Between C-4 and C-6, a very notable, abrupt change occurred in the observed curves, suggesting discrete effects of solutes below a certain critical size. Even more impressive are the frequency shifts in the Raman spectrum. The authors note that the Raman measurements show the occurrence of two different types of behaviors: for C-2 and C-4 sulfates, the behavior is notably different than for C-6 and C-8. The conclusion is that C-6 and C-8 sulfates create a more powerful H-bonded solution than water alone. As a result, the authors suggest that the Raman results are consistent with the NMR chemical shift data if one assumes an increase in the degree of covalency of the hydrogen bonding. The authors also observe that the

range between C-4 and C-6—the point where the abrupt changes are observed—corresponds to the carbon chain lengths where the transition to micelle-forming soaps occur.

#### D. SOME SPECIFIC STRUCTURAL MODELS

##### 1. *Hildebrand on "Icebergs"*

Not everyone agrees with the interpretation of water structure in terms of a clathrate or latent clathrate-like stability for water, nor is there unanimous agreement that some nonpolar solutes, such as methane, induce clathrate cagelike structures in their immediate vicinity. Few people have challenged the concept of clathrate stabilization in solution more succinctly and eloquently than Hildebrand (1969). This can be well illustrated by the following quotation from his paper concerning the relative diffusivities of methane in water compared to carbon tetrachloride:

- \* Methane diffuses in water at 25°C, 3/5ths as fast as it does in carbon tetrachloride. Since diffusivity depends mainly upon temperature, viscosity of the solvent, and molecular cross-sections of the diffusant, and since viscosities of H<sub>2</sub>O and CCl<sub>4</sub> at 25°C are almost identical, one may infer that molecules of CCl<sub>4</sub> in H<sub>2</sub>O are not imprisoned in "icebergs," and are retarded only by hydrogen bonds, not by encounters with "ice-like" aggregates.

Hildebrand's notion becomes particularly intriguing when compared to some recent neutron inelastic scattering experiments by Franks *et al.* (1971). The studies of dilute solutions of *t*-butanol in water have almost conclusively proven that the solute does, indeed, enhance the water structure. Whether or not the data can best be interpreted by invoking a clathrate hydrate structure remains to be seen. It is possible that the solute merely stabilizes (in time) whatever particular intrinsic water structure is present. Franks and co-workers introduce the term "glass-bergs" to connote that the structural entities are not stabilized Ice-Ih-like elements, but in a sense, perhaps, have rather "amorphous" structural arrangements. Stability then means merely that the time for exchange between neighboring positions in this "vitreous lattice" has been lengthened considerably. Franks and co-workers emphatically point out that the induced water structures bear no resemblance to (ordinary) ice. This should be compared to the unpublished X-ray scattering data by Lipscomb, quoted by Lumry and Rajender (1971), which suggest that the water associated with proteins may show Ice-Ih-like characteristics.

The existence of the crystalline solid clathrate hydrates has provided some of the most "inspirational evidence" for the possible occurrence of

clathrate-like entities in aqueous solutions—and possibly in pure water itself. This approach has been particularly advocated by Glew and co-workers (1968), who have studied the properties of aqueous solutions of acetone, ethylene oxide, propylene oxide, tetrahydrofuran, dioxane, and *t*-butyl alcohol. Anomalous properties of these solutions occur for solute concentrations between 3 to 6 mole percent. For all six solutes studied, notable downward field shifts were obtained in the NMR studies of the water, suggesting a stronger water–water hydrogen bonding. The water-to-solute ratios giving rise to maximum effects correspond to coordination numbers of the solutes of 24 to 28 water molecules similar to the structure-II clathrate hydrates. See also Section IV,C,4.

## 2. *NMR Information on “Icebergs”*

As mentioned in the introduction, NMR is playing an ever-increasing role in the study of the structure of water and aqueous solutions. To this field, Hertz and co-workers (see Hertz, 1970) have contributed notably. Recently, Hertz *et al.* (1969) have studied the tetraalkylammonium ions in aqueous solutions. These ions have commanded particular interest as they provide a water–nonpolar hydrocarbon interface. Thus, clathrate hydrates of the tetraalkylammonium halides are well known, and it would be expected that all such ions would be capable of engaging in clathrate-like formation in solution. Yet, from the study of the self-diffusion coefficient of the water in solutions of aqueous tetraalkylammonium chlorides, it was found that there was no evidence for rigid, long-lived hydration shells. Similar results were obtained with *t*-butanol, tetrahydrofuran, and acetone. In view of the extensive and very convincing studies by Symons, Blandamer and co-workers, and Arnett and co-workers in this country, it is difficult to resolve the conflict that by one measuring technique, no large hydration effects are obtained, whereas other techniques seem to give results which can be interpreted only in terms of rather extensive hydration effects.

## 3. *Clathrates versus Ice Structure*

As stressed before, it is remarkable that it has not been possible to prove or disprove firmly any of the various theories proposed for water structure. In particular, it would seem possible that discriminating experiments should be able to determine, for instance, whether or not the structure of bulk liquid water is best described in terms of a clathrate-like mixture model. The clathrate cage model is one of two different mixture models which provides for separate discrete voids or sites in a pseudo-lattice (short-ranged order) in water. The other model for water which allows for

discrete sites is the one assuming the existence of elements of Ice-Ih like-ness between adjacent hexagonal rings, forming a closed, hexagonal, cylindrical microprism; this model implies that discrete and rather large sites may occur. This view has been advocated and exploited by Samoilov (1965). Thus, experimental data tending to suggest discrete effects of solute size upon the properties of aqueous solutions might be taken as evidence for either a clathrate-like or an ice-like model. To distinguish between these two possibilities, however, it would be natural and necessary to look for other characteristics of the respective structured elements, such as the fivefold symmetry occurring in the individual sides of many polyhedra of the clathrates (for instance, in the pentagonal dodecahedron) compared to the hexagonal symmetry of the basal plane configuration in the hexagonal Ice-Ih lattice. Experimentally, a search for symmetry elements is not easy.

If anomalous, abrupt changes occur in solution behavior as a function of the size of the solute molecule, this may perhaps be taken as indication of a "fit" of the solute into a discrete void, but it must, of course, also be kept in mind that the effects observed must be distinguished from specific hydration effects, for instance, due to functional groups.

Helium apparently does not form a clathrate hydrate—undoubtedly because it is small enough to permit this atom to diffuse freely through the confining walls of a host lattice. On the other hand, whereas methane, ethane, propane, and butane form clathrate hydrates quite readily, pentane does not appear to form such compounds. This may be interpreted as due to the ability of the classically recognized clathrate cages to accommodate molecules as large as a four-membered ( $\text{iso-C}_4\text{H}_{10}$ ) hydrocarbon chain, but not a five-carbon atom chain.

### III. Structure of Water near Interfaces

#### A. TRADITIONAL VIEW

In the field of water structure, as in many other intellectual endeavors, some virtue has been ascribed to the adherence to the principle of maximum intellectual economy (*simplex sigillum veri*). This has led to the construction of many theories of interfacial phenomena, based entirely on the concept that the structure of water remains unchanged from the bulk up to say, one, or at most, two molecular diameters from the surface. In Section C below is summarized some of the available evidence which suggests that this assumption is untenable, even for such simple systems as

air-water, mercury-water, or "general solid"-water interface. Instead, there appears to be strong evidence for notable structural rearrangements of water adjacent to almost any interface be it a solid-water, water-immiscible liquid, or air-water interface.

### B. GENERAL APPROACH TO STRUCTURAL ORDERING NEAR INTERFACES

Most previous authors have not challenged the traditional view of aqueous interfacial structures; that is, they have ignored the possible existence of structural ordering in water near an interface. Among those who have taken issue with this oversimplified view are notably Derjaguin (1965) in Russia and Henniker (1949), Low (1961), and the present author (1969b) in this country. The traditional evidence for structuring in liquids, particularly for water adjacent to an interface, has come from measurements of viscosity of liquids in narrow pores, anomalous diffusion coefficients or energies of activation for ionic conduction in capillaries, etc. Recently, the present author has added to this type of evidence an additional, independent set of arguments for demonstrating the probable structuring of water near interfaces.

### C. EVIDENCE FOR VICINAL ORDERING BASED ON THERMAL ANOMALIES

#### 1. *Illustrative Examples*

The new evidence is based on the existence of thermal anomalies in the properties of vicinal water. It appears that these anomalies can best be interpreted as manifestations of cooperative phenomena (order-disorder phenomena), requiring the stabilization of structures which form cooperative, large-scale entities among the molecules involved. The cooperativeness is reflected in the abrupt temperature responses of these layers. Some typical examples will demonstrate this phenomenon.

*a. Disjoining Pressures.* Figure 4, taken from a study by Peschel and Adlfinger (1967), is an example; in this illustration the disjoining pressure of water between two quartz plates (separated 100 Å) is plotted as a function of temperature. The disjoining pressure is a measure of the repulsive forces between two surfaces and obviously depends on the structure and general properties of the intervening liquid layer. It is seen that the disjoining pressure does not vary in a simple, "regular" manner with temperature; on the contrary, notable anomalies are observed. It is of interest to call attention to the fact that the temperatures at which maxima are encountered in the disjoining pressure are close to 15°, 30°, and 45°C. As

will be discussed below, these temperatures do, indeed, have great importance for biological systems. We postpone at this time a discussion of the possible implications of this and proceed to discuss other examples of such thermal anomalies.

*b. Low-Frequency Mechanical Damping.* Figure 5 shows the half-life of mechanical vibrations of an oscillating quartz capillary filled with water. The data are from a study by Forslind (1966). It is seen that the half-life of the vibrations (of a capillary tube filled with water and oscillating in a vacuum) goes through a notable maximum near 30°C. Similar results have been obtained by Kerr (1970). In this connection, attention is

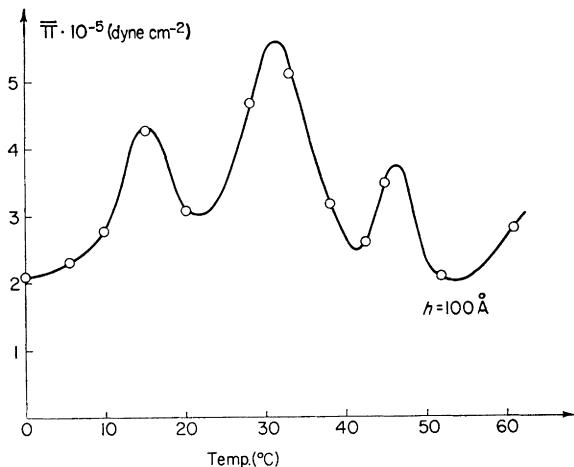


FIG. 4. Disjoining pressure of water between quartz plates: separation between plates, 100 Å. (Peschel and Adlfinger, 1967.)

called to the previously discussed values for the (apparent) entropies of surface formation of pure water (Drost-Hansen, 1965b). In the latter case we obviously deal with an interface which is vastly different from the quartz-water interface; however, the entropy of surface formation was shown to exhibit a notable maximum also near 30°C!

We are here dealing with three different, specific examples, all showing the existence of extrema near 30°C in the properties of vicinal water. The explanation of these anomalies may be relatively simple: below 30°, one particular structure of water is stabilized by the proximity to a surface or an interface (regardless of the nature of the interface), whereas somewhere above this temperature, a different structure prevails (i.e., a different structure is energetically favored). Around, say, 29–32°C, neither of these structures predominates, i.e., they lack stability. A transitional region

exists, most likely characterized by enhanced randomness due either to increased numbers of "monomeric" water molecules or, at least, to smaller structural entities. Thus, if this hypothesis is correct, we should expect increased disjoining pressure as the result of increased kinetic energy of a larger number of smaller, discrete, kinetic entities (due to the lower degree of structuring—possibly the enhanced concentration of monomers). Furthermore, we should expect the mechanical coupling to the wall of the vibrating quartz capillary to be greatly reduced because of the smaller, vicinal, anchored structural entities and, hence, expect the half-life of the vibrations to be notably increased, as is, indeed, observed. Finally, if more disorder prevails at this temperature, one must expect higher

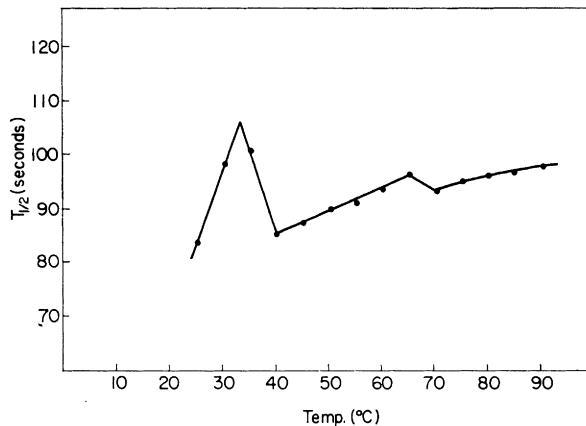


FIG. 5. Viscous damping of water in vibrating hairpin capillary. (Data by Forslind, 1966.)

entropy of surface formation and this has been reported earlier (see Drost-Hansen, 1965b; also Bordi and Vannel, 1958, 1962; Cini *et al.*, 1969; but compare Gittens, 1969).

c. *Surface Viscosity.* As a final example of the occurrence of a thermal anomaly in a relatively well-defined aqueous (nonliving) system, we mention some recent data by Peschel and Adlfinger (1967).

Using an experimental approach somewhat resembling that previously used for the study of the disjoining pressure, Peschel and Adlfinger measured the apparent viscosity of water between two closely spaced quartz plates. Their results are shown in Fig. 6. It is seen here that anomalies are observed in the viscosity of water near interfaces (determined for different plate separations) in the vicinity of 15°, 32°, and 45°C. Indeed, the present example, as well as the study of the diffusion constant for thiourea in water by Dreyer *et al.* (1969), discussed below, clearly indicate the oc-

currence of structural transitions in vicinal water affecting different transport properties.

*d. Diffusion Data and Activation Parameters.* Elsewhere in this chapter we mentioned that Hildebrand, on the basis of measurements of the diffusion constant for methane in water and carbon tetrachloride, concluded that there was no evidence for iceberg formation or other structuring of the solvent around the diffusing solute molecules. The measurements by Hildebrand refer only to one temperature; thus, the energy of activation for the diffusion process is not known. It is certainly conceivable that the process of initiating the displacement of the solute molecule—resulting in

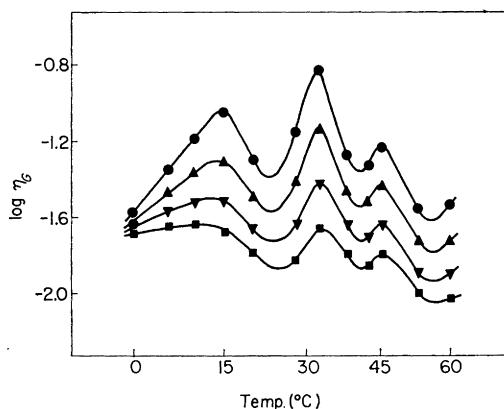


FIG. 6. Surface viscosity as a function of temperature for various separations between two adjacent quartz plates. Plate separations: 300 (●), 500 (▲), 700 (▼), and 900 Å (■). (Data by Peschel and Adlfinger, 1969.)

the diffusion process—hinges on the (rapid) breakdown of one hydrogen bond only in a structured cooperative entity. The subsequent events might well be the fast breakdown of the entire structured entity and the overall rate and its temperature dependence would, therefore, be dependent only on the one individual molecular happening—the initial breakdown of a single H-bond.

The statement made by Hildebrand regarding the diffusion mechanism in water obviously applies only to a bulk phase. Recently, very strong evidence has been obtained for anomalous diffusivities in water near interfaces. Dreyer *et al.* (1969) have measured the diffusion coefficient of thiourea in water over the temperature range from 2° to 65°C using a novel pulsation diffusion method. In this method, the system under study (i.e., the interface across which the diffusion takes place) is continually deformed and enlarged to enhance greatly the net rate with which the dif-

fusant is transferred in the concentration gradient. The results of this study are shown in Fig. 7. It is seen that anomalies occur near 17°, 28°, 43° and 60°C; that is, near the temperature where the thermal anomalies have been observed in general for vicinal water. It is obvious from this graph that four thermal anomalies are manifested in the data and that in this case it is useless to try a simple Arrhenius- or Eyring-type rate equation for the analysis of the activation parameters involved. We stress that the measurements are not representative of bulk water, but rather of a solution adjacent to an interface—the capillary wall (capillary diameter  $\sim 80$

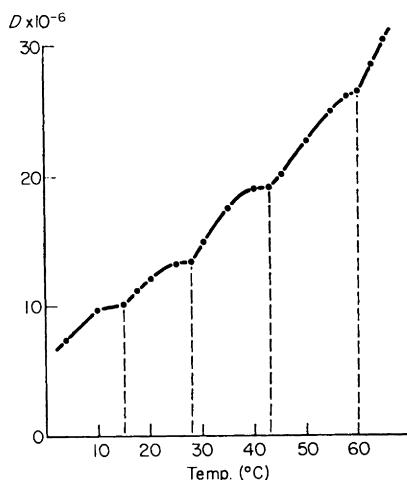


FIG. 7. Diffusion coefficient of thiourea (0.3 gm/liter) in water. Abscissa—temperature; ordinate—observed diffusion coefficient. (Dreyer *et al.*, 1969, with permission from the publisher of *Naturwissenschaften*.)

$\mu$ ). The authors correctly point to the importance of this phenomenon for the understanding of the properties and structure of water adjacent to membranes or macromolecules. (However, in the opinion of the present author, but contrary to that of Dreyer *et al.*, the results are not necessarily related to the anomalous water reported by Derjaguin.) Since the observed anomalous diffusion coefficients do, indeed, represent an interfacial phenomenon in an aqueous system, similar anomalies may well be expected to occur in many other systems including and, in particular in biological systems.

Elsewhere in this chapter, we shall point to the use and misuses of rate data in biochemical and biological studies. The data by Dreyer *et al.* strongly suggest that caution is necessary in the analysis of rate data on interfacial systems. The studies by Good (1960, 1961a-d, 1967) and Cold-

man and co-workers (1969a,b; Coldman and Good, 1967, 1968a,b, 1969; see below) appear to be reasonable attempts to use an Eyring rate expression for analyzing molecular aspects pertinent to the nature of the activated complex. Even so, in one of the graphs shown by Good (1960), it is also seen that the rate data themselves, in an Arrhenius plot, do not necessarily follow a straight line very precisely. On the contrary, there are systematic deviations, and, although it could be argued that these deviations individually might be within the experimental error, it is also possible that this is another instance where trends in the data cannot be neglected. As an example of an overt abuse of an Arrhenius rate expression, we discuss separately the study by S. M. Johnson and Bangham (1969) where it is obvious that very persistent, notable trends in the data have been ignored and obviate any detailed interpretation of the data, arrived at by averaging over a wide range of temperatures.

For many years, advances in the understanding of thermal anomalies in the properties of water were hampered by the erroneous notion that the anomalies were manifestations of sudden changes in the properties of bulk liquid water in general. Very careful measurements, for instance, by Senghaphan *et al.* (1969), Cini *et al.* (1969), Rushe and Good (1966), and Korson *et al.* (1969) proved that thermal anomalies are absent in the bulk properties of pure water. However, thermal anomalies do exist, but, as emphasized specifically by the present author (Drost-Hansen, 1969a), the anomalies are manifestations of changes in the properties of water near interfaces. Thus, those experiments which may conceivably be influenced by the proximity to a surface, such as viscosity measurements in narrow capillaries, are likely to show the existence of the unusual temperature dependencies, but bulk properties are undoubtedly not involved, at least not for pure water (for solutions, see discussions by the present author elsewhere 1967a). However, there is little doubt now that the thermal anomalies are, indeed, manifested in the properties of water near interfaces—the vicinal water. The origin, as discussed in the present chapter, is probably related to higher-order phase transitions. It is emphasized again that (in the absence of large solutes in appreciable concentrations) the phenomenon is probably restricted to water near interfaces, but such water may, indeed, be extensively structured.

*e. Influence of the Substrate on Vicinal Structure. (The “Paradoxical Effect”)* With regard to water near interfaces, it is important to distinguish between the different effects of various types of solids, just as the nature of hydration of solutes in aqueous solution depends on the nature of the solute. Structurally different types of arrangements are expected adjacent to nonpolar-, polar-, or ionic-type interface. The important aspect is the fact that in all cases, structural changes are apparently in-

duced and the structured zones may extend over many molecular diameters from the surface. It should be stressed also that these effects appear to be superimposed on the discrete separate effects of charged double layers (and other interfacial phenomena traditionally accounted for in colloid chemistry).

There exists presently a paradoxical state of affairs with respect to the occurrence of anomalies in the surface properties of aqueous systems. It is quite certain that anomalies occur in the temperature dependence of many properties of vicinal water, undoubtedly related to structural anomalies; however, these anomalies are observed in properties of water adjacent to highly dissimilar interfaces. Thus, thermal anomalies are found in the properties of water near the air-water interface, the mercury-water interface, the decane-water interface, many types of silica-water interfaces, the lead iodide-water interface, and the polyvinyl toluene-water interface, as well as the interface between water and many other substances. However, notwithstanding the enormous diversity of the chemical nature of the substrates, the anomalies are usually observed to occur at or near the same temperatures for all the systems under consideration. That the effect is, indeed, a surface effect rather than a bulk effect appears certain from the observation that the larger the surface-to-volume ratio of the system, the more pronounced are the observed anomalies. It seems paradoxical that the specific nature of the nonaqueous part of the interface should play practically no role in determining the temperatures of the thermal transitions. The only explanation which can be offered at this time is to assume that, at least for water near solids, the effect of the interface on the structure of the water is primarily to act as a "momentum sink." Thus, structures which are only latently present in bulk water may become stabilized near the interface; in other words, the interface may act as a momentum sink for thermal fluctuations which in the bulk would have led to the disruption of the structured entity. There are some indications, however, that specific, if minor, influences of the substrate are superimposed on the general tendency for structure stabilization near any water-solid interface. It would, indeed, be hard to see how the water immediately adjacent to, say, a lead iodide crystal (with its ion-water dipole interactions) could be identical to the nature of the water adjacent to a lipid or a hydrocarbon.

In connection with the "paradoxical effect" some recent NMR results by Woessner (1971) are of interest. For about a decade, Woessner (1966) and Woessner and co-workers (1963, 1968; 1969a,b; 1970a,b) have studied water near interfaces, particularly of minerals (quartz and clays). For such materials, Woessner has concluded that the water is preferentially oriented immediately adjacent to these surfaces—or within a few mo-

lecular layers from these interfaces. I have also discussed interactions at quartz and clay surfaces (Drost-Hansen, 1969b), but the conclusions by Woessner differ somewhat from mine, at least regarding the extent of the possible structuring of the vicinal water. However, it is of interest that Woessner noted some unifying features for the orientation of water near a variety of interfaces. The measure of orientation employed by Woessner is the quantity

$$(3 \cos^2\theta - 1)_{av}$$

where  $\theta$  is the angle between the molecular axis and the direction of the magnetic field. The systems studied by Woessner are such that this quantity is non-zero for sufficiently long times. Woessner observed that the ratio of values for  $\theta$  differs between  $D_2O$  and  $H_2O$ , but that the ratio is "very nearly the same for sodium hectorite clay, collagens, Li-DNA, and, within a large experimental error, for Rayon." This result appears to agree with the conclusion from the study of the thermal properties, namely that the anomalies occur in vicinal water at almost identical temperatures, regardless of the specific nature of the material in contact with the water (the "paradoxical effect"). Woessner also points out that the proton  $T_1$  minimum for water, adsorbed on montmorillonite, occurs at about the same temperature as it does for proteins. These results, therefore, appear to show that the presence of a surface is generally more important than its specific nature in determining some of the dynamic and structural aspects of the water at or near the interface (Woessner, 1971, personal communication).

Some anomalous temperature dependencies in the proton transverse relaxation times of water adsorbed on silica gel are shown in Fig. 2 and Fig. 6 from one of the papers by Woessner (1963). Without the notion that thermal anomalies exist in vicinal water, the observed results might easily have been attributed merely to experimental uncertainty and "noise." However, while the two examples from the study by Woessner certainly do not prove the existence of the structural transitions, the idea of thermal anomalies does offer a logical explanation for the observed results.

#### D. SOME SPECIFIC STRUCTURAL MODELS OF WATER NEAR INTERFACES

We have discussed some of the evidence for the existence of thermal anomalies in the properties of water near interfaces. This evidence suggests the occurrence of higher-order phase transitions which, in turn, are taken as evidence for the existence of large, ordered, cooperative arrangements of water molecules. It remains to speculate on the structure and

extent of such ordered groupings of water molecules. The reader is referred to Drost-Hansen (1969b) for a more detailed set of speculations; only a few paragraphs regarding vicinal water structure are quoted here:

The present author believes that the model for the structure of water which is most likely to prove correct is one involving the existence of discrete, structural elements. Most likely candidates as structural units are those associated with a mixture model containing structured clusters such as clathrate cages or high-pressure ice polymorphs in equilibrium with monomeric molecules. Here, as always, the reader is warned against the danger of generalizing the notion of structural entities to mean "discrete, permanent, microcrystalline chunks" of one form of lattice or other. Recently, Eisenberg and Kauzmann (1969) have given a good discussion of the very meaning of the term "structure" as it applies to liquid water. Suffice it to say that, both on general grounds, as well as in view of the structural implications of this paper, we shall adopt a conceptual model of water involving structured elements and discuss the likely occurrence of specific structured entities adjacent to various interfaces. Thus, notwithstanding the recent criticism of the utility of the concept of water structure in general (Holtzer and Emerson, 1969) the use of structural models appears highly practical for the discussion at hand. Again, it must be emphasized that the structural entities envisioned are not "stable, permanent, microcrystalline chunks"; rather, they are distorted appearances of elements with identifiable symmetries or other structural features, possessing the characteristics of flickering clusters. In other words, they may suffer frequent disruptions due to thermal motions, but when recreated elsewhere in the liquid volume under consideration, they again present a preponderance of one structurally characteristic element or another; for instance, the occurrence of pentagonal rings or similar geometric, identifiable features. Undoubtedly, the essential point in this discussion is the temporal stability of such identifiable characteristics. When discussing structure near an interface, the implication is only that the structure under consideration may have a lifetime which is notably longer than the corresponding lifetime of a structured entity in the bulk of the liquid. It remains to be determined whether this means an increase in the relaxation time by an order of magnitude (say, from  $10^{-11}$  to  $10^{-10}$  second), or from an increase corresponding to the increase in (dielectric) relaxation time on going from water to ice. Undoubtedly, future refinements of dielectric and NMR studies will throw considerable light on this question.

We finally attempt a "molecular" interpretation in terms of some conceivable vicinal structures. Figure 8\* shows a possible interfacial structure for water near a polar surface. The water molecules near the solid are oriented by dipole-dipole interactions. These interactions are not likely to propagate over very many molecular diameters. Sufficiently far removed from the surface are the structures in bulk water. In Figure 8 these are indicated by pentagonal circuits and partial pentagonal outlines. These outlines are intended to convey only the presence of geometrically identifiable structural entities (but not necessarily structures related to pentagonal dodecahedra or other clathrate-like units) be they clusters, clathrate cage-like or high-pressure ice polymorphs. It

\* Figure numbers have been changed to conform to the numbering in the present chapter.

is suggested that the structured entities are in equilibrium with monomeric water molecules (indicated by arrows) and possibly present voids or "sites" which may or may not be occupied by individual water molecules (the Pauling and the Frank-Quist model for water). The intermediate zone is the disordered transition between the ordered vicinal water structure and the differently structured bulk. Similar conceptual models can easily be constructed for the other cases discussed in the preceding paragraphs; one such case is shown in Figure 9.\*

It is seen that only limited progress can be expected regarding details of vicinal water structure until a general theory of bulk water structure has been

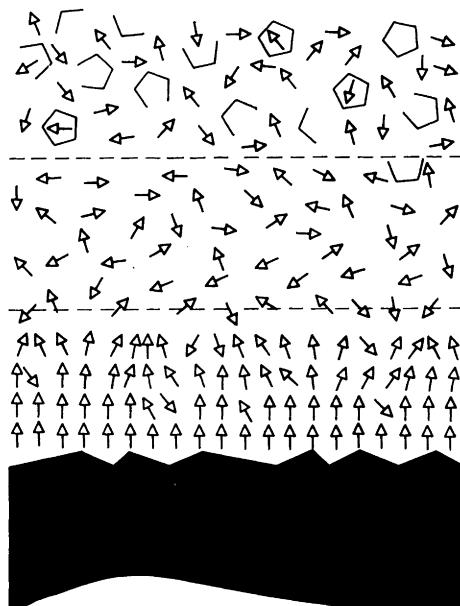


FIG. 8. Highly schematized model of water adjacent to a polar (or ionic) solid surface. Ion-dipole orientation in the vicinal layer. Note possible occurrence of disordered transition zone between ordered, oriented dipoles and bulk water. (Reproduced with permission from *Industrial & Engineering Chemistry*.)

reached. However, certain features of water near interfaces may still become apparent before a more complete understanding of bulk water is achieved.

#### IV. Structural Aspects of Water in Biological Systems

##### A. STRUCTURAL ROLE OF WATER IN GENERAL

###### 1. Szent-Györgyi on Vicinal Water

Historically, Szent-Györgyi was probably the first to lend stature to the notion of ordered water structures as important elements in biological

functioning. Earlier, Jacobson (1953, 1955) had been concerned with the possibility of ordered water ("liquid ice") in biological systems, but no one showed greater insight than Szent-Györgyi when he stated (1957):

We can thus suppose water structures to be built around dissolved molecules, structures which may have a different crystalline structure according to the polar or non-polar nature of the atomic groups on that molecule and the mutual distance of these groups in relation to the lattice constants of the different possible water crystals. It is believable that different spacing pro-

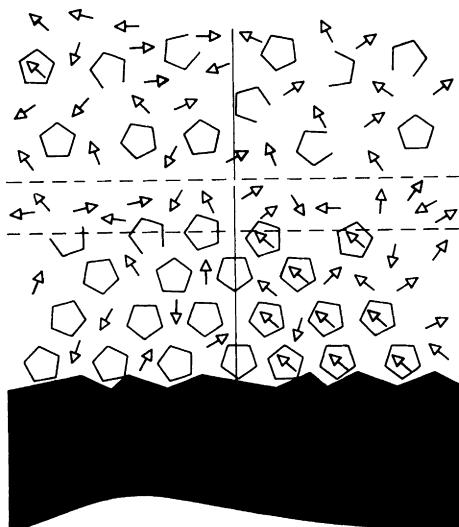


FIG. 9. Highly schematized model of water near nonpolar solid surface. Note the possible enhanced stabilization of clathrate-like entities. (Reproduced with permission from *Industrial & Engineering Chemistry*.)

motes different crystal forms or, if unfavorable, inhibits order and lattice formation. Possibilities are rich, relations complex.

Without ignoring the notable contributions of individual researchers (such as Jacobson and others), it is probably fair to say that in the period since World War II, the most inspired attack on the problem of water in biological systems was that led by Szent-Györgyi. Indeed, it became a great deal easier to pursue the idea of ordered water structures in biological systems under the banner of Szent-Györgyi's writings. Much has been added to the relatively simple, qualitative speculations of Szent-Györgyi—especially by Klotz, Lumry, Fernandez-Moran, Glassel, Warner, Hazelwood, and Ling. The writings by these authors have now become so numerous and detailed that the reader is directly referred to the more

important, individual contributions of these authors for details. In the following paragraphs, however, I have summarized some of the major facets of these studies.

Szent-Györgyi (1971) has recently speculated on the role of water structure in an article aptly entitled "Biology and Pathology of Water." The unique ability of water to form an impressive array of different structural arrangements has been stressed in this chapter, together with the use of such possible arrangements to accommodate the different environments possible in biological systems. Szent-Györgyi stresses essentially the same idea when he observes "water can thus play an important role not only in structure and physical properties, but also in function and in pathology. The wonderful many-sided reactivity of water, the ease with which it can form bonds of low energy, and the ease with which these bonds can be broken, may take us a long way toward the understanding of the wonderful subtlety of life and its reactions."

## 2. Dynamic Aspects; Flickering Clusters

In discussing structural aspects and ordering effects near interfaces, it is easy to lose sight of the truly kinetic nature of all molecular systems. Indeed, it is important to remember the "traffic" approaching and across an interface—not only of the solutes which may permeate the cell wall, but particularly of the solvent molecules. In the case of greatest imaginable stability of a possible (if unlikely) ordered, vicinal system, consider ordinary ice (i.e., the Ice-I<sub>h</sub> lattice). The separation between neighboring water molecules is 2.76 Å, but the thermal amplitude just below 0°C is 0.25 Å or approximately one-eleventh of the equilibrium distance! At the other end of the spectrum, compare, for instance, the traffic across an air-water interface of an area equal to the average area covered by one water molecule: the number of collisions per second is of the order of one hundred thousand (arriving at and being reflected or exchanged across the interface). This rapidly fluctuating environment does, however, still allow for the existence of structures and one may (and, indeed, one must) retain the concept of structures near interfaces; these structures probably differ from those which predominate in the bulk solution. However, we stress again that the situation is an exceedingly dynamic one with the vicinal structures suffering many types of distortions and fluctuations, consistent with the notions implied in the Frank model of the "flickering clusters." [In this connection, see the discussion of the concept of structure in the text by Eisenberg and Kauzmann (1969) and also the paper by Drost-Hansen, (1969b).]

### 3. Sizes of Clusters

Berendsen (1966) has made some simple estimates, based on fluctuation theory, of the probability of clusters of various sizes in water (at 0°C). The results are shown in Table VI. From the data in this table, Berendsen concludes that the probability is only of the order of one in thousands that clusters in the size range from 50 to 100 molecules will occur; thus, Berendsen concludes that the concentration of such clusters is probably insignificant. However, as mentioned elsewhere in the present chapter, the effect of the interface is likely to stabilize structural arrangement in the liquid through the influence of the substrate, acting as a momentum sink for thermal fluctuations which might otherwise disrupt the lattice stability. [In this connection, see also the brief discussion of the stabilization of cytostatic swarms in the monograph by Frenkel (1955).]

TABLE VI  
PROBABILITY OF "ICE CLUSTER" FORMATION IN WATER AT 0°C

Number of molecules $N$	Density			Enthalpy			
	Standard deviation (gm/cm <sup>3</sup> )	Ratio deviation to SD	Probability (%)	Standard deviation (kcal/mole)	Deviation $N \times 1.44$ (kcal/mole)	Ratio deviation to SD	Probability (%)
10	0.0815	1.0	32	10.5	14.4	1.4	16
20	0.0576	1.5	14	14.8	28.8	2.0	5
50	0.0365	2.3	2.2	23.4	72	3.1	0.2
100	0.0258	3.2	0.14	33	144	4.4	0.001

### 4. Hechter's Survey—The Best of Two Worlds

In this section we give a brief (and slightly dated) review of the progress along the lines presented by Hechter (1965) at the symposium on "Forms of Water in Biologic Systems". However, before summarizing Hechter's analysis, it should be noted that the advance made by Hechter is essentially in the nature of a compromise. Hechter proposed that it is necessary for the understanding of water in biological systems to operate conceptually with both (a) specific processes, such as in membranes (and in particular, with the role of water in an individual, specific, and, hopefully, relatively simple membrane transport process) and (b) a "general structuring" of all (or most of) the water in the interior of the cell. Thus, it is implied that all (or nearly all) the water of the cell is influenced by the proximity to an interface and that this water is more highly ordered than bulk water. As indicated, superimposed on this idea is the adherence to the possible existence of specific active transport processes across the cell

membrane. The idea that one may combine elements from either of the two (often sharply differing and at times, opposing views), is not acquired cheaply! As stressed elsewhere in this chapter, an allowance for an additional degree of freedom in model making must be paid for by an unusual degree of accomplishment by the proposed theory.

Hechter's basic view is that the "monolithic dichotomy of either/or" must be replaced by the conceptually far more difficult "also/and" pluralism. The either/or dichotomy refers to the membrane thesis of transport for cellular control as opposed to the "holist" theory which invokes the more-or-less complete intracellular ordering of the water of the cells and its ability to determine and effect the necessary thermodynamic and kinetic properties. Biochemically, the choice is to select between accepting an active transport, driven by metabolic energy through various transmembrane pumps, or simply to invoke changes in activities of solutes due to different aqueous environments. The latter would require far less metabolic energy; the energy requirements would now be primarily those needed initially to order the entire system under consideration, but would require very little energy expenditure for the continued maintenance of that state. In other words, the energy is not directly coupled to the transmembrane solute flow.

Having delineated the opposing views of the two major attitudes toward cellular functioning, Hechter makes the reasonable suggestion that facets of both approaches may be required for the complete description and understanding of cellular physiology. It is obviously difficult to argue against such a conciliatory attitude, but as stressed above, acceptance of a "combination approach" requires that the success of the approach be more complete than either of the individual avenues of approach.

The present author is, in a sense, willing to "pay the price" for the combination (unified) approach. He proposes specifically to pay in the currency of the ability, *sui generis*, of water to exist in vastly different physical forms under conditions that are physically almost identical. We mention briefly in this chapter the remarkably small energy differences between the high-pressure ice polymorphs, as one example of the versatility of water structuring, and added to this, the different types of clathrate cage structures available.

Another possibility has been advocated by Hazlewood (1971) who suggests that active transport may operate where ion transfer occurs from low to high concentration between two *bulk* phases, while transfer from the bulk outside solution to the intracellular protoplasma may be affected by the structuring of the water inside the cell. See the discussion in Section V, G.

We may conceptually combine (and likely confound) the approach to

the understanding of water structures in biological systems by considering a two-dimensional array of possibilities. Consider laterally displayed the possible major types of water structures (ice polymorphs, clathrates, etc.) which may be stable near an interface (even if not necessarily thermodynamically stable as bulk phases), while considering the various cellular environments vertically displayed, as envisioned by Hechter (1965):

... (a) water *within* the membrane structure; (b) one or more layers of water directly in contact with extended relatively immobile macromolecular surfaces, be it at the surface of membranes or highly ordered structural components of certain cell types (e.g., the contractile system in muscle fibers), or filaments of DNA in the chromosomes of the nuclei; (c) water *between* closely paired systems of unit membranes, as in the aqueous region between the interior and outer membranes of the mitochondria, or in the cisternae of the endoplasmic reticulum; (d) water within interior chambers of an organelle, like the mitochondrion or between the structural nucleoprotein components of the nucleus; (e) water in the hyaloplasm between the various organelles in the cell.

Consider further that this array should probably be extended in more than three dimensions, by adding, as independent variables, temperature, pressure, and the nature and concentration of solutes. Perhaps rather than despair over the fairly modest progress which has been made up to the present, one might be encouraged by the fact that—in the face of such enormous possible complexity—any degree of understanding has been achieved at all! Add to these problems the possibility that the systems may not be in thermodynamic equilibrium, but, at best, in a steady-state condition or merely in a diurnal metastable condition; it is possible also that hysteresis effects (thermal memory effects) occur (see Section VI,J), as well as, almost certainly, sheer rate-dependent phenomena.

For the other half of the combination approach, one must adopt the notion that active transport processes also play an important role in determining biological functioning. Here, the essential feature is that such processes are driven through metabolic energy. Note, however, that this does not exclude a secondary and possibly important role of vicinal water in this nonholist part of the combination approach—as stressed in Sections IV,A,9 and V,B,6,e, water structure undoubtedly plays a crucial role in membrane functioning because of the mutual interaction between the stabilized water structures (near the interface) and the substrate itself (the membrane).

##### 5. Ling's Theory

The studies by Ling clearly exemplify the holist attitude toward water structure in biological systems. Ling's theory is a more general theory based on an “association–induction” hypothesis. This has been particu-

larly elaborated on in the monograph by Ling (1962) and summarized in his contribution to the symposium "Forms of Water in Biologic Systems" (1965), from which we quote:

The association-induction hypothesis which deals with a broader topic agrees in essence with Troschin's sorption theory concerning ionic and non-electrolyte distribution problems, although the two theories were developed independently. The association-induction hypothesis offers, however, specific molecular interpretation of the differences in solubility properties of the cell water in terms of restricted rotation of poly-atomic non-electrolytes and de facto poly-atomic hydrated ions and of differences in the H-bond formed in the protoplasmic system. The theory also stresses that the living protoplasm and hence, protoplasmic water, does not exist in one single physical state, but as a rule, exists reversibly in more than one metastable cooperative state in the course of its normal physiological activity. Anticipating the evidence to be presented, we may state that it is our purpose in this paper to demonstrate that all or nearly all water molecules in a living cell can be considered to exist as polarized multi-layers oriented on the surfaces of cell proteins.

### *6. Derjaguin's Structured Water*

In connection with water in biological systems, Derjaguin (1965) reviewed some of the anomalous properties of water near interfaces in the symposium "The State and Movement of Water in Living Organisms." Derjaguin mentioned the measurements made by Metsik and Aidanova (1966) of the thermal conductivity of parallel stacks of mica sheets with layers of water of various thicknesses. Derjaguin points out that the average conductivity of these water layers increases as the thickness decreases, reaching a value "several dozen times higher" than the bulk conductivity for thicknesses of the order of  $0.1 \mu$ . Derjaguin also points to the dielectric studies by Zhilenkov (1963), in which it was found that the dielectric constant for the second layer of water on silica gels is only 8 to 10 and this value remains unchanged upon further addition of water up to as many as 10 or 20 layers. In other words, the water appears to have lost its orientational ability, thus, reducing the net dielectric permeability.

### *7. NMR Results*

Notable support for the holist theory has been obtained by Fritz and Swift (1967). These authors studied the state of water in the polarized sciatic nerve of the frog using an NMR technique. The results from this study suggested that "very marked changes in the state of the intracellular water accompanied nerve polarization. If this change does occur in untreated nerves it is of great potential significance in the theory of neural phenomena...." The authors call attention to the nearly simultaneous

article by D. Chapman and McLauchlan (1967) who studied the NMR spectra for various orientations in the magnetic field of the rabbit sciatic nerve. These authors found clear indications of two types of proton environments consistent with the findings of Fritz and Swift.

An early study of water in biological systems by NMR techniques was reported by Odeblad (1959). Assuming that various possible disturbing influences are not operating, Odeblad contends that "there may be a mechanism that makes possible the arrangement of the order of, for example,  $10^2$  layers of water molecules on the cell surfaces."

Recently, Damadian (1971) published an interesting paper on the application of nuclear magnetic resonance to biological problems. He demonstrated that the proton relaxation times in malignant tumors are notably different from the values obtained for normal tissues, thus suggesting an increase in the motional freedom of tissue water. This is in agreement with the observations and speculations presented in Section VI, H, 2, in which the effectiveness of the hyperthermia therapy of cancer is related to the probable greater disorder of the water in malignant cells.

Other NMR studies on biological systems were reported in a symposium "Magnetic Resonance in Biological Systems," edited by Ehrenberg and co-workers (1967). A more detailed discussion will be presented in Section V, F.

#### *8. Limitations on Role of Vicinal Water Structure*

For an excellent introductory review of the more classic aspects of water in biological systems, see, for instance, the small monograph by Dick (1966). References to more complete treatments can be found in this book. Dick mentions briefly the possibility that the structure of water in cell systems may be different from that of bulk water. However, not everyone has recognized the probable occurrence of phase transitions in vicinal water. Even those in other fields who have contributed notably have occasionally overlooked details of some importance—at least so far as surface and interfacial effects are concerned. Thus, Cole had shrewdly observed that "if we fail to look carefully and worry effectively about the exceptions, we may too long postpone the appearance of some radical, undreamed of, unifying concept." However, Cole (1968) appears to overlook the likely existence of abrupt changes in vicinal water (near the transition temperature) as reflected in dielectric properties. Cole reports that "there have not been many or extensive measurements of temperature coefficients of membrane capacities, probably because they have seemed rather dull and not very important," and notes further "it seems safe to assume that within the range of 0 to 40°C, there is no phase change such as

those which produce dramatic effects in many dielectrics." In view of what is reported in the present chapter, this certainly seems to be a notable oversimplification or omission. However, some additional comments are appropriate here. There is little doubt that water of hydration may exist adjacent to many biologically interesting interfaces, ranging from the cell surface (membrane interfaces in general) to the "interface" between the water and the dissolved macromolecules (such as the proteins and enzymes). To demonstrate unequivocally the existence of such hydration structures (ordered structures of vicinal water) is by no means simple. Very few direct approaches are possible (see, however, discussion of recent NMR results above). More often, our evidence is only indirect and sometimes tenuous. In some cases the thermal anomalies are probably good indicators of the effects of cooperative phase changes of the structured, vicinal water; at the same time it must be kept in mind that the effects of temperature may not always produce anomalous behavior (a "kink") as the details of vicinal water structure do not play a predominating role in all reactions or equilibrium properties of the biological system under consideration. Certainly, some changes will be relatively independent of whatever the attendant water structure may be. At the same time it is undoubtedly also worth while to pay careful attention to temperature effects in biological systems and this is only possible through very careful studies, that is, studies of high precision, carried out at closely spaced temperature intervals. The effects of the thermal anomalies are often seen superimposed on those general processes which are partially understood in terms of our general insight into the structural and kinetic aspects of molecular biology. A large amount of such evidence is available but has not been exploited. Again, it is important to recognize that the interaction between the water and the macromolecules is a mutual interaction, that is, different water structures are stable in different temperature regions, and these hydration structures, in turn, influence and modify the underlying substrate—be it, for instance, a membrane or a protein. We stress again that the temperatures at which the structural changes occur in the vicinal water are very likely "invariants," that is, relatively independent of such variables as, say, electrolyte and nonelectrolyte concentration and (perhaps to a lesser extent) pressure. On the other hand, the conformation of the macromolecules is obviously very sensitive to environmental changes in the form of electrolyte concentration or hydrostatic pressure. Thus, again we emphasize that the effects of the structural changes in vicinal water are sometimes merely superimposed upon the behavior of the biological macromolecules per se, rather than determined through the overall temperature-solute-pressure dependencies.

### 9. Mutual Effects—Structure of Substrates and Solvent

It is important to stress the fact that the macromolecules in biological systems significantly influence and probably order the structure of the water (at least the vicinal water) in the cell. However, this water, in turn, imposes some restrictions and conditions on the properties and structure of the substrates. Indeed, this is one of the reasons why notable effects may be observed in biological systems at discrete temperatures—the proteins (and other macromolecules) in the system will be influenced by any drastic structural change in the water of hydration. Similar arguments must apply to the water vicinal to a membrane and in the membrane pores. Thus, order-disorder transitions in vicinal water introduce an additional element of cooperative behavior of the macromolecules. Cooperative phenomena are the type required in order to have all-or-none responses, i.e., triggering phenomena. For an interesting example of exceedingly abrupt temperature effects in biological systems, see, for instance, Section V,E where some neurophysiological responses to temperature are briefly outlined; see also the examples of electric potential responses of marine algae as described by Drost-Hansen and Thorhaug (1967) and the studies of Thorhaug quoted by Drost-Hansen (1969c).

### 10. Review by Tait and Franks

For a general survey and introduction to the problems of water in biological systems, see the article by Tait and Franks (1971). These authors eloquently point to many unsolved problems regarding water in biological systems. Throughout the article the authors describe the urgent need for more detailed studies, but note the apparent futility of the typical X-ray diffraction studies (which have incidentally come mainly from England). Such studies have indeed led to "exciting discoveries of biopolymer structures," but appear not to be well suited to a study of the dynamic behavior or other characterization of the aqueous component of the macromolecular systems. Tait and Franks end their article on a rather discouraging note regarding the study of water in biological systems: "It is not anticipated that much progress can be expected in this area, while the intermolecular nature of bulk water itself is still a matter of lively controversy." The article by Tait and Franks should be studied carefully by anyone interested in the topic of water in biological systems. A few specific points will be noted here, while other points will be discussed elsewhere in this chapter. The authors emphasize the studies by Warner (1965) and Berendsen (1967) on the "lattice fit" between water molecules and the unique 4.8 Å spacing of oxygen atoms in many bio-

logically active molecules, observing that water "can discriminate between molecules as similar as  $\alpha$ - and  $\beta$ -methyl pyranosides, which differ only in the position (axial against equatorial) of one hydroxy group". They also note that the effect of carbohydrates are of a short-range nature as opposed to the structuring that results in the case of hydrophobic solutes.

## B. BOUND WATER

### 1. Szent-Györgyi on Semantics

A matter of semantics might be mentioned here briefly. Biologists and biochemists have long realized the existence of bound water. Bound water has always been a rather ill-defined term, probably owing its origin to water not readily removed by "reasonably mild drying action." However, it may possibly be necessary to distinguish bound water from the type of water with which we are primarily concerned in this chapter, namely, structurally modified water. Szent-Györgyi has suggested the distinction between bound water and oriented water in terms of energetics. He (Szent-Györgyi, 1957) states:

The formation of such water structures should not be confused with the old idea of "bound water." "Binding" involves rather the idea of energy than that of structure. "Binding" means a certain force, energy needed to remove a molecule from its site. Such "bound" molecules, having their dipole forces engaged, are also unfit to serve as solvents for other molecules. Such a binding is especially strong around free charges, as those of ions. The order thus produced is "short-range order" the number of more firmly held layers of molecules being very small, 1-2. Contrary to this the building of lattices means "long-range order" in which the single molecules collaborate collectively.

Indeed, we owe Szent-Györgyi a great deal of gratitude for his contributions to the general question of the role of water in biological systems as exemplified in his small book "Bioenergetics" from which the above quote was taken. However, it should be obvious from the present discussion that the distinction between bound water and the long-range ordered or structured water may not be as simple and clear cut as implied in the statement by Szent-Györgyi.

### 2. Nonsolvent Water

Methods for studying water in biological systems, and particularly those for determining the amounts of bound water, were reviewed in a paper by Higasi (1955). The older methods were primarily based on measurements of water loss upon dehydration; dielectric measurements; and

water which does not possess the "normal" solvent properties of water. The idea of nonsolvent water implied that water which was sufficiently intimately tied to the underlying substrate would not be able to act as ordinary water with its usual solvent properties. Conceptually, this is a reasonable, if somewhat crude approach, and only because of the availability of more refined methods of physical measurements, such as dielectric studies, NMR spectroscopy, and other techniques, has the approach via nonsolvent water slowly been abandoned. However, attempts are still being made to discuss the apparent nonsolvent character of water, and recently de Bruijne and van Steveninck have applied the same approach (1970). These authors studied the permeation into the eventual equilibrium (or at least steady-state) concentrations of nonelectrolytes in yeast cells.

Among the experimental approaches which have played a role in the understanding of the problem of the structure of water in biological systems are dielectric studies. However, in this field great problems are encountered even in very simple, physicochemical systems. These problems are in part experimental (electrode polarization) and in part conceptual and fundamental owing to the difficulties of measuring dielectric properties of a very "leaky condenser" which, furthermore, is heterogeneous, thus giving rise to various charge separation and accumulation phenomena (Maxwell-Wagner polarization). Regarding the utility of dielectric studies as a means of studying water near interfaces, the reader is referred to the article by the present author on the nature of water near solid interfaces and, particularly, to the references therein. With regard to water in biological systems, see, for instance, the articles by Schwan (1965) and the monograph by Cole (1968). See also Section V,D,2 for an interesting study of dielectric properties of a lipid membrane.

## C. POSSIBLE WATER STRUCTURES NEAR BIOLOGICAL INTERFACES

### 1. *Stabilizing Effect of an Interface*

Before proceeding, attention is called to the fact that, although in the past the existence of ordered water structure at interfaces has often been doubted, it is certainly important to recognize that there is no lack of available "structured" species of water which can be invoked for such vicinal stabilization. Thus, we have mentioned (and will discuss in more detail below) the ice polymorphs and the clathrate hydrates (both the simple and the mixed clathrate hydrates). In addition, one can certainly envision water structure stabilized by dipole-dipole interactions without conforming to either of the above-mentioned categories of stable struc-

tures. Finally, a solid interface may act as a momentum sink serving to stabilize what might possibly be only intrinsically latent (metastable) structures in the bulk by removing some of the thermal fluctuations which would disrupt the structured units in the bulk phase. We also call attention to the fact that electric double layers certainly play a large role in the stability of colloid systems; it is the contention here, however, that such electric double layers may exist independently of the aqueous structures imposed for the reasons discussed above (rather than that they involve the primary mechanism giving rise to stability).

Perhaps the ultimate in "long-range effects" at an interface was discussed by Schulman and Teorell (1938). These authors measured the amount of water carried from one compartment to another in a trough by a moving monolayer of oleic acid. The thickness of the water layer, moving with the monolayer, was found to be of the order of 30 microns! Exactly how much of this can be explained in terms of some "classic hydrodynamic" effects and how much is due to vicinal water structuring must await a more detailed analysis.

Subsequent to the study by Schulman and Teorell (1938) Pak and Gershfeld (1967) carried out somewhat similar type measurements and observed the effect of steroids on the thickness of the water layers carried along with a monolayer. These authors conclude "if the monolayer is taken as a model for a biological receptor site located at an interface, the possibility must be considered that the steroid hormones exert their influence indirectly on the receptor site by altering the aqueous environment." This suggestion is similar to the idea mentioned briefly in Section IV,6,C: namely, that some of the biological effects of drugs may be due to types of interactions other than direct chemical bond formation between the solute molecule and the receptor site. See also the discussion of the paper by DeHaven and Shapiro (1968).

## 2. Solution Aspects of Cell Fluids

It is important to note that in biological systems we are dealing with rather concentrated solutions. Biological fluids are approximately  $0.1\text{ M}$  in electrolytes and quite concentrated in nonelectrolytes (about 20% of the net weight is proteins). It is almost certain that many of the difficulties which have been encountered in general physiology and related areas stem from the fact that the aqueous phases have often been treated as mere electrolyte solutions without proper regard to the nonelectrolytes present. Particularly, the Russian school of physical chemists has demonstrated the profound structural changes that take place in water and aqueous electrolyte solutions upon adding nonelectrolytes. Various spe-

cific aspects of this problem have also been surveyed recently by Franks (1968), by Franks and Ives (1966), and by Symons, Blandamer, and co-workers (for a review of the papers by the later authors, see the recent review by Blandamer and Fox, 1970). The systems which have been studied most intensively are aqueous solutions of the lower aliphatic alcohols and, to a somewhat lesser extent, of organic solutes such as acetone, dioxane, urea, and reducing sugars. It is interesting that somewhat less attention has been paid to solutions of amino acids as far as the influence on structural properties of solution is concerned. Solutes such as the alkaloids, clathrate hydrate formers, and hormones will be discussed separately, but briefly in the present chapter. Finally, a vast literature exists on the polypeptides and proteins, but these systems are poor as model systems insofar as vicinal water structures are concerned because of the intrinsic complications which derive from the larger size of the solutes, the simultaneous presence of nonpolar, polar, and ionic groups, and conformational changes.

### 3. "Ice-Likeness" of Vicinal Water

As discussed in various papers by the present author, it appears likely that Ice-Ih-like structures may not play an important role in the structure of bulk (pure) water nor may such Ice-Ih-like structured units be present in ordinary solutions. However, due to the uncertainty regarding the structure of water, the preceding statement is merely a tenable hypothesis. The absence of elements of Ice-Ih in bulk water obviously does not preclude the possibility that Ice-Ih might be stabilized by proximity to a surface. This idea has been advocated by those who have envisioned nucleating agents for supercooled water (for instance, in supercooled clouds), as promoting the growth of ice by epitaxy from oriented Ice-Ih-like layers; that is, induced solidification caused by the proximity to a suitable substrate with a lattice configuration in the basal plane similar to that of ordinary ice. This problem is far from resolved and a possible alternative to the simple notion of epitaxy has been presented by the present author. The suggestion is almost exactly contrary to the "conventional wisdom." In this new (very tentative) hypothesis it is proposed that nucleating agents are capable of inducing a rigid structure of water adjacent to the substrate, but this structure is significantly different from the bulk structure. In the transition zone between the two aqueous structures (the stabilized, vicinal structures and the bulk structure) may possibly exist a layer of enhanced disorder—the disordered layer in the three-layer model of vicinal water (see Section III,D). It is certainly conceivable that it is this disordered water which facilitates nucleation, i.e., permits the easy re-

arrangement of the water molecules (by random, thermal fluctuation) into nuclei for the ordinary Ice-Ih lattice.

In molecular biology, considerable attention has been paid to the possibility that Ice-Ih-likeness may play an important role. One of the first to advocate this idea was Jacobson (1953) who proposed that the spacing of turns in helical proteins might be stabilized by an ice-like structure of the vicinal water (water of hydration) of the proteins. This basic idea has been further pursued by Warner (see the symposium on "Forms of Water in Biologic Systems," 1965).

An impressive example of the utility of hexagonal ice-likeness as an element in the stabilization of biologically interesting macromolecules is the tobacco mosaic virus (TMV) protein. Warner has shown with models how hexagonal conformation can be built from subunits and such units serve as building blocks for the morphologically hexagonal TMV rods; however, HOH bond angles and hydrogen bond angles are not yet sufficiently fully determined to make the proposed model more than an interesting possibility. Recall, for instance, that the difference in free energy between Ice-Ih and Ice-II is only 19 cal/mole. Ice II is not hexagonal, but rhombohedral; Ice-Ih is merely stabilized by the positional disordering of the protons. In passing, we might note also that, although many of the high-pressure ice polymorphs do, indeed, require inordinate (and unphysiological) pressures, the energies of transformation from one to another of the polymorphs is quite low, ranging from 19 cal/mole (Ice-Ih to Ice II) to approximately 550 cal/mole with most of the transitions around 200 cal/mole. Obviously, in the range of temperature of physiological interest, 200 cal/mole is only one-third of  $kT$ —in other words, relatively small compared to thermal energy fluctuations. This, indeed, makes it unlikely that Ice-Ih should be the only preferred or particularly stable form of all the ice polymorphs.

#### 4. Clathrate Structures

The strongest impetus to study clathrate hydrates in connection with biological systems was the nearly simultaneous and independent studies by Pauling (1961) and by S. L. Miller (1961) on "hydrate microcrystals" as an important element of the mechanism of anesthesia.

Clathrate hydrates have been known for more than 150 years; Faraday prepared and described a chlorine hydrate (stable at temperatures below 28.7°C; dissociation pressure, 252 mm). The name "clathrate compound," however, is far more recent—it appears to have been introduced by Powell (1948). Over the past two decades, clathrate compounds have come to play an important role in technology as well as in biology.

The crystalline clathrate hydrates are solid solutions of a minor component in a water lattice. The essential feature is the existence of a "host lattice," which by itself is thermodynamically unstable, but which becomes stabilized by the presence of a "guest molecule": the water lattice becomes stabilized upon forming cages around the guest molecules. Usually, though not invariably, the solute molecules act as guests in the host lattice and are small molecules without a large dipole moment. (Molecules with large dipole moment generally tend to interact strongly with specific water molecules, thus destroying the symmetry required for the stabilization of the host network.) The interactions between the guest molecules and the host lattice are of the van der Waals-type forces, and no chemical bonding (generally) is incurred. The solutes that do not form clathrates are those with high charge density (small ions), molecules with large dipole moments, or molecules that are either too small (helium) or too large. However, it is important to recall that, although a number of substances do, indeed, tend to form clathrate hydrates with water (for instance, argon, krypton, xenon, methane, ethane, propane, chlorine, methyl chloride, methyl bromide, bromine, or ethyl chloride), no direct evidence is available as yet to demonstrate that an aqueous solution of these compounds in water induces clathrate-like entities in the solution. Glew (1962a,b), among others, has advocated on an indirect basis, that such structuring of the solution undoubtedly takes place and this view seems to have been adopted by such authors as Franks, Blandamer, Symons, and others, as well as by the present author.

In connection with the clathrate hydrates, particular interest attaches to the so-called "mixed clathrate hydrates." These are clathrates in which there are two types of guest molecules. Since clathrate hydrates in general may occur in several forms, consisting of polyhedra of different sizes, different-sized cavities are available to serve as host voids. Thus, typical examples are double hydrates containing Ar, Kr, or Xe with either  $\text{CH}_2\text{Cl}_2$ ,  $\text{CHCl}_3$ , or  $\text{CCl}_4$  or  $\text{SF}_6$  with  $\text{H}_2\text{S}$ .

The existence of such mixed clathrates may be particularly important in connection with biological systems. In the cell, the water adjacent to a protein molecule or a cell membrane may be stabilized in part by some of the solutes in solution with the help of the smaller, nonpolar side chains of the proteins (see Klotz, 1958). Thus, it is of interest to speculate that nitrogen, which forms a hydrate only at physiologically "extreme" pressures (160 atm), may possibly be able at moderate pressures to form a clathrate hydrate vicinal to a protein molecule or a membrane surface by the formation of mixed clathrates (through the effects of the smaller, nonpolar groups mentioned above). This type of reasoning is of importance in connection with the problem of solubility of hydrocarbons in protein solu-

tions or deep-diving physiology where "nitrogen narcosis" has been observed (see Section VI, E, 3).

For a review of anesthetic effects in terms of clathrate hydrate formation, the reader is referred to the original articles by Pauling and by Miller and, more recently, to the papers by Catchpool (1966) and the discussion in Section IV, C, 4.

In the pentagonal dodecahedra, a Type I clathrate, the polyhedra are regular twelve-sided structures with pentagonal faces. However, these cannot fill space (as required in order to form an extended three-dimensional crystalline solid hydrate). The cavities between adjacent dodecahedra are formed by polyhedra with fourteen sides; twelve are pentagonal, and two are hexagonal. The smallest of the units in the unit of structure contains 46 water molecules, a total of six, larger, fourteen-sided cavities, and two twelve-sided cavities. Another type of clathrate is the so-called Type II (or 17-Å type) consisting of 136 water molecules per unit cube with sixteen dodecahedra and eight hexakaidecahedral cavities. We point to these crystallographic facts to stress the likely existence of different types of clathrate cages in our total inventory of possible stabilized water structures which can be induced near solid surfaces. Note that these clathrate hydrates have near tetrahedral arrangement of the water molecules, but fivefold symmetry (pentagonal faces). Bernal (1960) has asserted that fivefold symmetry may be more commonly encountered in nature than normally assumed—a fact which Berendsen has also emphasized from his studies on water near various protein molecules, particularly, collagen and silk fiber.

If and where extensive networks are formed of clathrate hydrates, it is undoubtedly worth noting that the bond angle in the clathrate hydrate is  $108^\circ$  compared to the  $109^\circ 28'$  encountered in the tetrahedral arrangements necessary to make hexagonal structures. The details of the potential energy curve as a function of the HOH bond angle remain uncertain. However, it would be remarkable if the free water molecule bond angle (of approximately  $105^\circ$ ) could be opened up through a rehybridization by the mere presence of neighboring water molecules to give exactly the tetrahedral bond angle of the hexagonal structure,  $109^\circ 28'$ . Hence, whether or not the potential energy as a function of the bond angle shows a deep or a relatively flat minimum, it is certainly energetically more favorable to require only to open the bond angle from the vapor phase bond angle to  $108^\circ$  rather than to  $109^\circ 28'$ .

An example of a clathrate hydrate in which one part of the guest molecule is also part of the host lattice is hexamethylene tetramine, studied by Mak (1965). In this compound, the water cage is somewhat different from the other clathrates with slightly puckered, six-member rings of water

molecules. Other interesting hydrate forms, which may play a role in connection with water in biological systems, are the hydrates of ether, ethylene oxide, and acetone. These will be discussed in Section V,B,2.

### *5. Enhanced Reaction Rates*

In connection with the problem of ordered structures, attention is called to the fact that proton mobility is very high in ice. A proton is readily passed from one water molecule to another, facilitated by the rotation of the water molecules in the lattice. Similarly, facilitated conduction may possibly take place in other types of "rigid water." This probably plays a role in various biological systems, although at the present time, the evidence for this conjecture is rather weak. It has been alleged that certain reactions of biochemical interest progress faster in ice than in solution; in other words, reaction rates in some aqueous systems have shown an increase in rate upon freezing. In terms of a facilitated proton mobility, this proposition does, indeed, seem plausible. It is not yet certain that experimental artifacts could not play a significant disturbing role in these cases. Thus, the freezing of ice leads to the exclusion of most electrolytes (and nonelectrolytes) which may subsequently concentrate in small "brine pockets." Although the temperature is lowered, the reactions may proceed more rapidly merely because of the vastly increased concentrations. This field, however, deserves considerable further study. For enzyme reactions in frozen systems, see the article by Bruice and Butler (1965) dealing with two specific examples of relatively simple reactions in ice formed by the freezing of various aqueous solutions of the reactants.

In connection with the effect of ordered structure on proton mobility, see the article by Privalov and the present author's comments on this notion (Drost-Hansen, 1967b).

### *6. Effects at Low Concentrations*

One of the obvious problems in research on the effects of various pharmacological agents on cellular functioning is the problem of the activity and concentration of the solute. As discussed elsewhere in this chapter, there are reasons to believe that the activity of the solute may be different from that which a classic physicochemical study would suggest on the basis of equilibrium measurements on the separate bulk phases. Vicinal water may be so highly organized that the ordinary calculations of ionic activities are inapplicable, or at best, unknown. The other disturbing factor is obvious—the concentrations reported are generally the concentrations calculated on some volume, or mass, bulk basis. Such concentration units may have little relation to physiological effects; in the classic study

by Franks and Ives (1960) on the interfacial tension between water and hexane, notable increases in interfacial tension were observed for methyl and ethyl alcohols in concentrations of less than  $10^{-10} M$ . This simple example clearly shows that even for an "unsophisticated" system, a concentration of bulk solute so low as to leave completely unaffected the overall bulk structure may play an enormous role in interfacial phenomena. Obviously, the problem has not escaped the attention of biologists, to wit, the concern of the toxicologist who may be dealing with lethal effects of selected toxins in concentrations of less than 1 part in  $10^9$  (calculated on a per weight basis of body fluids). However, only rarely has the possibility been considered that these effects may reflect phenomena that are manifestations of induced water structure changes rather than specific, chemical bond-type interactions between functional groups of biochemically important substrates and the toxic compound.

### *7. Relative Size Effects*

As discussed above, it is likely that different types of structural entities may be stabilized in water adjacent to a solid surface, depending upon the nature of the solid. It is probable that a nonpolar, low dielectric material (such as a monolayer on an air-water interface or hydrocarbon-water interface) is able to stabilize structured units similar to or identical with clathrate hydrate structures. On the other hand, in the case of strongly polar or ionic surfaces, such as cellulose, quartz, or lead iodide, other types of interactions between the water molecules and the solid substrate should lead to other types of stabilized, vicinal structures.\* In these cases, dipole-dipole and ion-dipole interactions will play the dominant role. In either case it is possible, and in some cases very likely, that a disordered zone may exist between these vicinal, ordered structures and the bulk-like structure. At this time the suggested existence of a disordered zone remains a mere hypothesis based on indirect evidence and physical intuition rather than on concrete, experimental evidence.

Relative sizes very probably play a prominent role in biological systems; thus, it is possible that in small cells all the water present is "near" a cell surface, and, in addition, there is likely to be a sufficient concentration of macromolecules to bring all the water under the influence of some type of "surface entity," thus giving rise to a predominant water structure throughout the entire cell. At the same time, recall that in relatively concentrated solutions of both electrolytes and nonelectrolytes, there appear to be elements of water structure which retain the structural attributes of the original ordinary, bulk water (unaffected by the proximity to the

\* See, however, discussion of the paradox of surface influences (Section III,C,1, e).

solute) as suggested by the neutron inelastic scattering results by Safford (1966; see, also, Drost-Hansen, 1967a). However, the smaller the cell, the less bulk structure would be expected to exist (as the ordered structures would tend to exclude more of the bulk-like regions and, in some cases, possibly also the disordered region should such exist). No wonder, then,

TABLE VII  
FIT OF NATURAL MOLECULES TO WATER LATTICES

Molecule	Repeat in direction of best fit (Å)	Number of water repeats	Deviation from fit (%) based on	
			4.74 Å	4.52 Å
Collagen	28.6	Axial	6	+1      +5
Deoxyribonucleic acid	34	Axial	7	+2      +7
Feather keratin	23.6	Axial (quasi- repeat <sup>a</sup> )	5	0      +4
Also observed by X- ray diffraction	18.9	Axial	4	0      +4
Tobacco mosaic virus	23	Axial (quasi- repeat <sup>a</sup> )	5	-3      +2
Cross-β-protein	4.65	Axial	1	-2      +3
β-Protein				
parallel-chain pleated sheet	4.73	Perpendicular to fiber	1	0      +5
antiparallel-chain pleated sheet	9.46	Perpendicular to fiber	2	0      +5
chitin	4.69		1	-1      +4
apatite	9.43		2	0      +5
gramicidin S				
etamycin			1	≈+1      ≈+6
actinomycin	4.8	(Model)		
Circulin				(Fit in hexagonal pattern)
Staphlomycin				
repeat in myelin	4.7		≈-1	≈+4

<sup>a</sup> Not precisely in axial direction.

that biological systems have escaped any profound or accurate quantitative description up to the present.

### 8. Berendsen on Vicinal Water Structures

Berendsen has considered repeat lattice distances for various molecules of biological significance and the number of water molecules required to repeat the patterns in the direction of best fit. Table VII from one of Berendsen's articles (1966) shows the various repeat numbers proposed and their degree of fit. It is worth noting that Berendsen observes that the

fit of the water to the fibrous molecules to be barely on the border of significance because five to seven water-repeat distances are involved.

Berendsen makes an interesting observation regarding the surface entropy of various structures. He concludes that water structure near a (flat) hydrophobic surface is characterized by a relatively large positive entropy. However, in the case of the clathrate hydrates, a stabilization and decrease in entropy is observed, and he, thus, proposes that the entropy change upon cavity formation in water depends on the radius of curvature, suggesting a changing of the sign of the entropy at around 3.5 Å.

Berendsen (1967) has further commented on the model proposed for (vicinal) water by Warner, particularly in connection with the stabilization of the TMV protein subunits. Berendsen notes:

It is not at all sure, and not even very likely, that Warner's models represent protein conformation in solution or even in crystals, but the proposed models are very interesting with respect to biological activity. The marginal stability of each of the possible conformations of proteins relative to each other makes the actual structure critically dependent on the nature of the surroundings. The occurrence of a "hydrophobic" side make Warner's structure very acceptable in conjunction with hydrophobic surfaces, as lipid layers.

As stressed throughout this chapter, there is little doubt that the substrate influences the structure and properties of the vicinal water and that the converse holds true also, namely, that the particular water structure, intrinsically stable within a certain temperature interval, influences the structure of the underlying macromolecular substrate or membrane.

Berendsen (1966, 1967; also see Berendsen and Migchelsen, 1966) has long been advocating the idea of orientation of water molecules around the collagen molecule and contributed significantly to this through NMR studies. Figure 10A shows some of the results obtained by Berendsen and Migchelsen (1966). The line width (in reciprocal milliseconds) for the outer peaks of the proton resonance in a collagen sample (45 gm water/100 gm collagen) is plotted as a function of the reciprocal absolute temperature. The general shape, with the broad minimum near 0°C, is that expected as the result of the superposition of two straight lines in the composite relaxation process. Figure 10B shows the data points obtained by Berendsen and Migchelsen, replotted, and the curve used by these authors has been omitted. It is now seen that an additional definite trend exists in the data. For temperatures above 16°C (for the reciprocal of the absolute temperature less than  $3.47 \times 10^{-3}$ ), all data points (except one) fall on a relatively straight line with far smaller slope than the limiting slope (line E). It is obviously not possible to delineate specifically what this anomaly implies for the structural arrangement of the water adjacent to the collagen strand. The coincidence between the observed temperature of this change

and the thermal anomaly in the vicinity of 13° to 16°C strongly suggests that the anomaly owes its origin to a structural change in at least part of

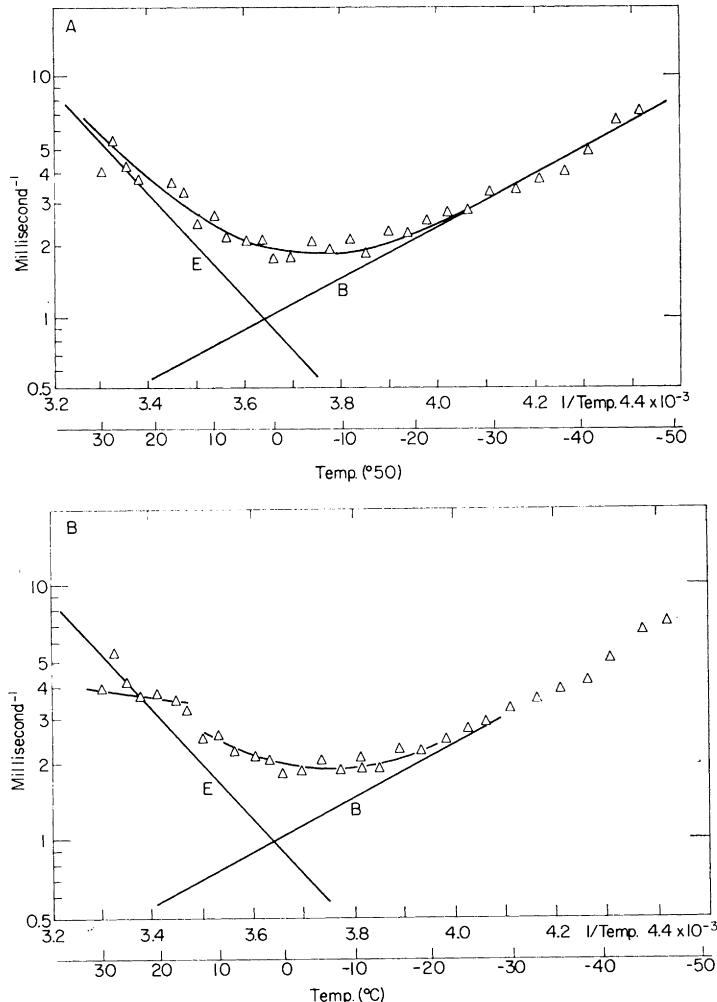


FIG. 10. (A) Line width (in reciprocal milliseconds) for the outer peaks of the proton resonance spectrum in a collagen sample. (Berendsen and Migchelsen, 1966, with permission from the publisher.) (B) Proton resonance line width in collagen sample. Same data as in Fig. 10A but with curve redrawn by the present author.

the water associated with the hydration of the collagen. It is worth noting that, upon careful inspection, a considerable number of such thermal anomalies can be seen in other published data for collagen properties.

Among more recent studies of water in biological systems, special at-

tention should be paid to the proceedings of a symposium held in Russia (Kayushin, 1969).

### *9. Positronium, Anomalous Water, and Other Exotic Aspects*

Among the more unusual techniques which have been applied to the problem of the possible existence of structured water in biological cells is a recent report by Gustafson (1970) who studied the positron annihilation radiation from the water in biological cells. Gustafson used this approach based on the observation that in ordinary ice the rate of positronium formation is far higher than in liquid water. Thus, it was to be expected that if the water in a cell were essentially ice-like, a notable increase in positronium formation from such cells would be observed. The results obtained by Gustafson were negative in the sense that there was no significant increase in the positronium formation rate in the cells studied (abdominal muscle from white rats); thus, the results obtained suggest that the water in the biological cell is not in an ice-like state ( $+4^{\circ}\text{C}$ ). Gustafson points out, however, that the results cannot exclude the possibility that other structured forms of water exists. Water structures with densities greater than that of water may still be present but might not result in enhanced positronium formation rates. Gustafson considers the possibility that anomalous water ("polywater," "Derjaguin water") might play a role in the cell, but the present author is certainly more than willing to adopt Gustafson's general feeling that "practically nothing is known about the behavior of positrons in polywater." In fact, the likelihood of the existence of anomalous water in biological cells is vanishingly small (a possible, but improbable exception—active silicosis).

We might draw an analogy (or, perhaps more precisely, a parable) regarding anomalous water in biological systems. Just as it is known that xenon is a potent general anesthetic, exerting its effect without forming any chemical bonds, it is also known that chemical compounds of xenon do exist. Similarly, anomalous water may exist (rather than being an experimental artifact, such as a silica gel). However, anomalous water appears only to form on freshly prepared glass or silica surfaces, preferably from a subsaturated vapor phase. This suggests that the formation requires the presence of a very high-energy surface site—a very unlikely part of a biological system. Hence, it is reasonable to assume that should anomalous water truly represent an intrinsic new form of water, it has probably as little bearing on biological systems as does the ability of xenon to form genuine chemical compounds have on its ability to induce narcosis.

### 10. Note

We conclude this section by noting that Clifford and Pethica (1968) have discussed the nature of water near a variety of interfaces. Table VIII from their paper shows the types of water-solute interactions considered and the corresponding sizes of the water domains—essentially the amount of water present compared to the amount of solute or matrix. This table does not give any quantitative indication of the extent of water structuring, but it does provide a rather inclusive survey of the types of

TABLE VIII  
OVERVIEW OF WATER STRUCTURES IN SURFACE SYSTEMS

Type of system	Size of water domain		
	I Essentially as single molecules	II Small groups of molecules	III Large enough for bulk water structure
(A) Water-solid particle	Water physisorbed on solid surfaces less than 2 monolayers or water in micropores	Water in the smaller “intermediate pores” or in concentrated colloidal dispersions	Water in dilute dispersions of particles or in dilute colloidal dispersions
(B) Water-macromolecule	Water adsorbed in small amounts on macromolecules	Systems containing similar amounts of water and macromolecules	Dilute solutions of macromolecules
(C) Water-oil emulsions	Dilute solutions of water in oil	Water in oil emulsions (very small droplets)	Water in oil emulsions (large droplets) and oil in water emulsions
(D) Water soap systems	Hydrate water in solid soaps	Water in concentrated micellar systems and in reverse phase micelles	Dilute solution of single molecules of micelles in water
(E) Small molecules	Dilute solutions of water	Solutions of intermediate concentration	Dilute solutions in water

systems most likely to be encountered in biological systems (especially if one substitutes the phrase “water-membrane” for “water-solid particle.”

The conceptual approach represented by Table VIII regarding water structuring and order as related to solutes in general is similar to that of this chapter.

## V. Possible “Sites of Action” of Water Structure Effects

### A. INTRODUCTION

There is little doubt that the structure of vicinal water may undergo abrupt changes at or near a number of discrete temperatures, primarily

in the ranges between 13° to 16°C, 30° to 32°C, 44° to 46°C, and 60° to 62°C. All of these temperatures are in the range of physiological interest. In view of the fact that all biological systems possess a large surface-to-volume ratio, it is not surprising then that some biological systems reveal abrupt changes at or near these temperatures. The question arises, At what specific point in the total biological system is the site (or sites) of action of the water structure effects? It will undoubtedly be some time before this question will receive a final answer; however, in this section attention is directed to a few of the probable major sources of influence of water structure changes. Among the possible sites are macromolecules in the biological systems (polypeptides, proteins, and enzymes) and lipids, membranes, nerves, and muscle. The effects of water structure on metabolic processes are in all probability primarily through the activity and functioning of adenosine triphosphate (ATP) and enzymes. Electrolytes and gas exchange rates are probably affected through the influence of the water structure of membranes. Consciousness and other nervous activity are affected through the structural changes of the water in or near the nerve fibers and synapses—a problem in lipid-water and membrane interactions. Only relatively crude speculations as to details are possible at this time. On the other hand, as illustrated in the following section, the evidence is overwhelming that the structural role of water in biological systems does, indeed, play an important role in the overall response of the cellular processes to temperature.

## B. SOLUTES

### 1. *Hydrophobic Bonding*

To explain the interactions of many biologically interesting molecules, as well as to describe the behavior of even simple alkanes in solution, the concept of hydrophobic bonding has found considerable utility. Objections have been raised, yet, it appears that the concept is useful and has at least operational significance. Hydrophobic bonding is essentially the stabilization (of the water structure) which occurs as two nonpolar entities in solution approach each other. In this process, the intervening water structure experiences an increase in nearest-neighbor coordination number. The classic treatment of hydrophobic bonding is the one by Nemethy and Scheraga (1962a,b,c; see also Nemethy, 1967), but other quantitative and semiquantitative treatments have been attempted. See also the extensive studies by Ben-Naim (1965, 1969a,b, 1971a,b,c). The hydrophobic interaction does not result from unfavorable energetics for the nonpolar group in contact with the water, but, instead, from the ordering experienced by

the water molecules, lowering the entropy. Much evidence suggests that the effective (average) number of H-bonds (per water molecule) is increased for the water adjacent to (and between) nonpolar moieties; the effect is due to the possibility of increasing the number of attractive intermolecular contacts (due to van der Waals forces). Other interpretations of hydrophobic bonding have been proposed by different authors. Thus, Franks (1970) notes that:

Small angle x-ray scattering on some aqueous solutions shows sharp maxima in the scattering intensity at certain concentrations, e.g., mole fraction 0.12 in the case of *t*-butanol. It is also known that this peak intensity increases with rising temperature so that we have the indication of a lower critical demixing behavior. It is also known that, in the system water-tetrahydrofuran, demixing actually occurs at 72°. It is conceivable, therefore, that this lower critical demixing phenomenon can be identified with the hydrophobic bonding effect observed in dilute aqueous solutions.

From purely geometric considerations of hydration structures around the nonpolar solutes, Stillinger (1970) has tentatively proposed a water stabilization effect due to a preponderance of "eclipsed orientations" of hydrogen-oxygen lone pairs in the vicinal water structures (without invoking any specific geometric structures for this vicinal water, such as clathrates). It can be seen that, although no definite unique model is yet available, it may still be useful to employ the concept in discussing the behavior and structural properties of aqueous solutions of nonpolar solutes.

## 2. *Organic Hydrates*

Before discussing the hydration of macromolecules (of biological interest) in solutions, attention is called to the structure and properties of organic hydrates. Although crystalline inorganic hydrates have been the subject of much study, this topic is of only limited interest to the biophysicists. In electrolyte solutions the hydrations of the ions, particularly in the primary hydration shell, is dominated completely by ion-dipole interactions, and the structure of the pure solvent itself plays but a minor and indirect role. However, in the case of the organic hydrates the situation is sometimes considerably different. Recently, Jeffrey (1969) has reviewed the question of water structure in crystalline solid organic hydrates. Jeffrey has classified organic hydrates and other water structures according to the degree to which the ordering is determined by the water-water interaction. First, the epitome of control by water-water interactions, is, of course, the various forms of ice (Ice-I and the high-pressure ice polymorphs). Second, Jeffrey considers clathrate hydrates to be essentially of two types—the simple gas hydrates with nonbonded guest molecules

stabilizing the voids of the host lattices and the peralkylammonium salt hydrates where water and anions form closely related hydrogen-bonded structures with the cations occupying the polyhedral voids. Third, Jeffrey introduces the term "semiclathrate hydrates" in which the water-host structure has polyhedral clathrate voids, occupied by hydrogen-bonded alkylamine molecules. In addition to these three groups, water structures may be three dimensional, two dimensional, or one dimensional and correspondingly form hydrogen-bonded nets, sheets, columns, ribbons, or chains. These structures contain no identifiable clathrate cagelike voids, and the main characteristic is the strong interaction between the functional group of the organic molecule and the water molecule. Finally, Jeffrey briefly considers hydrates with isolated water molecules. For the latter, it suffices to note that there is a very large number of ways in which water molecules can adapt themselves to fit the lattices in hydrates of organic compounds or inorganic salts.

Of interest to biological systems are those hydrates where the ratio of water molecules to solute molecules in the solid state is large. One may reasonably expect that the major contribution to the lattice stability derives from the water-water interaction. Were it not for the formation of voids which leads to a low density relative to ice, the water in the structures of the gas hydrates would be energetically competitive with ice!

Jeffrey has also pointed out that in the solid crystalline hydrates the ratio of solute to water is an order of magnitude higher than the solubility of the solute in liquid water! Of particular interest to biological systems are the alkylamine hydrates. The studies of these materials were begun toward the end of the last century by Pickering, but little attention has been paid to his very extensive studies. In these hydrates a large number of different ways of hydrogen bonding occurs between the functional groups, in competition with the tendency for clathrate hydration of the hydrocarbon (nonpolar) groups. The alkylamine hydrates have hydration numbers (as reported by Pickering) ranging from 0.5 molecules of water per organic molecule to 36 or 37. The list of polyhedra present in some of the alkylamine hydrates is most impressive; only a few of these are irregular polyhedra. Many eight-, eleven-, twelve, fourteen-, fifteen-, sixteen-, seventeen-, eighteen-, and twenty-six-sided polyhedra are reported. Thus, there is no shortage of crystalline equivalent arrangements of water into lattices which could conceivably occur in the water vicinal to the side chains of the proteins of biological interest. It is also worth noting that the host lattice in some of these structures may accommodate larger molecules. The reason that pentane, for instance, does not form a clathrate hydrate, whereas some of the larger amines do, is simply due to the additional com-

ponent of lattice energy in the amine hydrate as the result of dipole interactions between the amines and water-host lattice.

Jeffrey has also pointed out that with one exception (hexamethylene tetraamine hexahydrate) all clathrates and semiclathrate hydrates are polyhedral. This should be considered in connection with the recent theoretical calculations by Del Bene and Pople (1969) which suggest considerable hydrogen bond stabilization through resonance effect in cyclic structures of water molecules, including tetramers, pentamers, and hexamers.

TABLE IX  
HYDRATION PROPERTIES OF ORGANIC MOLECULES IN THE  
CRYSTALLINE STATE

Hydrogen bonding  ↓ Hydrophobic clathration	Carbohydrates, polyols, amino acids, peptides, pyrimidines, purines, urea, ureides, acids and salts, amides, nitriles, aldehydes, phenols, <i>quaternary methylammonium ion</i> , glycols, polyoxymethylenes, alcohols, ketones, cyclic amines, aliphatic amines, cyclic ethers, <i>quaternary n-butyl- and iso-amylammonium ions</i> , mercaptans, alkanes, alkyl halides (rare gases, halogens, $\text{ClO}_2$ , $\text{SO}_2$ , $\text{H}_2\text{S}$ , $\text{N}_2\text{O}$ , $\text{CO}_2$ , $\text{CS}_2$ , $\text{COS}$ )	Low hydrates  ↑ High hydrates
--	---	--

Returning briefly to the general problem of hydrates, Jeffrey (1969) has presented a table in which the participation of the water ranges from low hydrates, determined by direct hydrogen bonding between solute and the water structure, to the high hydrates characterized by hydrophobic clathrates. Table IX shows the results presented by Jeffrey. Finally, we quote Jeffrey on the multitude of possible interactions between amines and the water lattice:

In summary, then, the amine molecules have been observed to interact with surrounding water framework in the following ways: (1) forming no hydrogen bonds with the water structure, as in  $16(\text{CH}_3)_3\text{CHN}_2 \cdot 156\text{H}_2\text{O}$ ; (2) forming one donor and one acceptor hydrogen bond which bridges across the water oxygen vertices at opposite sides of a void, as in  $12(\text{CH}_3\text{CH}_2)_2\text{NH} \cdot 104\text{H}_2\text{O}$ ; (3) forming two donor hydrogen bonds bridging across two adjacent oxygens which would form an edge in a regular gas hydrate type of polyhedron, as in  $10(\text{CH}_3)_2\text{CHNH}_2 \cdot 80\text{H}_2\text{O}$ ; (4) forming two acceptor hydrogen bonds from a bridging water oxygen, as in  $4(\text{CH}_3)_3\text{N} \cdot 41\text{H}_2\text{O}$ ; (5) replacing a water oxygen

vertex and bridging across a void by a hydrogen-bonded dimer of two amine molecules, as in  $16\text{CH}_3\text{CH}_2\text{CH}_2\text{NH}_2 \cdot 10\text{H}_2\text{O}$ .

There is good reason to believe that further studies of the remaining hydrates reported by Pickering will each reveal a new water framework suitably engineered to fit the particular stereochemistry of the alkylamine.

### 3. Gels

Gels play an important role in biological systems, but the role of water structure in gel formation is only poorly understood. Essentially, the problem is that gels can be formed at very low concentrations of solutes with viscosities which are several orders of magnitude larger than the viscosity of the pure solvent. On the one hand, it appears that in some gels rigidity (or at least, greatly increased viscosity) is achieved for solute concentrations so low that the viscosity increase cannot merely be the result of polymer "entanglement." This, then, would suggest a structural role of vicinal water, stabilized by the proximity to the gel-forming solute molecules. On the other hand, there is evidence that the microscopic viscosity in such systems is only slightly influenced by the presence of the gel-forming solute. Thus, ionic conductivities in gel systems appear almost identical to those in bulk solutions of the pure electrolytes (in contrast to what would have been expected if Walden's rule were obeyed), and ion diffusivities also appear relatively unaffected. Furthermore, the proton relaxation times appear almost unaffected; mobilities, diffusion coefficients, and relaxation times differ only by factors of about 2 for viscosity changes up to  $10^5$  to  $10^6$ . Obviously, in some cases, including in particular some of biological interest, sufficient gel-forming material is present to impart overall lattice rigidity due to simple polymer entanglement. However, systems have been described (such as the cetyltrimethylammonium bromide- $\beta$ -naphthol complex) for which the viscosity is highly shear rate-dependent in concentrations of solute of less than 0.02% (corresponding to a ratio of water molecules to solute of approximately 1:50,000). As mentioned by Kruyt (1949), the copper salt of cetylphenylether sulfonic acid imparts a detectable elasticity to water at a concentration of only 0.0002%. In this case the average separation between individual solute molecules is approximately 200 water molecules—indeed long-range ordering. See also the discussion by Glasel on macroscopic viscosity and NMR relaxation in protein solutions (Section V,B,5), both of which again confirm that little, if any, relation exists between local and macroscopic viscosity.

Cerbon (1967) has studied immobilized water in lipid systems using an NMR technique. Vastly different effects were observed for the influence of the relative viscosity on the proton transverse relaxation time ( $T_2$ ,

seconds) in the case of dextran, yeast, ribonucleic acid (RNA), and an aqueous lipid system. Thus, the increases in relative viscosity decreased the relaxation time in the RNA solutions markedly, whereas dextran (commercially used as an effective water thickener) had only a minimal influence on the relaxation times.

#### 4. Peptides and Nucleotides

The most obvious link between water, on the one hand, and biological systems, on the other, is via the role of water in determining protein properties. Indeed, "polypeptides plus water equal proteins," and it appears that only through the action of water do proteins and lipids hang together in lipoproteins, etc. Great advances have been made in the understanding of the structure of RNA, deoxyribonucleic acid (DNA), and other nucleotides; yet, hardly anyone has begun to comprehend, or even describe, the role that the water must play in the stability of these macromolecules and in the functioning of these biomolecules. As an example, it is known that DNA requires 30% water to stabilize the double helix; yet, details of the structural functioning of this water remain almost completely obscure. Among the many other biologically important compounds in which the role of water is still poorly understood are also the polysaccharides and mucopolysaccharides (which play an important role in gel formation).

#### 5. Glasel's Studies

Glasel (1970a,b) has recently studied some fundamental questions regarding the role of the water structure in connection with the problem of conformational changes of biologically interesting molecules (also see Glasel, 1968). In the first of the papers by Glasel, a number of relatively small molecular weight solutes was studied, including dimethylsulfoxide (DMSO), *t*-butanol, and urea, as well as some quaternary ammonium compounds and some nonionic surfactants. Particular interest attaches to the study of urea as a denaturant of proteins since urea has long been studied in considerable detail. Glasel writes:

For relatively large  $\tau_e$  this indicates that the number of waters hydrated is small and hence the interaction with water is not a long range phenomenon.\* For urea, the interaction at *any* concentration is weaker than for any of the other molecules studied, and is the same order of magnitude as for simple electrolytes. Therefore, explanations of the denaturing properties of urea must lie elsewhere than in a change in the structure of the solvent. That is, an explanation in terms of the stability of the denaturant-polymer-hydration complexes must be sought.

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\*  $\tau_e$  = rotational reorientation time.

This statement deserves considerable amplification. It should be noted first, however, that the denaturing effects of urea are not perhaps as astounding as may be implied by Glasel. In fact, it is customary in practical protein chemistry to use very high concentrations of urea solutions for denaturing the proteins (in the concentration range of 2 to 8 M). This is undoubtedly important as it clearly suggests that the denaturation does not depend on the individual single effects of the denaturant on a specific singular binding site in the protein. However, the fact that the urea does not appear to be very highly hydrated is consistent with the findings by Frank and Franks (1968); indeed, the urea appears not to interact with the water structure at all, but behaves merely as a "perfect solute." It is proposed here that, although urea may fit into the (bulk) water structure without notably affecting this structure (neither through reorganization of the solvent nor through extensive, direct hydration), the urea may not fit at all in the structure of the vicinal water associated with the macromolecules! Thus, the suggestion by Glasel that "an explanation in terms of the stability of the denaturant-polymer-hydration complexes must be sought" may well be related to an inability of the urea as a solute to fit into the rather unusual solvent, consisting of the differently structured, vicinal water.

From the studies on the NMR relaxation rates, Glasel makes a number of general conclusions, of which we quote a few:

1. Organic molecules and cations can best be described in solution by association of water molecules with their surfaces, and not by long-range effects on water structure.
2. The association of water molecules with organic solutes of the type studied here is not especially strong, compared with ionic effects of the same concentration. The observed effects on magnetic resonance relaxation in water are dominated by the longer times of reorientation of the larger solute molecules. Thus, the magnitude of the observed effect is very large in micelle suspensions of surfactants.

In addition, Glasel notes,

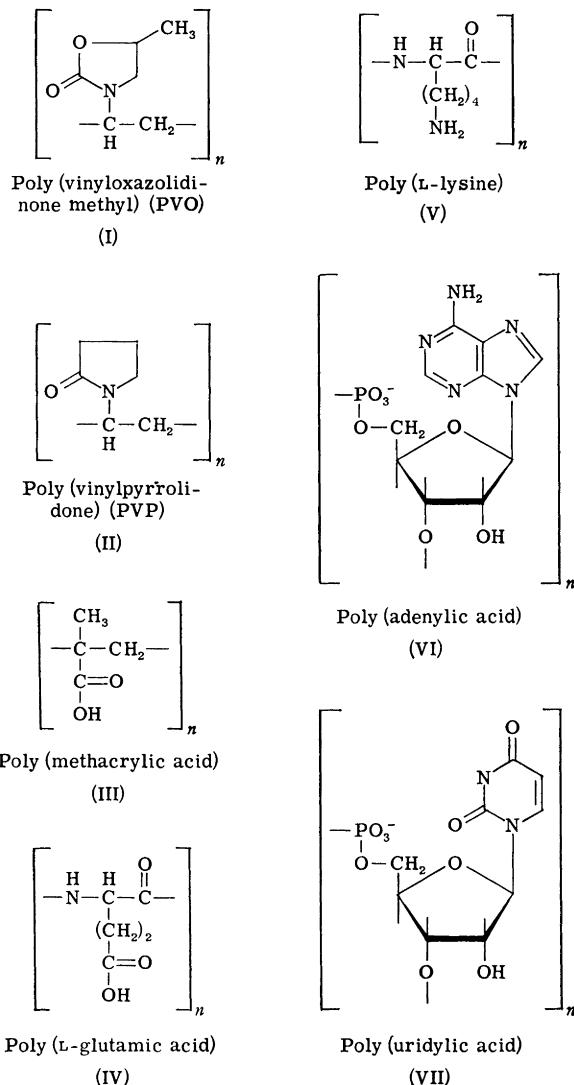
3. DMSO-H<sub>2</sub>O solutions are composed of mixtures of molecular complexes in the ratio 1:2 and 1:3.

Finally,

4. The biophysical activity of these compounds in promoting denaturation depends upon the competing interaction with polymers.

Glasel proceeds to propose a simple means of hydration of the polymer, the counterions, and the solute. This study by Glasel was followed by a second contribution in which the deuteron spin relaxation rates were studied on a number of heavy water solutions of some macromolecules of gen-

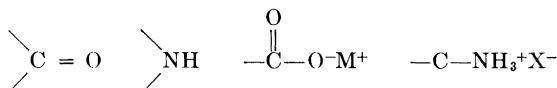
TABLE X  
Molecular Structures and Abbreviations of Polymers Used in Study by Glasel



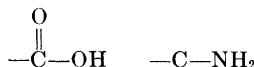
eral interest in connection with biopolymers. These polymers (see Table X) were chosen as representative of various elements of typical macromolecules, having a number of properties in common with the larger biopolymers including cloud points at high temperatures and/or high pH and

phase behavior similar to the proteins (induced by the presence of urea and ammonium sulfate, etc.).

The results of these studies are rather remarkable. One of the most important observations is undoubtedly that the polymers that interact relatively strongly with water are those for which the dominant interactions take place between the unpaired species of the polymer and the water. Poly(methacrylic acid) is mentioned as a case in point. Glasel notes that phenomenologically, the following functional groups do not form strong interactions with water:



whereas



do form strong interactions with water. Furthermore, strong interactions between water and polymers occur only in those charged polymers where there is total or at least partial intramolecular or intermolecular neutralization, i.e., when the counterion effects are eliminated through the polymer-polymer interactions. Finally, no interaction with water occurs in polymers where the geometric (helix-coil) fluctuations of intramolecular structure are large and with characteristic times of the order of a millisecond.

Two important statements from Glasel's study (1970b) should be noted and a comment will be made on these in connection with the occurrence of thermal anomalies in aqueous solutions of biologically interesting macromolecules. Glasel states:

Rule 1: evidence for this is based on the behavior of polymers I, II, III (basic), V (acidic) and VII. This and Rule 2 point out clearly the importance of counterions in shielding polymers from water interaction. When counterion shielding is present, conformational changes in the polymer do not effect water-polymer interactions because there are no interactions. This is illustrated by the behavior of poly(methacrylic acid) as shown in Figure 5. Similar conclusions on the basis of partial molal volume changes have been made for this polymer.<sup>19</sup> The results obtained from Figure 6 indicate *specific* interaction of water molecules with at least this one polymer. For poly-U, where the pyrimidine *pK* is of the order of 9.5, neither the charged nor the uncharged form shows any interaction with water. This and the similar absence of interaction for the acid form of poly(L-lysine) indicate the weakness of imide-water interaction. In addition, all of the polymers studied having simple carbonyl functions display no observable interaction. To summarize: in order to display interaction, there must be a proton donor without counterion shielding.

Rule 2: The coverage of Rule 1 is supported by the observations on III (acidic), IV, V, and VI. All of these have labile proton donor groups which may hydrogen bond to water in their uncharged form.

Finally, we quote:

Evidence as obtained from high resolution NMR experiments on polymers undergoing helix-coil transitions (26-28) suggests that such fluctuations exist and that their lifetimes are  $10^{-8}$ - $10^{-3}$  seconds. The experiments described here indicate that these fluctuations destroy the interaction of water with poly-A and poly(L-glutamic acid). The interaction of water with polymers is, in those polymers where it exists, remarkably stable as long as the topology of the polymer is stable. In the case of poly(L-lysine) the effect is not noticed because there is no interaction with the charged form and, hence, no mimima can be observed.

In his conclusions, Glasel notes that not only is there, contrary to expectation, no evidence for the interaction of water with imide or carbonyl functional groups, but, furthermore, no hydrophobic interactions are found where expected. Also, in those cases where functional groups could hydrogen bond to water, it appears that such bonding is very sensitive to counterion shielding.\*

Thermal anomalies are sometimes observed in physical properties of polypeptides (and many other biopolymers) in aqueous solutions, while at other times anomalies appear to be completely absent. This observation is not the result of experimental uncertainty; instead, in view of the findings by Glasel, it appears more likely that some biopolymers are, indeed, interacting with and inducing notably different and extensive vicinal water structures, whereas in other cases, no such ordering occurs. Thus, we should expect only those processes and phenomena that are governed by the water-interacting polymers to exhibit the thermal anomalies. Hence, a possible rationale may be sought along these lines to explain the fact that some biophysical and biological systems show pronounced thermal anomalies, whereas other systems equally clearly demonstrate the absence of any thermal transitions.

## 6. Proteins

a. *Two-State Processes.* As mentioned elsewhere in this chapter, polypeptides, enzymes, and proteins in general are obviously among the most important biologically interesting macromolecules. However, this topic

\* It is unfortunate that in the otherwise impressive study by Glasel, a temperature of  $31^\circ \pm 1^\circ\text{C}$  was employed throughout. Considering the amount of evidence to indicate a structural change may take place at around this temperature, some of Glasel's conclusions may need to be rechecked at different temperatures, or preferably, over an extended temperature range.

can only be given a most cursory treatment here; the reason for this is obviously the immense complexity of the problem. In general, changes in temperature will have obvious consequences for the state of aggregation of any macromolecule, especially those with the complexity of enzymes and other proteins. The amino acid sequences of the proteins play an enormous role in determining the conformation of these molecules. In a sense, it is only secondarily that the solution structure plays a role; yet, it is beyond question that the secondary and tertiary hydration structures do significantly interact with the proteins and, thus, jointly determine thermal stability properties. Variations in ionic strength obviously will be reflected in the properties of the protein through the influence on the ionizable groups of the protein. However, as stated before, although the proteins themselves in part influence the structure of the vicinal water, this water, in turn, impresses constraints and conditions for stability upon the protein. Because of the complexities involved, the proteins are poor model systems with which to probe the role of water in biological systems.

The nature of proteins is such that over narrow temperature intervals rather abrupt conformational changes may occur. These have been described as all-or-none processes or two-state processes in the cases where essentially only two possible states occur—namely, the initial and the resultant state (corresponding to a higher temperature). The transitions, however, are not truly discontinuous; they are, indeed, abrupt and thus somewhat resemble all-or-none processes. However, superimposed upon these transitions, all-or-none transitions in the water structure must also be taken into account. Recall in this connection that thermal anomalies are frequently observed in water structure vicinal to vastly different types of water-solid interfaces, be they hydrophobic (nonpolar), hydrophilic, or ionic surfaces. Thus, in studies of temperature effects on proteins, one must be equally concerned with the detailed structural aspects of water.

*b. General Reviews.* One of the classic papers on protein hydration and protein properties is the contribution by Klotz (1958). There is little doubt that this paper must be considered among the first serious attempts to investigate the extensive hydration of proteins and the functional role of this hydration phenomenon; this places the paper in the class of the contributions by the early pioneers Jacobson and Szent-Györgyi. Lumry and Biltonen (1969) have reviewed various aspects of protein conformation, including water structure effects, in an article "Thermodynamic and Kinetic Aspects of Protein Conformation in Relation to Physiological Functions." The authors have presented an extensive review of protein conformation; they summarize other earlier contributions and specifically

implicate the structure of water in the stability and nature of the conformation of the proteins.

Privalov, of the Institute of Proteins, Academy of Sciences of the USSR, has also long been concerned with the problem of the structure and properties of water in biological systems. Some interesting suggestions were made earlier by Privalov (1958), whereas another study of the role of water structure in thermal denaturation of macromolecules appeared in a symposium, "Water and Biological Systems," edited by Kayushin (1969). Privalov makes the important point that it is specifically necessary to take into account the superimposed effects of the tertiary hydration structures of the proteins in considering thermal denaturation of proteins. The reader is referred for details to the rather brief note by Privalov. Sidorova (see, for instance, Kayushin, 1969) and co-workers have written extensively on the state of water in biological systems. Sidorova has, in particular, applied infrared techniques to study the changes in water structure of macromolecules as a function of temperature.

A slightly dated review of the structure of proteins was presented by Richards (1963). The review still deserves careful reading, particularly the discussion on apolar bonds (i.e., hydrophobic interactions). Specifically, Richards reviewed the controversy between the ideas of Kauzmann and those of Klotz. The present author sympathizes with the excruciatingly honest observation by Richards to the effect that "On reading the papers and listening to talks by these various people, this reviewer finds himself in the embarrassing position of being convinced by each one in turn. He would like to suggest that perhaps there is a measure of truth all around."

Von Hippel and Schleich (1969) have presented an extensive review of the effects of various salts on the structure and stability of biologically interesting macromolecules in solution. This study is, without a doubt, one of the most extensive on the problem. The authors have paid particular attention to the effects on water structure by the presence of the electrolytes; as such, the chapter by von Hippel and Schleich represents a readable summary. However, it is unfortunate that it is yet too early to attempt to correlate the effects of electrolytes on water structure with the effects of ions on the water structure near most biopolymers. Only the last section of their extensive chapter even attempts to approach the problem. Von Hippel and Schleich note that the ions will affect the water structure, as will the nonpolar groups of the macromolecules; in addition, the "local water" (undoubtedly the water referred to in this chapter as vicinal water) will also be perturbed by the ions present, and this, in turn, will influence or determine the extent to which the water can become organized by the

exposed nonpolar groups of the macromolecules. Thus, it will also determine the free energy of transfer of these groups from the hydrophobic macromolecular interior to the aqueous environment. One can view the mechanism of the effects of ions (and other perturbants) on  $T_m$  as caused by a "tripartite competition" between the organizing forces, all of which impose a particular (and different) type of order on the local water. These three competing, organizing forces are the nonpolar groups of the macromolecule, the ions (or other nonaqueous additives), and the unperturbed water lattice itself. According to von Hippel and Schleich, structure destabilizers make less water available to Frank-Evans icebergs around the exposed macromolecular nonpolar groups than is available in the presence of unperturbed water lattice; whereas structure stabilizers must have the properties of somehow loosening the unperturbed water lattice, thus making more water available for organization about the nonpolar groups. See also the recent article by Schleich and von Hippel (1970).

For an excellent survey of the use of infrared spectroscopy with biomolecules, see the article by Susi (1969), dealing particularly with the study of polypeptides, protein structure, and various nucleotides.

*c. Relation to Bound-Water Problem.* The problem of the concept of extensive stable hydration hulls has been neatly summed up by Lumry and Rajender (1971) in their discussion of the subject:

For some years it was customary in studies of protein hydrodynamical properties to incorporate into the calculations a shell of water which was treated as moving with the protein. There was considerable experimental justification for this course of action provided by the results of drying experiments, calculated diffusion coefficients, transport of solvent by protein, buoyancy measurements and so on, but the use of partial specific volumes computed on a dry weight basis makes it unnecessary to examine this problem in every hydrodynamic or equilibrium sedimentation experiment.

Recent history of the bound-water problem seems to stem from specific suggestions made by Jacobson (1953) about the structure and extent of the bound-water shell. Since that time there has been much argument about the matter, but no solutions have been forthcoming. Thus, Lumry and Rajender (1971) point out that:

The idea of stable hydration shells has not been very popular in recent years despite the skillful championing of the idea by Klotz (289-292). It is probable that there are some very unusual features of water at protein surfaces and the experiments by Lauffer and co-workers on TMV protein (332-334) are particularly important in this connection; but the idea of a structured shell with either ice-like or clathrate character has not been generally appealing for reasons which have been presented by Kauzmann (177). However, if such shells exist they are the most obvious places to look for the source of solvent-dependent compensation behavior and should have top priority in studies of compensa-

tion. Although the estimates of "hydration" of water differ markedly from method to method, a frequent figure deduced from different types of experiments is 30% of the weight of the protein (see Glasel, 293, 294 for a recent experimental approach to the problem).

Lumry and Biltonen (1969) have also discussed various aspects of bound water. These authors include in the category of bound water those molecules that, at the surface, interact strongly with the charged groups of the proteins and those molecules that are found in deep indentations (caves) and large holes (if such ever exist). They also point out that this water probably is not a stabilizing factor in conformation configuration and also that such surface and "cave" water appears to be the only part of the protein hydration about which everyone seems to be in at least partial agreement.

Meryman (1966) has also discussed the problem of bound water in his introductory article to the volume "Cryobiology." The definitions of bound water reviewed by Meryman range from water that does not freeze (of the order of 5 to 10% of the total amount of water in animal tissue) to water that does not act as a normal aqueous solvent. Meryman also quotes Bull (1943) who compiled a review of no less than fourteen different methods for measuring bound water, which, as Meryman points out, give rise "to almost as many definitions."

*d. Enthalpy-Entropy Compensation Phenomena.* Lumry and co-workers, and most recently Rajender, have carefully reviewed enthalpy-entropy compensation phenomena in aqueous solutions of both small molecules and proteins. In this section is summarized briefly a few of the many important suggestions made by Lumry and Rajender, and speculations are made upon some possible basic, unifying features. In the treatment of membranes as a site of the action of the vicinal water structure, the linear compensation effect will be discussed again in connection with the hemolysis of erythrocytes (the studies by Good and co-workers).

The compensation process implies that there is a linear relationship between the entropy change and the enthalpy change; this is specifically discussed by Lumry and Rajender for a number of processes of small solutes in aqueous solutions. The proportionality constant between the enthalpy and entropy is referred to as the "compensation temperature." In other words, in the general expression

$$\Delta G^0 = \Delta H^0 - T \Delta S^0$$

$\Delta H^0$  and  $\Delta S^0$  may be related in a compensatory fashion, such that

$$\Delta H^0 = \alpha + T_c \Delta S^0$$

This latter equation is an extrathermodynamic statement and no clear-

cut explanation can be offered for the relative constancy of observed values for  $T_c$  (wherever water is the solvent). The remarkable fact is, however, that the values for  $T_c$  all fall in a relatively narrow range from about 250° to 320°K for processes as dissimilar as solvation of ions and nonelectrolytes, hydrolysis, oxidation-reduction, ionization of weak electrolytes, and the quenching of indofluorescence, among others (see Lumry and Rajender, 1971, for details). The interpretation of the existence of this enthalpy-entropy compensation is that it is a manifestation of an intrinsic aspect of the water structure itself. Regardless of the solutes and the solute processes studied (thus, including both thermodynamic equilibrium properties and transport phenomena), it has been discovered that similar enthalpy-entropy compensation regularities are observed among the proteins of functional importance in physiological processes. Hence, it is suggested by Lumry and Rajender that the enthalpy and entropy relationship may be used as a diagnostic test for the participation of and, in fact, the controlling role of the water in the protein processes. The enthalpy-entropy compensation phenomena discussed by Lumry and Rajender certainly emphasize the probable critical and crucial role played by the structure of water in protein and enzyme processes. For a list of authors who have contributed to the subject of linear compensation phenomena, see the article by Lumry and Rajender.

Obviously, temperature plays an enormous role in any consideration of protein and enzyme stability. For an excellent survey of the effects of temperature on the proteins in solution and in biological systems, see, for instance, the recent monograph edited by Poland and Scheraga (1970). There is little doubt that a study of temperature effects on the properties of proteins will suggest a wide and almost continuous range of temperatures of importance in protein chemistry. Thus (apparent) melting temperatures,  $T_m$ , may fall anywhere in a large range—usually between 10° and 95°C. However, without a more detailed look at the underlying processes, these temperatures are of little immediate use in an attempt to elucidate the interplay between protein properties (for instance, the helix-coil transformation) and the water. The reason for this is the fact that (apparent) melting temperatures,  $T_m$ , will be strongly influenced by other factors, such as the ionic strength, pH, and the concentration and nature of nonelectrolytes present in the medium. Not only does this obscure any specific role that the water might play, but it makes the application to living systems far more difficult because of the delicate and minute differences in pH and ionic activity which must occur in different living cells within the same organism at any time. It is in this connection that the real advantage of the study of the enthalpy-entropy compensation phenomenon becomes obvious. As discussed in this section it is by means of

the compensation phenomenon that it is possible to identify a single characteristic temperature. It is this temperature (actually a proportionality constant), the compensation temperature  $T_c$ , that reveals far more directly the role of the water structure. Nevertheless, it is possible in some cases to see anomalies in protein behavior, as a function of temperature, superimposed upon the general trend of the data in graphs of properties such as optical circular dichroism versus temperature (to mention but one example), due to the water structure changes. Although a sigmoid shape curve is generally anticipated (as the result of the phase cooperative behavior of the proteins themselves), evidence is sometimes present for smaller, but notable, deviations at or near the temperatures of the thermal anomalies. Again, this is a matter of paying attention to and accepting trends in data even when the total deviations may appear to be within the experimental error on an individual determination.

Values for  $T_c$  generally fall within the range of 250° to 320°K, but far more often the values cluster between 270° and 290°K—notably between 285° and 290°K (i.e., 12–17°C). It is interesting to speculate that the compensation temperature  $T_c$  may be the “critical temperature” for the stability of one or another type of vicinal water structure, stabilized by the proximity to the interface. In this connection, recall the discussion in Section III,C,1,e of the paradox of the invariance of thermal anomalies with the specific nature of the substrate and also the suggestion that superimposed upon this invariant behavior there must be (perhaps minor) specific influences, for instance, due to a polar versus a nonpolar interface.

As an example of the entropy–enthalpy compensation, Fig. 11 shows the data collected by Arnett (quoted by Lumry and Rajender, 1971). It is worth mentioning that the following passage from the discussion by these authors:

The aggregate of evidence thus far presented not only emphasizes the dominant role of liquid water but also indicates that neither the type of small solute nor the type of processes is very important. There seems to be a wide variety of perturbations of liquid water which evoke the same type of response in water. Even the alcohol-perturbed examples can be included in these blanket statements since Rajender has found that the characteristic near-infrared spectrum of water in these solutions retains its distinctive isobestic pattern up to the alcohol mole fractions which produce extremum behavior in the examples we have discussed....

Recall here, as discussed by the present author, that even in relatively strong aqueous solutions there is evidence for structural arrangements characteristic of the bulk structure of water which are unaffected by the presence of the solute, even in rather high concentrations of electrolytes. The same notion has been stressed by Safford (1966).

e. Mutual Effects—Solute-Solvent Interaction. In connection with the mutual interaction of water and the substrate, recall again that not only does temperature change the water structure in general, but also the water structure near an interface and the latter more or less abruptly. Also, the changes in the vicinal water structure must, in turn, influence the nature of the (solid) substrate. Thus, if the ordering in water near an interface changes at one of the transition temperatures, changes may then occur in the properties of the underlying substrate. This may be an overriding

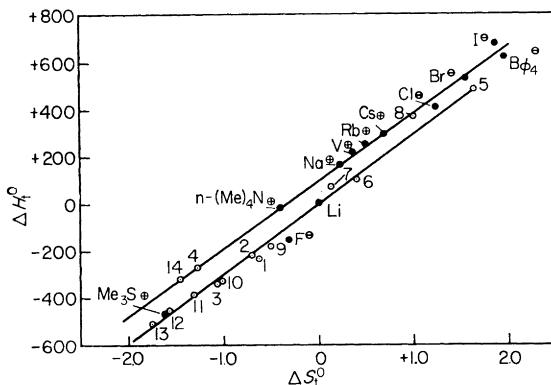


FIG. 11. Enthalpies and entropies of transfer for nonelectrolytes and individual ions from  $\text{H}_2\text{O}$  to  $\text{D}_2\text{O}$  at  $25^\circ\text{C}$ . Nonelectrolytes are shown with open circles ( $\circ$ ) and ions by filled circles ( $\bullet$ ). The two lines are drawn arbitrarily through what seem to be the most "typical" members of each series and their relative positions are not significant although their slopes are. The ion correlation line could be shifted merely by using some other ion than  $\text{Li}^+$  as reference. (1) Argon, (2) propane, (3) butane, (4) iodine, (5) methyl fluoride ( $30^\circ\text{C}$ ), (6) methyl chloride ( $30^\circ\text{C}$ ), (7) methyl bromide ( $30^\circ\text{C}$ ), (8) methyl iodide ( $30^\circ\text{C}$ ), (9) glycine, (10) DL-alanine, (11) DL- $\alpha$ -aminobutyric acid, (12) DL-norvaline, (13) DL-norleucine, (14) L-phenylalanine. (Arnett and McKelvey, 1969, with permission of Marcel Dekker.)

principle in connection with protein conformation and should perhaps be related to what has been mentioned by Lumry and co-workers as "subtle and not so subtle effects of the water on the protein structure."

With regards to the interaction between water and some of the macromolecules of the biologically interesting systems, particularly the proteins and enzymes, we again quote Lumry and Rajender (1971):

The similarity of  $T_c$  values for small solute and protein compensation processes in water has provided the basis for the proposal that all Vaslow-Doherty processes have a common source. Systematic studies of the effects of solvents on the linear compensation processes of proteins now underway should provide definite evidence for or against this generalization, which is only a hypothesis at the present time. In the protein cases there are other alternatives. For example,

it is a natural consequence of any cooperative structure that enthalpy and entropy changes must to some extent compensate each other. Protein unfolding processes are weak first-order phase transitions, weak because the cooperative unit is very small so that the transition-temperature range is very much broader than that for the melting of ice. Compensation behavior due to the protein conformation is clearly apparent in the thermodynamic changes in protein unfolding which are discussed at length elsewhere. However, this type of compensation process is not quantitatively similar to Vaslow-Doherty compensation, except for changes involving the exposed nonpolar groups as demonstrated in the ribonuclease and trypsin examples.

Lumry and Rajender note the fact that the compensation process results in observed values of  $T_c$  for small solutes which are nearly identical with the values for proteins; they also note that the most likely explanation for this behavior is that water alone is involved as the underlying agent. This, in turn, is astutely correlated with the "fitness of the environment for life," as discussed by Tracey (1968). (See Section VI,A,2 of the present chapter.) Earlier, Lumry and Biltonen (1969) emphasized the probable importance of the enthalpy-entropy compensation phenomenon with the statement, "Indeed, it is probable that the enthalpy and entropy change during conformational changes of proteins is the single most important physical-chemical characteristic of protein function."

*f. pH Effects.* The apparent melting temperatures for proteins yield an almost continuous distribution of values. Hence, the obvious advantage of a determination of enthalpy-entropy compensation behavior is to simplify complexities otherwise encountered in the "point-by-point" description of the effects of temperature upon different protein properties in various media—strongly dependent upon the ionic strength and other parameters (such as pressure). However, the effects of pH are more difficult to deal with and the compensation phenomenon probably cannot be obtained from conventional rate data alone in most instances where the pH is varied greatly. Lumry and Rajender feel that the degree of complexity in such systems must be considerable and "compensation may be hidden."

*g. Protein-Hydrocarbon Interactions.* In connection with the mixed clathrate hydrates and the possibility that the nonpolar side chains on the proteins in solution may act as "help-gas" (*Hilfsgase*), it is interesting to consider some of the studies by Wishnia (1962) and by Wetlaufer and Lovrien (1964). Wishnia demonstrated that the solubility of ethane, propane, and butane is greatly enhanced in solutions of bovine serum albumin, hemoglobin, and lysozyme. The increase in solubility was several-fold above that in pure water. It was shown that the solubility enhancement was almost temperature independent over a wide range of temperature (25°), suggesting that the enthalpy of bonding is small, and, hence, that the solubilization process is determined primarily by entropy changes.

Wetlaufer and Lovrien observed reversible changes in viscosity, optical rotation, and other properties of bovine serum albumin (at alkaline pH) and  $\beta$ -lactoglobulin (for neutral pH) in the presence of various hydrocarbons. This suggests then that the hydrocarbons readily become associated with the protein and that the mechanism most likely depends on the incorporation of the hydrocarbon into cavities—probably clathrate or semi-clathrate hydrate-like entities—induced in the vicinal water of the macromolecules. As mentioned previously, the structure of the vicinal water will again determine, in part, the conformation of the macromolecules, and, conversely, the macromolecule will influence the structure of the water. Hence, it is not surprising that changes occur in properties such as viscosity or optical rotation of the protein solution upon addition of hydrocarbon moieties into the vicinal water structure. See also the discussion by Schreiner (1968) of the effects of various inert gases on some properties of purified enzymes (such as tyrosinase and acetylcholinesterase).

*h. Notes.* In addition to Lumry and Rajender and Lumry and Biltonen, Brandts (1969) has stressed the need to consider carefully the nature of vicinal water in determining the conformational stability of macromolecules in solution. Brandts notes, "Thus, a complete description of the denaturation process must take cognizance not only of the order-disorder transition of the polypeptide chain itself, but also of the order-disorder transition associated with the solvent in the mode of accommodation of non-polar side chains in the denatured state."

A useful review of the behavior of nonelectrolytes in water is presented somewhat incidentally by Brandts (1969) who discusses the properties of aqueous solutions of nonelectrolytes as a function of the nature and concentration of the dissolved electrolytes. The reader is referred to this article not only for the review of the underlying theory, but particularly for the attempt to apply these ideas to the problem of the stability of biologically interesting macromolecules.

Sidorova and co-workers (see Kayushin, 1969) have studied the state of water in biological tissues by infrared spectroscopy. The article by Sidorova and co-workers should be consulted for some useful suggestions regarding the utility of infrared spectroscopy as a tool in the study of water in biological material. The authors point out that the interpretation of the vibrational spectrum of water and the assignment of certain frequencies is still somewhat ambiguous and involves the use of other methods, such as neutron inelastic scattering, NMR, and dielectric studies for additional structural information. Among the interesting conclusions which were drawn by Sidorova and co-workers is the suggestion that because the data for eggs (egg yolk and egg albumin) differ so little from those obtained for pure water, the water in eggs is identical in structural properties

to ordinary water. They further note that the water appears to freeze at the same temperature as pure water and "has the same solvent action." The present author finds this statement somewhat difficult to accept and particularly takes issue with the statement by Sidorova and co-workers that "this conforms to the widely held view that most of the water in protein solutions is in the free state," although this latter statement is possibly correct for globular proteins. Attention is called to the statement in Section V,D,6,b that limitations exist on the use of infrared as a discriminating tool in biological systems because of the relatively poor resolution of the broad bands due to any hydrogen-bonded systems. However, the paper by Sidorova should also be studied for further information because it contains a separate section covering the near-infrared spectra of water, alcohol solutions, and the influence of urea on the solubility of hydrocarbons in water. With regard to urea, Sidorova and co-workers conclude that water-urea clusters probably exist in solution; this should be compared to the opposite conclusion reached by Glasel (1970a) and by Frank and Franks (1968).

For a recent infrared spectroscopy study of the hydration of DNA, see the article by Falk *et al.* (1970). These authors proposed that even when the surrounding water has frozen into ice (ordinary Ice-Ih) an inner layer of ten water molecules per nucleotide does not freeze. The authors conclude that the biopolymer hydration shells are not "ice-like" in the sense of possessing a crystalline Ice-Ih-like structure; specifically: "The present results demonstrate that, at least for DNA, there is no such ordering. The innermost, least mobile part of the hydration shell is in fact, entirely incapable of crystallizing into the ice structure. Evidently, the preferred configuration of water next to the biopolymer is incompatible with the structure of ice. There is no spectral evidence of 'quasi-crystallization' of any part of the hydration shell at room temperature, and at low temperature it is the water *far* from the biopolymer surface which freezes into ice. The hydration shell of DNA is definitely not 'ice-like' in the structural sense."

## 7. Enzymes

a. *Dixon and Webb's Review.* For a general review of enzymes, as well as a brief review of the effects of temperature on enzyme activity, see, for instance, the monographs by Dixon and Webb (1960) or by F. H. Johnson *et al.* 1954). Dixon and Webb note the occurrence of several abrupt changes with temperature in enzyme activities (particularly in quoting data by Massey). Although the authors allow for the possibility that a phase change could occur in the solvent, they have apparently only concerned

themselves with a first-order transition (water–ice). Apart from this possibility, Dixon and Webb summarize the following possibilities for the origin of abrupt changes in Arrhenius plots.

1. Anomalies may be present due to the existence of two parallel reactions with different temperature coefficients. The authors note that it is very difficult to get anomalies as sharp as are often observed unless the enthalpies of activation differ by a very large amount.

2. The occurrence of successive enzymic reactions with different enthalpies of activation.

3. Different forms of the enzymes with different activities. These forms would both be active, with different enthalpies of activation. Again, however, this requires that the activation parameters differ greatly.

4. Dixon and Webb quote Kistiakowsky and Lumry (1949) who proposed that the enzyme may exist in two forms, one of which is inactive.

5. The simultaneous occurrence of forward and reverse reactions, but only with the forward reaction exhibiting an anomaly. This, of course, does not explain the occurrence of the anomaly in the forward rate of the reaction.

There is certainly no lack of suggestions to explain the occurrence of thermal anomalies in enzymic rates. However, it seems that a less forced and far more likely explanation for the anomalies may be sought in a change in the structure of the vicinal water, manifesting itself either through the general change in vicinal properties (including entropy, viscosity, and diffusion rates), or resulting from conformational changes of the enzyme, “impressed” upon the macromolecule due to the different states of the vicinal water above and below the thermal transition points.

*b. Rate Expressions.* As mentioned in the preceding section, Dixon and Webb reviewed some of the possible causes for the occurrence of abrupt changes in slopes in Arrhenius plots of enzymic reaction rates. More recently, Brandts has been concerned with this phenomenon (1967; also see F. H. Johnson *et al.*, 1954). One of the most likely mechanisms for achieving notable changes in slope is through a reaction catalyzed by an enzyme which may exist in two forms (a native and a denatured form). Thus, in the expression for the rate of the process, one must allow for a temperature effect on the equilibrium constant between these two forms, characterized by a free energy of transformation. This generally will give a rate expression of the form:

$$\text{rate} = \frac{CT \exp(-\Delta E^\ddagger/RT)}{1 + \exp(-\Delta F^\circ RT)}$$

where it is customary to assume the free energy is composed of two temperature-independent terms, namely, the enthalpy ( $\Delta H^\circ$ ) and the entropy

term ( $T\Delta S^\circ$ ). The result of such an approach was reviewed by Brandts (1967) and is shown schematically in Fig. 12. The different curves in this hypothetical illustration refer to enzymes of varying thermal stability. The less thermally stable the enzyme catalyzing the reaction, the more truncated does the rate expression become. The present controversy is whether or not this approach (and an extension of this approach) offers an explanation of the observed anomalies in well-known enzyme systems, particularly when extremely abrupt changes in slope are observed. It must be remembered that the equilibrium distribution between the native and

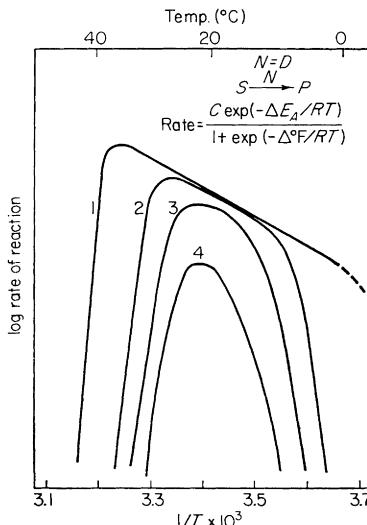


FIG. 12. Schematic representation of rate of enzyme-catalyzed reactions; see text for explanation. (Brandts, 1967, with permission.)

denatured form of enzyme will follow a normal free energy-equilibrium constant (exponential) distribution, thus limiting the abruptness of the transition. The more abrupt the transition, the larger the free energy difference between the two forms of the enzyme must be; but this intuitively seems energetically contrary to the expected behavior of biological systems in general. It is for this reason that the present author proposes the introduction of another factor, namely, vicinal water structure which can act to sharpen the transitions, recalling the mutual interaction between the structure of the vicinal liquid and the molecular conformation of the interface presented to the vicinal water.

*c. Examples of Marked Anomalies in Enzyme Reactions.* A typical, although certainly not a forceful example, of the occurrence of an anomaly in the properties of an enzyme has been reported recently by Lehrer and

Barker (1970). These authors studied the thermal stability and reactivity of aldolase obtained from rabbit muscle. (Previously, Massey *et al.* (1966) had reported the enzyme to undergo a temperature-dependent transition, and Lehrer *et al.* studied this in some detail.) A possible anomaly was observed in the vicinity of 30°C; a typical result is shown in Fig. 13 as an Arrhenius plot for the cleavage reaction of aldolase. This clearly indicates a nearly linear behavior above and below 28° to 30°C, respectively. The

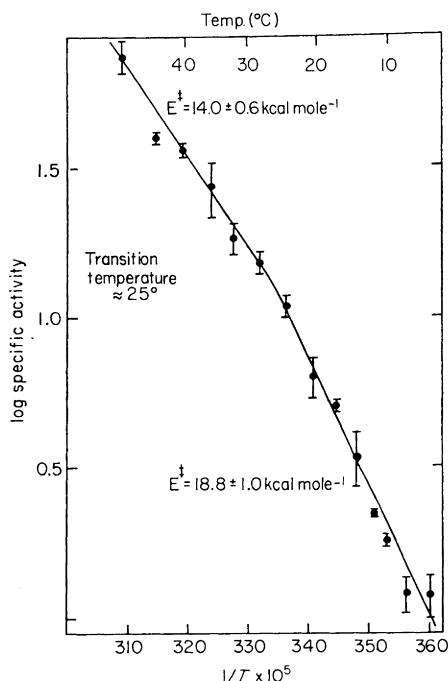


FIG. 13. Arrhenius plot of rate of cleavage reaction of aldolase. (Lehrer and Barker, 1970, with permission.)

authors tentatively ascribed the change to the mechanism proposed by Massey, namely, that the enzyme may exist in two different conformations above and below the transition temperature. However, it is suggested here that the change is a manifestation of water structure; it is possible that there is a conformational change in the enzyme to conform to whatever is the stabilized, vicinal water structure in the different temperature intervals (changing at 30°C).

Another typical example of a remarkably abrupt change in enzyme property with change in temperature was reported by Takahashi and Oshaka (1970). These authors studied the proteinase from the venom of

the snake *Trimeresurus flavoviridis*. Again, very notable thermal anomalies were observed. Thus, the enzyme activity of the enzyme on casein was decreased by two orders of magnitude on increase in temperature from 60° to 63°C approximately.

Henn and Ackers (1969) have recently provided another example of an abrupt temperature anomaly of an enzyme property. These authors have observed a very abrupt change between 12° and 14°C in the association constant for the dimerization of the subunit of D-amino acid oxidase. The authors specifically propose that the variation in the enthalpy of the reaction reveals a reversible change in the heat capacity of the protein. This is

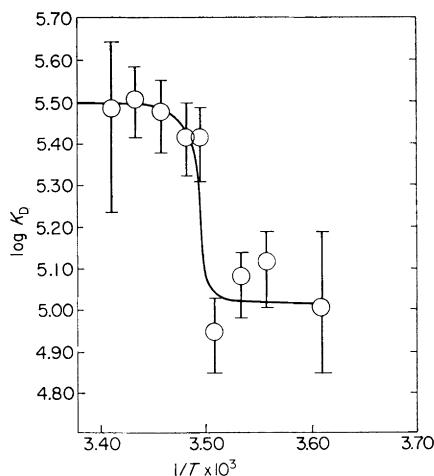


FIG. 14. Van't Hoff plot for dimerization of D-amino acid oxidase apoenzyme. (Henn and Ackers, 1969, with permission.)

interpreted as an isomerization reaction between different conformational states (in addition to an association of the subunits). Figure 14 shows the results of the van't Hoff plot for the dimerization of the apoenzyme. Again, we suggest that the vicinal water associated with the amino acid oxidase apoenzyme may play a crucial role in determining the transition temperature.

A full discussion of the general problem of the structure of water adjacent to proteins in general and enzymes in particular is outside the scope of this chapter. However, a number of other examples of highly anomalous temperature dependencies of enzyme properties are mentioned below; it is proposed that these reflect the discrete and specific influences exerted by structural changes in the water vicinal to these macromolecular solutes. In other words, various types of phase transitions are, indeed, to be ex-

pected on purely energetic grounds in the proteins per se, but at least some of the thermal anomalies reported in the literature for protein properties are likely manifestations of changes in the vicinal water structure.

Fischer and co-workers (see Piguet and Fischer, 1952; Meyer *et al.*, 1953) have demonstrated the occurrence of abrupt changes (kinks) in the properties of  $\beta$ -amylase. These authors obtained kinks between 17° and 19°C with differences in apparent energies of activation ranging between 9.3 (0–18°C) and 13 kcal/mole above this temperature range. Anomalies were obtained both for barley and wheat  $\beta$ -amylase.

Subsequent to these initial studies, Markovitz *et al.* (1956) studied the properties of  $\alpha$ -amylase isolated from *Pseudomonas saccharophilia*. Again, it was found that the rate of enzyme activity exhibited notable anomalies in the vicinity of 15°C. The difference in the apparent energies of activation for the  $\alpha$ -amylase was almost 6000 cal (8500 kcal/mole between 15° and 40°C and 14,400 cal/mole between 0° and 15°C). Similar differences in energies of activation were obtained for  $\alpha$ -amylase isolated from another bacterium; but the authors point out that the amylase of malt, swine pancreas, human saliva, and human pancreas show only a single energy of activation over the same temperature interval.

A further example of abrupt temperature changes in another enzyme system has been reported by J. J. Baldwin and Cornatzer (1968). These authors studied the glyceryl phosphorylcholine diesterase. This enzyme shows extremely abrupt inactivation at approximately 60°C.

It is worth mentioning in passing that the enzyme data which in general tend to show the most distinct evidence for abrupt thermal transition are those for which the properties are the most sensitive to changes in pH! Undoubtedly, a very careful study of this effect would throw considerable light on the general phenomenon. The observation of notable changes with pH corresponding to the largest sensitivity to temperature is likely a manifestation of the same underlying cause: as discussed elsewhere, hydrogen ions appear to upset the water structure more than any other single ion and whenever extensive vicinal structuring is present (and thus, able to manifest most clearly changes with temperature), the effects of hydrogen ions are most pronounced. Compare this effect with the results obtained by Glasel discussed in Section V,B,5.

In addition to the few examples mentioned here specifically of abrupt changes in enzyme properties at the temperatures of thermal anomalies, see also, for instance, the article by Kemp and co-workers (1969) who observed a rate change in the succinate oxidation by rat liver mitochondria near 17° to 18°C. See also the article by Staal and Veeger (1969) describing a thermal anomaly near 15°C in the glutathione reductase.

It should be noted that particularly in the literature on enzyme kinetics

the existence of thermal anomalies has often been discussed, although no agreement currently exists as to the origin of these phenomena. As a matter of semantics, it is important to note that these anomalies have often been referred to as discontinuities (in Arrhenius plots, for instance). Actually, overt discontinuities are observed only very rarely in log-rate plots. For a discussion of possible types of thermal anomalies in rate processes, see Drost-Hansen (1967a).

*d. Cold Inactivation.* Graves and co-workers (1965) have studied the cold inactivation of glycogen phosphorylase. The authors found that storage at temperatures less than roughly 15° to 20°C (in a buffer at pH

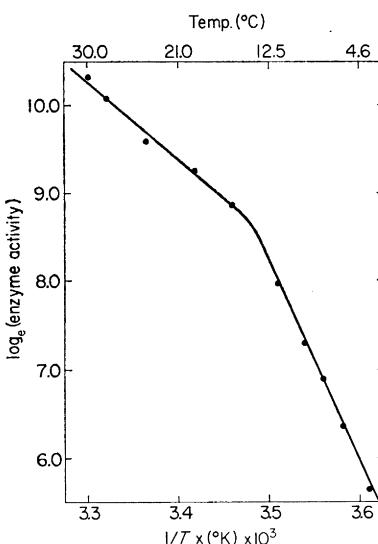


FIG. 15. Arrhenius plot of rate date for phosphorylase *b* activity. (Graves and co-workers, 1965, with permission.)

6.0) led to more rapid inactivation than storage at higher temperatures. The cold inactivation could be reversed upon rewarming. Figure 15 shows the effect of temperature on the enzyme activity of the phosphorylase *b* activity. It is seen that a notable anomaly occurs in the vicinity of 13°C. Again, we propose that the occurrence of the thermal anomaly is a manifestation of the attendant change in water structure at this temperature. Particular attention is called to this example because of the likely importance of the general phenomenon of cold inactivation on the process of vernalization (see Section VI,C,2). For additional discussions of the phenomenon of cold inactivation, see the recent article by Kuczenski and Suelter (1970); the authors observe:

Low temperature instability of proteins indicate that associations between

apolar groups, significantly weakened at low temperatures (Kauzmann, 1959; Scheraga *et al.*, 1962), are important in these proteins. The temperature dependence of inactivation both in the presence and absence of FDP suggests a first-step dissociation involving such apolar groups. In the presence and absence of FDP, the initial rates of inactivation,  $k_2$ , decrease as the temperature is raised. Although the data are insufficient to quantitate the effect of temperature on the equilibrium concentration of the dimer, examination of the shape of the curves suggests that the concentration of the dimer decreases with increasing temperature but that the rate of dissociation of the dimer,  $k_3$ , increases. This suggests a heterologous interaction between sub-units, i.e., hydrophobic forces predominating between the dimers, and electrostatic forces predominating between the sub-units of the dimer.

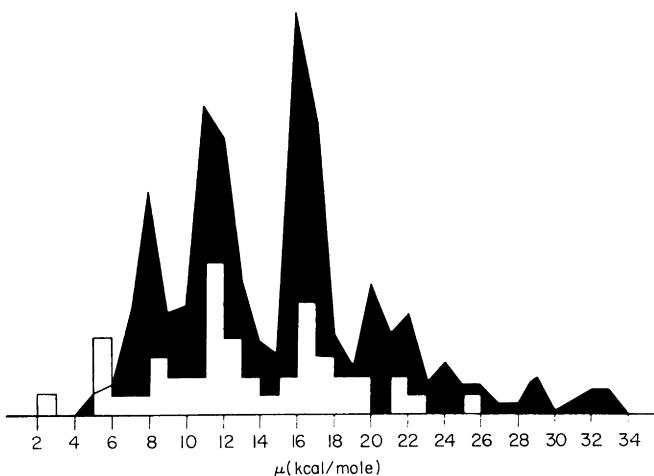


FIG. 16. Distribution of energies of activation of biologically interesting processes. (F. H. Johnson *et al.*, 1954, with permission.)

Finally, we mention the studies by Brandts and Ting (see Brandts, 1967) and Biltonen and Lumry (1969). These studies clearly show maximum stability of chymotrypsin, chymotrypsinogen, and dimethionine sulfoxide chymotrypsin near 10° to 15°C! This observation may be purely accidental (although this is perhaps not entirely likely) in view of the fact that RNase, for instance, has maximum stability below 0°C. Brandts (1967) has reviewed other cases of cold denaturation of proteins. The phenomenon of low-temperature denaturation will no doubt eventually prove of considerable interest in connection with hypothermia, hibernation, and vernalization.

e. *Distribution of Activation Energies.* It is interesting to compare the relatively narrow range of compensation of temperatures which is normally encountered with the notably discrete distribution of apparent

energies of activation for various biological and biochemical rate phenomena. Figure 16 shows the highly nonuniform distribution of energies of activation as reported in the monograph by F. H. Johnson *et al.* (1954). At first sight, this illustration might tentatively be interpreted to indicate that the rate-controlling process in a large variety of biological and biochemically interesting systems is determined simply by a rather small number of discrete specific enzyme reactions with characteristic energetics. However, the possibility should also be considered that this discreteness is a further manifestation of those processes which are determined, or at least notably influenced, by a relatively small number of distinct states of the water of solvation of the macromolecules involved. Unfortunately, it does not seem possible to extract from the presently available data any further information to corroborate this tentative suggestion.

### C. LIPIDS AND LIPOPROTEINS

#### 1. *Lipid-Water Interactions*

Lipids, lipid-water, and lipid-polymer interactions in general are exceedingly complex and outside the scope of the present chapter. However, it is necessary to point briefly to a number of phenomena related to lipids (including the phospholipids and lipoproteins) in order to appreciate an origin of thermal anomalies in biological systems which may not be related to the anomalous thermal properties of vicinal water. Higher-order phase transitions—not related to water structure—do certainly occur in proteins and (for other reasons) in pure (anhydrous) lipids. This is not to imply that other phase transitions involving proteins as well as lipids and lipoproteins may not, in addition, reveal phase transitions influenced by or possibly determined by the nature of the vicinal water structure. The possible occurrence of transitions in lipid-water systems caused by the changes in water structure (for which only a very limited amount of evidence will be presented) is not a highly useful piece of information and, in fact, confounds the issue rather than shedding light on the general problem of the temperature response of biological systems. However, one must not despair because of the extreme complexity which is implied in this connection. With the availability of more and more discriminating techniques (such as NMR), it is very likely that the environments and motions of water molecules vicinal to, say, a phospholipid membrane may be delineated with some degree of certainty. For a comprehensive review of lipids, see the article by Shah (1970).

An excellent discussion of an example of a ternary system of lipids has been presented by Mandell *et al.* (1967). This study has resulted in very

detailed phase diagrams for some three-component systems (e.g., sodium caprylate-decanol-water). The corresponding visually observed microscopic phase appearances are also shown in this article and a number of suggestions made to explain structuring in such systems. The behavior is truly complex, and it is somewhat distressing that in biological systems the mesomorphic phases are undoubtedly even more complex. In connection with this study, it is also notable, as pointed out by Mandell *et al.*, that water and decanol, which have practically zero mutual solubility, become mutually soluble in all proportions in the presence of a critical concentration of bile acid salts. What is desired to stress here is, of course, the active part which the water may play in this process, namely, through the ubiquitous ability to form bonds in a great variety of ways through the multitude of possible modes of hydrogen bonding (see, for instance, the discussion of the organic hydrates by Jeffrey in Section V,B,2).

For a more recent review of the study of lipids and lipoproteins, particularly in membranes, see the article by D. Chapman and Salsbury (1970). This review is mainly concerned with the use of NMR as a tool for the study of the lipid system. In connection with the phospholipids, see also the discussion by R. A. Chapman (1967). The great complexity observable with anhydrous lipids is amply illustrated in the article by Barrall and Guffy (1967); see also the article by Cyr *et al.* (1967, p. 13) and the article by Flautt and Lawson (1967, p. 26). The complexity of molecular interactions in some mixed lecithins was studied recently by Phillips *et al.* (1970). Finally, see Luzzati and co-workers (1968) regarding water/lipid interactions.

An interesting note on the temperature stability of high-density lipoprotein from egg yolk has been published by Franzen *et al.* (1970) who reported on data for the specific optical rotation of  $\beta$ -lipovitellin as a function of temperature. The data shown have, according to the authors, been "arbitrarily fitted to a linear function. The dashed lines represent one SD from the least squares mean value of the specific rotation which is represented by the solid line." This is a remarkable approach as the data themselves show an impressive trend of temperatures above 30°C; thus, although the authors have suggested an "average" straight line fitted to the data with a positive temperature coefficient, an inspection of the data (above 30°C) clearly indicates a negative temperature coefficient. (Apart from this trend, other trends may possibly be present in the data, such as a minimum in the specific rotation in the vicinity of 15° to 20°C.) If the changes at 15° and 30°C are caused by vicinal water—certainly not an unlikely suggestion—the results are of further interest as specific rotation for other optically active, aqueous systems have also shown anomalies at 15° and 30°C. Compare Section V,D,10 for a discussion of the optical ro-

tation of D-glucose (as studied by Martin-Löf and Sörenmark, 1969b,c) and the measurements by Kendrew and Moelwyn-Hughes (1940; see Drost-Hansen, 1965a) on some reducing sugars.

## 2. *Lipids in Membranes*

A review of the chemistry of membranes, including lipids, is presented by Van Bruggen in Chapter I of Part A. One of the most obvious needs for studies of lipids and lipoproteins is in connection with the very question of the cell interface. This interest stems from the pioneer studies of Gorter and Grendal (1925) and Danielli and Davson (1935) and co-workers (see Davson and Danielli, 1943) who devised the concept of bimolecular lipid leaflets as a possible model of the cell membrane.

Various authors have devised different lipid-water systems amenable in principle to theoretical treatments. However, the treatment of each is often tenuous. Ohki (1970) has considered the following possible forms of phospholipids dispersed in aqueous solution: (1) spherical micelles, (2) cylindrical micelles, (3) bilayers, and (4) lamellar and "bubble" membranes (vesicles). (The latter two are both related to the bilayer structure.) The essential point is that in all cases mentioned the surface-to-volume ratio is large, and it is to be expected that vicinal water structures may play an important role in determining the overall properties of the systems. Differential thermal analysis has proven a discriminating tool by which to study phase transitions in phospholipids—both in the anhydrous state as well as in the lipid-water systems. Again, the complexity is enormous and the reader is referred to articles such as the recent one by Abramson (1970). Other references to the problem of phase transitions in lipid systems, and particularly in connection with membranes, are found in Ladbrooke and Chapman (1969); this article is a lucid survey of lipids as well as proteins and biological membrane material by thermal analysis and contains an excellent review of the extensive writings by Chapman and co-workers on the subject of lipids. See also the monograph on liquid crystals edited by Porter and Johnson (1967).

An interesting discussion of the behavior of lipid systems was presented by Small (1970). Again, the use of tools such as differential scanning calorimetry and petrographic microscopy have assisted greatly in delineating the complexity of cholesterol esters. Notable transitions were observed at a number of different temperatures. However, it is emphasized that while the anhydrous lipids do reveal abrupt, anomalous changes at a number of discrete temperatures, biological systems most likely involve aqueous lipid systems. It is very possible that some, if not all, of the anomalies observed in biological systems originate in lipid-water transi-

tions rather than transitions which occur in the anhydrous lipids [see the discussion of thermal transitions of phospholipids in water presented by Abramson (1970, p. 37)]. Because of the complexity referred to above, it remains to be determined which of the anomalies owe their existence primarily to the water structure changes and which, if any, are primarily determined by the nature of the pure lipid transitions.

Various effects of lipids in membranes have been discussed in a number of articles published in the recent monograph "Biological Membranes," edited by D. Chapman (1968). Particular attention is called to the chapters by Rouser *et al.* (1968, p. 5), Luzzati (1968, p. 71), D. Chapman and Wallach (1968, p. 125), and Dawson (1968, p. 203).

Recently, Bean and Chan (1969) have studied thermal transitions and electrical conductivity of ultrathin lipid membranes. While recognizing that thermal transitions may occur due to changes in water structure, Bean and Chan seem to prefer an interpretation (in the case of those lipid bilayer membranes which are modified by proteins) in terms of a shift in equilibria between two conductive states in a protein "pore." The discussion by these authors is interesting; they state:

...both of the anomalous transitions observed here are in the direction of decreasing conductance with increasing temperature, which would be more compatible with an increase in molecular organization or interaction leading to a decrease in diffusion coefficients for ions in the region of the membrane. Such reorganization might be within the lipid bilayer, at the bilayer interface, or even in the surrounding thickened torus of retracted lipids at the edge of the bilayer. Interfacial reorganization might include changes in water structure (icebergs) associated with the membrane, as suggested by Drost-Hansen and Thorhaug but could also be the result of reorientation of the polar groups of the lipids in the interface, which would also create a shift in water structure.

In connection with the question of lipids as a membrane constituent, it is of interest to note that D. Chapman [1965, quoted in Rose's book (1967, p. 131)] has observed endothermic transitions at temperatures of about 33° and 48°C by differential thermal analysis with human cerebral and central nervous system myelin.

Various aspects of bimolecular films have been reviewed by Tien and James in Chapter VI of this volume (Part A). As mentioned in the following section, other resistance measurements on bimolecular leaflet films have suggested strong temperature-dependent anomalies (T. E. Thompson, 1964). More recently, Ting *et al.* (1968) have studied black lipid membranes prepared from chlorophyl and chloroplast pigments in sodium chloride solutions. Properties of these chlorophyl lipid membranes are notably different from those of black lipid membranes prepared with naturally occurring phospholipids, synthetic surfactants, and oxidized choles-

terol. However, it appears that temperature plays a notable role in the stability of these films. Thus, Ting *et al.* note that, whereas the resistance decreased with increasing temperature (as would be expected) over the range of 16° to 30°C, the membrane was stable only for short periods of time above 34°C while below 16°C a precipitation took place in the membrane.

## D. MEMBRANES

### 1. *Introduction*

The general problem of morphology, structure, and functioning of cellular membranes is outside the scope of the present chapter. We are concerned here only with the role which water may play in membrane stability and functioning. However, in order to appreciate this problem it is necessary to have some acquaintance with the nature of cellular membranes, and the reader is referred to the monographs edited by Järnefelt (1968), Kavanau (1965), D. Chapman (1968), New York Heart Association (1968), several of the symposia published by the Society for Experimental Biology (see S. E. B., 1965), Schlögl (1964), Clark and Nachmansohn (1954), Kleinzeller and Kotyk (1961), Schoffeniels (1967), Snell *et al.* (1970), and particularly Lakshminarayanaiah (1969). The last-mentioned volume (dealing especially with transport phenomena in membranes) contains an extensive bibliography, including an excellent annotated bibliography of recent work; see also the earlier work of Davson and Danielli (1943). Among other reviews of biological membrane structure, see "Recent Progress in Surface Science" edited by Danielli *et al.* (1964, 1970) and also the selection of papers by Branton and Park (1968). Two symposia on membrane properties deserve special mention: "Membrane Phenomena" (see Faraday Society, 1956) and "Biological Membranes: Recent Progress" (New York Academy of Sciences, 1966). See also "Symposium on Cell Membrane Biophysics" (1968).

### 2. *Vicinal Water in Membranes*

In terms of transport processes the thermal anomalies may be seen as manifestations of water structure changes, resulting in vastly different mobilities of solutes (ions and low molecular weight nonelectrolytes) in the vicinal water in membranes. Again, a change in the vicinal water structure may, in turn, result in a change in the conformation of the substrate. In terms of the holist theory, the thermal anomalies are manifestations of changes in the thermodynamic activities of the solute as the

vicinal water structure suddenly changes. It is also possible, of course, that the influence of the changes of the water structure simultaneously affect both thermodynamic equilibrium and transport properties. Thus, by itself, the occurrence of thermal anomalies does not distinguish between active and passive transport, on the one hand, and change of the cell water *in toto*, on the other hand.

A recent study of membranes by dielectric measurements will illustrate the likely (or perhaps maximal) extent of water structuring in membranes. Coster and Simons (1970) have used a Wayne-Kerr conductance bridge to study the capacitance changes in membranes. The main result from this study was the discovery of layers of water near lipid membranes with properties notably different from the properties of bulk water. The apparent thickness of the water layers adjacent to the membrane appeared to approach 40,000 Å! Such a layer (4  $\mu$  thick) would obviously have a very pronounced effect on the overall electrical properties of all types of cells if these do, indeed, have characteristics similar to the lipid membranes proposed initially by Danielli and Davson (1935). However, Coster and Simons (1970) call attention to the need to examine carefully some of the underlying assumptions required to extract the value for the thickness of the changed water layer from the experimental data; it is to be hoped that this type of study will continue as it appears to offer a very promising approach to the understanding of vicinal water in biological systems.

More than anyone else, it is probably Stein (1967) who has contributed the most systematic analysis of membrane functioning in terms of the underlying molecular processes, especially transport theories. Stein seems particularly attuned to the possible structural roles of water in the morphology and functioning of membranes.

### *3. Examples of Thermal Anomalies in Membranes*

Remarkably sharp anomalies in membrane properties have been observed by Dalton and Snart (1967). It is interesting that in these studies one of the characteristic temperatures—at which an abrupt change in the energy of activation for the conduction through the toad bladder membrane occurs—is very close to 28°C ( $28.2 \pm 0.4^\circ\text{C}$ ). It seems reasonable to propose that this change is a manifestation of a change in water structure associated with the vicinal water of the membrane. However, a second (and often very abrupt) anomaly occurs in the vicinity of 37.2°C. The origin of this anomaly is far more difficult to explain within the framework of what has been discussed in the present paper as this temperature does not coincide with any of the known thermal anomalies due to water

structure changes. However, as pointed out in Section V,C an anomaly near 37°C could be due to a transition in the lipids of the membrane.

As demonstrated in this chapter, many data from the biochemical and biological literature show evidence of thermal anomalies, but the anomalies have frequently been overlooked or ignored by the authors themselves. A striking example of the recognition that not all functional relationships are straight lines (or, at best, simple smooth curves) is reported in the discussion "Cellular Dynamics" (Peachey, 1968). When Booij (see Peachey, 1968) became aware of the data on the anomalous phospholipid membrane resistances as a function of temperature, as presented by T. E. Thompson (1964; also see Drost-Hansen and Thorhaug, 1967), he reported an observation which he had previously considered too unimportant to publish, namely, that in the range from 17.5° to 27.5°C the permeability of onion scale was relatively temperature independent. However, below this temperature the water permeability dropped, apparently significantly, and above 27.5° the permeability increased rapidly. Booij conjectured (undoubtedly correctly) that these "breaks" might be related to the anomalies reported by Thompson for the electrical resistance of the phospholipid membranes as a function of the temperature.

Siegel (1969) has studied the excretion of  $\beta$ -cyanin by beet roots as a function of temperature in an oxidizing environment. An Arrhenius plot of the  $\beta$ -cyanin rate of discharge versus reciprocal absolute temperature shows two distinct limiting curves—changing from one curve segment to another at 60°C. The difference in the apparent energies of activation is considerable (93 kcal/mole below 60°C and 18 kcal/mole above 60°C).

Among the anomalous, abrupt changes in membrane properties reviewed by Drost-Hansen and Thorhaug (1967) were the rates of diffusion of sodium and potassium chloride across a "butanol membrane" (data from Rosano *et al.*, 1961). Mention was also made of the temperature dependence of the resistance of a phospholipid bimolecular membrane studied by T. E. Thompson (1964) and the highly anomalous conductance of a barium stearate multilayer membrane studied by Nelson, and Blei (1966). Additional data on collodion-potassium oleate membranes were reported by Nelson, and these frequently exhibited maxima near 15°C in bi-ionic potentials. Particularly interesting results were obtained (using 1 M solutions of KCl and NaCl, separated by the membrane) at high temperatures. The results, reversible below 50°C, suggest that significant, anomalous changes occur in these membranes as a function of temperature.

Some interesting results obtained by differential scanning calorimetry were discussed by Steim (1968). The study by Steim involves spectroscopic and calorimetric measurements of biological membrane materials, as well

as of some simple aqueous systems, particularly lipid-water systems. The results on the materials from the membrane of *Mycoplasma laidlawii* are especially interesting as they clearly show several transitions near the temperatures discussed in the present article and here ascribed to the changes in vicinal water structure. It is not presently possible to correlate all of the observations made by Steim with the changes discussed in the present chapter; however, the study is important in demonstrating some possible advantages of differential calorimetry over other tools such as high-resolution NMR or optical rotation dispersion studies. Incidentally, Steim concludes from this study that his data can be interpreted most readily in terms of the membrane bilayer hypothesis, but Steim does not emphasize the role of water structure changes in this connection.

Chaudhry and Mishra (1969) measured the diffusion of  $^{24}\text{Na}$  across atrial wall segments from the rat heart. The measurements were made with equal sodium ion concentrations on both sides of the wall of the tissue studied. The authors observed transitions in the diffusion versus temperature at 15°, 32°, and 42°C. Because these values are close to those described by the present author, suggesting the occurrence of higher-order phase transitions in water near interfaces, Chaudhry and Mishra observe that this may be taken as evidence for the diffusion of the sodium ion through water-filled pores. While the experiments by Chaudhry and Mishra are remarkable, caution must be exercised in the interpretation. Thus, conceivably the transport through the membrane might still take place via mechanisms other than diffusion in water-filled pores; the notable temperature effects might be due to structural effects in the layers of water adjacent to the membrane rather than *in* the membrane itself. However, the study by Chaudhry and Mishra certainly deserves careful consideration and emphasizes the utility of further detailed studies along these lines.

#### 4. Permeability Studies

In an impressive series of studies, Wright and Diamond (1969; also see Diamond and Wright, 1969) have studied the membrane permeability of various nonelectrolytes. The study is not only a profound and extensive, comparative study of various permeability coefficients for a large number of nonelectrolytes, but it is based on a surprisingly simple approach—the nonelectrolyte movement is monitored through the attendant osmotic flow through the membrane, giving rise to a streaming potential.

In connection with osmotic flow, it is important to consider the existence of an unstirred layer; this was discussed in some detail by Wright and Diamond. They correctly point out that the unstirred layer may play a

dominant and, in fact, destructive influence on the interpretation of data aimed at calculating activation energies of permeation (from permeability measurements at different temperatures), solute-solvent interactions in membrane permeation, and comparisons of measured and observed reflection coefficient values in connection with different kinetic models of membrane structure and permeation processes. However, in the study of the rabbit gall bladder membrane by Wright and Diamond the effect of the unstirred layer played a considerably less crucial role. According to these authors, the unstirred layer effect is simply to shift the observed reflection coefficients since the method is essentially a comparative method. Nonetheless, it is obvious that the effect can hardly be completely neglected since the unstirred layer will, if structured, obscure differences between the structured elements of water in the permeated membrane material and in the unstirred layer.

Many nonelectrolytes may be diffused through cell membranes, but vast differences are observed in the permeation rates. Thus, Wright and Diamond (1969) note that molecules of approximately the same size and molecular weight and diffusion coefficients in bulk solution may differ by as much as a factor of  $10^8$  in the rate of diffusion through various membranes.

Wright and Diamond measured the permeability of some sixty non-electrolytes and correlated the permeabilities with the oil-water partition coefficient (and with the ethyl ether-water partition coefficient). Similar studies have been carried out by Collander (see, for instance, Stein, 1967). Phenomenologically, the data from these studies (as well as many others; see the two foregoing works for references) have provided a large amount of information. However, it appears very difficult to elucidate systematically the relation between the nature of the nonelectrolyte and its permeability. Obviously, size alone will play an important role as will the presence of functional groups, steric aspects, etc. Yet, undoubtedly superimposed on these facets is the probable importance of the hydration of the nonelectrolytes, the hydration of the membrane matrix, and the detailed structure of the vicinal water of the membranes. Unfortunately, it will no doubt be some time before these relationships will begin to be understood quantitatively. Wright and Diamond refer to "anomalous" and "normal" permeability components; there is little doubt that the detailed nature of the matrix will influence relative permeabilities of the solutes. It is interesting that in spite of all the information and its correlation, for instance, with lipid solubility, it is still impossible to make significant statements regarding the likelihood that permeation (especially for water) is primarily via a continuous phase (water-filled pore) or a solvent process in the membrane material.

Among the several significant facts noted by Wright and Diamond is the possible important effects of unstirred layers on tracer permeability when compared to osmotic permeabilities. The difference was tentatively identified with momentum transfer between solute and water molecules. The articles by Wright and Diamond (1969; Diamond and Wright, 1969) should be consulted for details in connection with this discussion; the authors point out that:

All these properties of water in narrow channels are intermediate between properties of bulk water and of ice, indicating that water near a charged surface assumes a more ordered, ice-like structure. Most of this experimental work has been in channels with diameters in the range of 100–1000 Å, whereas anomalous non-electrolyte permeation in the gall bladder disappears for solutes with more than about three carbon atoms (hydrated diameters larger than about 5 or 6 Å).

Further permeability studies have been published by Wright and Prather (1970; also, see, Prather and Wright, 1970). Finally, it should be noted that Wright and Diamond (1969) analyzed the available data in terms of molecular models, taking into account the various types of forces affecting the nonelectrolytes, such as the permanent dipoles, induced dipoles, van der Waals forces, including short-range repulsive forces and inductive effects.

##### 5. *Unstirred Layers*

For some interesting measurements and, particularly, some interesting speculations regarding the effects of unstirred layers (in connection with transport numbers), see the article by Barry and Hope (1969). These authors are not concerned with the possible structuring of any vicinal water in or adjacent to the cell wall and cell membranes, but draw attention to the charge accumulation effects which may occur in such regions due to superimposed electric fields.

The thickness of unstirred layers is usually taken to be more than a few microns and often as much as 20–50  $\mu$  (see, for instance, Curran). For a discussion of the effect of unstirred layers on the determination of apparent permeability coefficients, see the discussion by Goldup *et al.* (1970, p. 244). Recently, another study has dealt with the problem of the effects of the unstirred layer. Green and Otori (1970) have studied by a direct optical method the thickness of the unstirred layer of fluid adjacent to two solid interfaces. The surfaces observed were, respectively, the posterior surface of the rabbit cornea and a glass surface (a contact lens). The procedure was to study the movement of various types of small, discrete light-scattering particles, such as polystyrene (less than 0.25  $\mu$  in

diameter) or carmine particles. The thickness of the layer in which there was no notable movement of the suspended particles (other than that which could be ascribed to diffusion) was determined optically. In the unstirred case, the layer thickness appeared to depend on the nature of the solid material, being about  $350 \mu$  thick on the cornea and  $150 \mu$  thick on the contact lens. The thickness was also measured with vigorous stirring, which reduced the stagnant layers to  $65 \mu$  and less than  $20 \mu$ , respectively. It is unfortunate that not quantitative estimates were presented for the shear rates. Thus, it is relatively uninstructive to know the motion in the cell was achieved by stirring at 400 rpm with a Teflon-coated magnetic stirring bar. However, even without a quantitative estimate of the degree of agitation at the interface, it does appear as if very notable thicknesses must be allowed for in diffusion studies at membranes.

#### *6. Comparison of Membrane Matrix Effects*

In connection with the structural characteristics of the cellular plasma membrane, Schultz and Asunmaa (1970) have studied the possibility of ordered structures of water. The article reviews some of the evidence available for ordering of water near solid surfaces in general and near membranes in particular. The authors appear to adopt the notion of a well-defined thickness of very highly structured water. One of the most interesting observations from the studies of these authors is the suggestion that "It has been demonstrated that the characteristics of ordered water in a porous glass desalination membrane are very similar to those in a cellulose acetate desalination membrane. This result is very surprising and further experimental work is required to see if the same is true in other strongly hydrophilic membranes."

The present author does not necessarily subscribe to the notion of a very sharply defined, structured layer near any solid interface nor to the experimental foundation on which the proposed similarity in water structure between the highly different substrates has been based. At the same time, however, as discussed elsewhere in the present chapter, there is evidence that the detailed chemical nature of the substrate may play only a secondary role. In other words, the proposal by Schultz and Asunmaa does agree qualitatively with the observation that similar, ordered structures appear to be induced by the proximity to different solid interfaces, as evidenced by the occurrence of thermal anomalies at the same temperatures, regardless of the nature of the substrate.

*a. Spectroscopic Studies.* Only relatively few systematic studies have been made on membranes by spectroscopic means. However, Zundel (1969) has recently published a monograph devoted to the study of hydration and intermolecular interactions with polyelectrolyte membranes by

infrared spectroscopy. Although this book deals with physicochemically well-defined types of membrane materials, it is very likely that the general approach will become a model for spectral studies of other types of membrane materials, including biological materials. As could be expected, a large fraction of this study is devoted to the spectroscopic properties of the water of such membranes and membrane materials. It should be stressed, however, that the approach, while sound and extremely fundamental, does not allow for more sophisticated aspects, such as modified or extensive hydration structures. This is quite natural, of course, considering the relatively insensitive technique used, namely, the spectral changes primarily effected through the hydrogen bond with its attendant large bandwidth. Those aspects that result from the ion hydration or the specific polymeric contributions from the membrane substrate will not readily reflect the details of "secondary hydration structure" effects (see the discussion of Sidorova's studies, Section V,B,6,h).

*b. Study by Resing and Neihof.* Resing and Neihof (1970) studied the nature of adsorbed water on bacterial cell walls by an NMR technique. These authors worked with a "representative bacterium" (*Bacillus megaterium*) over a wide temperature range with isolated cell walls containing approximately 33% water. The authors found no evidence that the water of these cell walls was "ice-like" nor did they find that the mobility of the water was of the order of magnitude expected for "solid-state like" water (in the temperature range in which the bacterium grows). Specifically, the authors found that "the distribution function necessary to fit the cell wall relaxation time data is so broad that it reaches, with appreciable amplitude, from the liquid value to the value in ice. Nevertheless, the median is much closer to the jump time for liquid water than for ice. The conclusion is clearly that the water in cell walls is not 'ice-like' in terms of mobility."

A number of comments are appropriate regarding the study by Resing and Neihof (1970). These authors did not discover any notable reduction in the mobility of the water near the membrane surface. On the other hand, as discussed in the section dealing with NMR studies by Glasel, it has been clearly shown that some macromolecules do not appear to possess extensive hydration structures. Again, notable differences are frequently observed between the properties of water in living organisms and in dead organisms (and, incidentally, between dormant and active states). The argument by Resing and Neihof, that removing some of the water (from "at least 90% of the volume") to 33% water (by lyophilizing and rehumidifying) should not necessarily be expected to result in a water structure resembling that in the living organism. However, it might still be argued that since most any solid interface apparently tends to induce

vicinal water structures, some kind of structure should still have been observed—even though it might have no relation to the original water structure of the living bacterium. It seems at this time that the study by Resing and Neihof is more an anomaly than a general finding. As such, the study deserves careful consideration but the results certainly cannot readily form the basis of more generalized statements about absence of ordered water structure near biological interfaces!

The membrane samples used by Resing and Neihof (1970) were prepared by storing the specimens in an evacuated desiccator over saturated sodium acetate solutions, corresponding to a relative humidity of 76% (at 20° C). Such drying may significantly influence the nature of the adsorbed macromolecules. Wetzel and co-workers (1969), for example, have shown that pronounced changes in the UV absorption spectrum and the dichroism of oriented films of calf thymus DNA occur as a function of relative humidity. Particularly, these authors found that notable hysteresis effects were observed in the range of relative humidities from 0 to 65% and called attention to the structural changes proposed by Falk and co-workers (1962, 1963) in the structure of DNA in the range from 55 to 75% relative humidity. Other authors have demonstrated changes in configuration in the range from 75 to 92% relative humidity in DNA. Thus, the absence of evidence for structuring at the cell walls studied by Resing and Neihof (in the range of water contents employed by these authors) does not rule out the possibility that structuring may indeed occur in the living cell.

### *7. Studies by Good*

An important contribution to the understanding of the state of water in membranes has come from the extensive studies by Good (see also Coldman) on the hemolysis of mammalian erythrocytes. For over a decade Good has studied the details of hemolysis of erythrocytes as a function of temperature in the presence and absence of various pharmacons. For this study, Good and co-workers have based their approach entirely on the kinetics of the hemolysis of the erythrocyte membranes. The results have been cast in the form of the Eyring rate equation and the results interpreted in terms of the apparent enthalpies, entropies, and free energies of activation (and the activation equilibrium constant  $K$ ).

The most obvious results obtained by Good and Coldman is the impressive degree of linearity between the apparent entropy of activation as a function of the apparent enthalpy of activation. Stressed by several authors as well as elsewhere in this paper, the detailed information about the apparent enthalpies of activation and the entropies of activation are

far more revealing than merely the apparent free energy of activation ( $\Delta G^\ddagger$ ). The linear relationships between  $\Delta S^\ddagger$  and  $\Delta H^\ddagger$  is often encountered in chemical kinetics. Good points out that the proportionality constant in the expression

$$\Delta H^\ddagger = \Delta H_0^\ddagger + T_c \Delta S^\ddagger$$

can usually be related to the influence of the solvent on the kinetic process in question (see the detailed discussion of the enthalpy-entropy compensation phenomenon in Section V,B,6,d).<sup>\*</sup> Coldman and Good (1968b) interpret the compensation phenomenon as implying that the hydration of the membrane plays the dominant, controlling role in the hemolysis of most of the erythrocyte cells (with the exception of cells from cattle and dogs). The authors further go on to note that the value for the apparent entropy of activation ( $\Delta S^\ddagger$ , extrapolated to 0°K) is practically equal to the standard entropy of water and suggest that the process appears to take place in a wholly ordered water structure environment. This is interesting in connection with the recent NMR studies discussed in Section III,F. Coldman and Good (1968a) summarize some of the pertinent conclusions:

It is concluded from these results that linearity between the Arrhenius activation parameters depends more on cell membrane hydration than on any other single factor.

Furthermore,

It has recently been postulated that the cell membrane contains an interconnected hydrogen-bonded framework—a hydrate continuum—that permeates the ordered lipoprotein structure (16) and it has also been proposed that changes in the configuration of the lipids may determine the water content of the membrane structure (17). More recently a model has been put forward (18) which assigns to water an important role as an integrated structural component of the membrane protein, and which provides also for extensive cell surface hydration. There are, therefore, grounds for supposing that ordered water of hydration is just as important a constituent of the cell membrane as it is of the intracellular phase, and it may be that cell swelling in a hypotonic medium is not due merely to an increase in volume of the intracellular phase, but also to an increase in volume of the membrane.

In a subsequent series of papers, Good and co-workers studied the effects of various pharmacons on the hemolysis of erythrocytes. Particular interest attached to the study of the effects of the barbituates [also, see, the article by Tracey (1968)]. Again, it was found by Good and co-workers that the kinetics conform with the compensation law; that is,

\* Note that in Section V,B,6,d we were concerned mostly with equilibrium properties, whereas Good's studies are of rate processes.

that the apparent entropy of activation is linearly related to the apparent enthalpy of activation and thus reveals the likely importance of hydration effects on the mechanism of the process. Furthermore, Coldman and Good (1969) note that the results suggest that the nonpolar side chains of the barbituates play an important role through the attendant hydration phenomenon and relate this observation in terms of the pharmacological activity. Specifically, with regard to hydrophobic hydration (referred to by Good and co-workers as "apolar hydration"), the authors note that:

... it is well known (21), however, and its occurrence depends on the structure of the solute and the absence of a direct interaction between solute and water; the solute behaves essentially as an inert support that maintains the first layer of surrounding water molecules in tetrahedral configuration, thus favoring the formation of pentagonal polyhedral hydrogen-bonded structures or clathrate cages of water. The capacity of water to form such cages is almost limitless, and studies with alkyl-substituted ammonium salts show that entities as large as the tetraisobutyl group (22) and the benzene ring (23) can be enclosed by water in this way.

Finally, in a separate study, Coldman and Good (1969) studied the hydralional effects of leptazol and concluded "... that the convulsions induced by leptazol, insulin hypoglycemia and electric-shock treatment may depend on the disruption of cerebral hydration structure."

In conclusion, there is little doubt that the studies by Good and co-workers will become classics in their approach to the understanding of the kinetic behavior of biological systems (through careful studies of temperature effects) and through the information which may be obtained from such studies regarding the role played by the solvent—the water.

### 8. Other Studies on Erythrocytes

Controversy continues as to whether or not the permeation of water through red cells is through individual pores (by a flow mechanism perhaps resembling Poiseuille flow) or through some "solubility matrix effect" (implying the absence of discrete pores). Recently, Solomon and co-workers (see Vieira *et al.*, 1970) studied the hydraulic permeability of erythrocytes of humans and of dogs as a function of temperature. Based on their measurements, the authors concluded that the product,  $L_p \times \eta_w$ , was constant over the temperature range studied. This, in turn, was interpreted as supporting the view that over the temperature range of interest (5–39°C), temperature induced no restraints on the equivalent pores. Though it could not be ascertained that Poiseuille flow did, indeed, take place in such small pores, speculations were advanced that at least for the dog erythrocyte membrane, the diffusion of water occurred through a

diffusion mechanism similar to that occurring in free solution. Figure 17A shows the normalized hydraulic conductivity coefficient for canine erythrocytes, as shown by Solomon and co-workers. Figure 17B shows the same data redrawn by the present author. It is immediately obvious

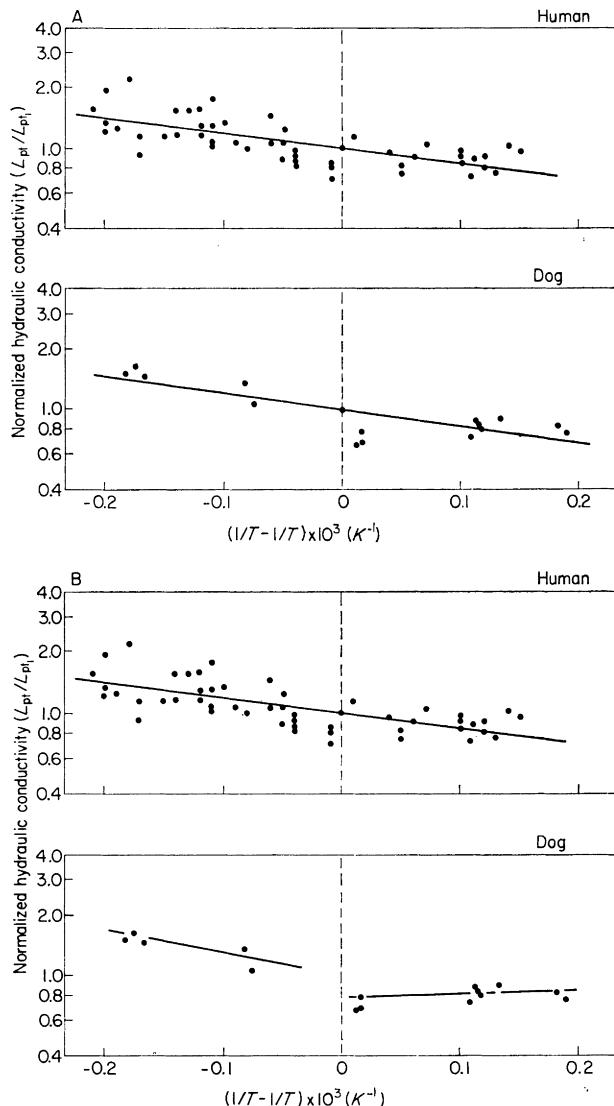


FIG. 17. (A) Normalized hydraulic conductivity coefficient for erythrocytes. (Solomon and co-workers, see Vieira *et al.*, 1970, with permission.) (B) Same data points as in Fig. 17A but curve redrawn by present author.

that the points below approximately 27°C are more-or-less temperature independent, and thus the product of the hydraulic permeability coefficient and the viscosity of the bulk water will not be temperature independent as required for Solomon's interpretation. The difference in the two sets of curves (A and B) comes, of course, from the different ways of assessing experimental errors: Solomon and co-workers merely drew a straight line, fitted by a least-square best fit, through all the data points obtained without any regard for trends. The redrawn illustration (Fig. 17B), however, becomes just as probable when the possibility of a structural change near 30°C has been accepted.

The data obtained by Solomon and co-workers for the hydraulic conductivity coefficient in human erythrocytes may or may not show the same effect, namely, that below 27°C the hydraulic conductivity is essentially temperature independent but increases slightly above this temperature. However, in the case of the experiments with the human erythrocytes, the experimental scatter is too large to draw any conclusions with certainty. The large scatter in the data in the latter case by itself does obviously not prove the converse hypothesis, namely, that the least-square best fit adopted by Solomon and co-workers is the proper analytical functional representation of the temperature dependence of the hydraulic conductivity. For a discussion of the pore concept in membranes, see also Solomon (1968).

The studies by Solomon and co-workers have in the past contributed significantly to our understanding of membrane functioning and particularly to the problem of the mechanism of solvent and solute transport across membranes. The example discussed here is in no way meant to belittle the signal contributions of Solomon and co-workers, but rather to call attention to the dangers inherent in overlooking the importance of trends versus experimental errors in experimental data.

The possible existence of actual water-filled "pores" in cell membranes (of lipidic nature) compared to other possible mechanisms for transport, especially for water, has been discussed by several authors. In addition to the contributions by Solomon *et al.*, see also the article by Ilani and Tzivoni (1968) who suggest that the water, at least in the simple hydrophobic membranes studied by these authors (prepared by impregnating filter matrices with toluene or other organic liquids), does not possess actual "open pores"—hardly a surprising conclusion. The inferences from this study were tentatively considered in connection with the general cell membrane. In this connection, see also the studies by Ting *et al.* (1966). These authors have considered the possible existence of "soft ice" at the interface between butanol and an aqueous salt solution. The butanol was chosen as an experimentally convenient and conceptually reasonably

simple model system of a lipid by Schulman and co-workers (see Rosano *et al.*, 1961). Ting *et al.* note, incidentally, that at least for rubidium, the Arrhenius plot of the rate of transfer of the ion across the interface suggests a break at 15°C. Compare in this connection the note by Drost-Hansen and Thorhaug (1967). Among the many other studies concerned with the problem of the possible existence of water-filled pores in membranes, attention should be drawn to the study by Gutknecht (1968) who concluded that, at least for *Valonia*, it appears unlikely that the protoplast contained specific, water-filled pores.

### 9. Bangham's Studies

S. M. Johnson and Bangham (1969) have studied the effects of anesthetic agents on the phospholipid membranes; Bangham and co-workers (1965a,b, 1966) have contributed greatly to the understanding of membrane properties and processes (as well as anesthesia) over several years. Johnson and Bangham (1969) specifically studied the permeability to potassium ions of 4% phosphatidic acid—96% phosphatidylcholine liposomes. Experiments were performed in the presence and absence of anesthetic agents, including ether, chloroform, and *n*-butanol; the effect of valinomycin was also studied. The rate data obtained were exploited in terms of apparent enthalpies of activation in an Arrhenius equation. Among the results obtained were (1) the observation that a cation permeability barrier was located at the water-lipid interface, (2) "the anesthetics increased the freedom of movement of groups in the lipid molecules near the interface," (3) the increase in permeability of K<sup>+</sup> in the presence of valinomycin was due to an entropy increase in the activated state, approximately 35 cal × mole<sup>-1</sup> × deg<sup>-1</sup>, and (4) "the increased freedom of movement in the interface when the anesthetic was present allowed the valinomycin to adopt a more favorable orientation in the interface for the exchange of K<sup>+</sup>."

The study by Johnson and Bangham is a relatively straightforward application of an Arrhenius equation to the experimental rate data observed. However, the study is a typical example of the dangers which result from neglecting obvious anomalies, such as trends in experimental data. Figure 18A (Johnson and Bangham, 1969, p. 93, Fig. 3) shows the liposome permeability in the presence of chloroform. Valinomycin was present in a mole ratio of 1:10<sup>6</sup> (lipid). Characteristically, the trends in the data were ignored. Figure 18B shows the same data as reported in the original article, but with the straight lines deleted and more realistic curves drawn in. It is seen that, indeed, rather abrupt anomalies do occur, and these reflect vastly different slopes, i.e., very different activation parameters. Hence,

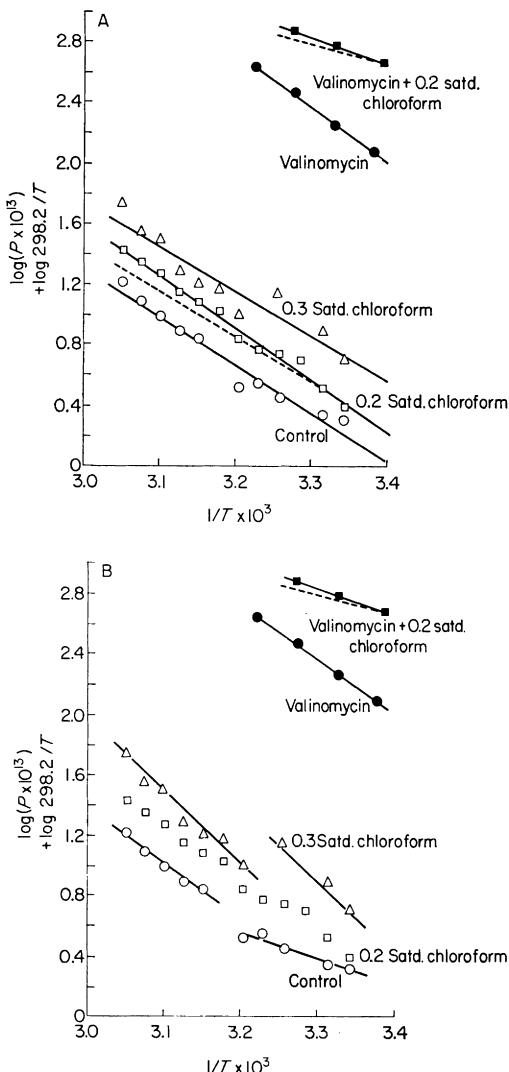


FIG. 18. (A) Liposome permeability; Arrhenius plot. (S. M. Johnson and Bangham, 1969, with permission.) (B) Same data points as shown in Fig. 18A but with curves redrawn by the present author (note—one curve deleted for clarity).

although the present author does not take issue with the general attempt to interpret molecular happenings in terms of a simple kinetic rate expression, he does take issue with the frequently practiced custom of ignoring realistic error limits and the forcing of all experimental data to

fit straight lines in order to obtain, by "brute force methods," data for the apparent activation parameters.

In connection with the study by Johnson and Bangham, notice in Fig. 18B that the failure to produce a reasonably smooth curve (i.e., free of thermal anomalies) increases as the concentration of chloroform increases. A tempting suggestion here is that the chloroform stabilizes vicinal water structures through clathrate hydrate formation—the more pronounced this structure is, the more abrupt the attendant thermal transitions will be (compare the studies by Nelson and Blei, 1966). Note also, however, that chloroform is readily soluble in the lipid.

There is little doubt that the curves shown in Fig. 18B fit the experimental data considerably better than the straight lines proposed by Bangham and Johnson. However, it should be noted in Fig. 18B that the anomalies do not appear to occur near 30° and 45°C as would be expected, but rather tend to show an anomaly in the vicinity of 38°C. In this connection, compare the discussion of the phase transitions of the lipids and particularly the studies by Chapman and co-workers, Sections V,C,1 and 2, and the studies by Steim, Section V,D,3.

#### 10. Cellulosic Membranes

The problem of water in and adjacent to cellulose has been a subject of a great deal of research because of the obvious industrial importance, as well as the role cellulose plays in understanding the physiology of plants; many of the temperature-sensitive phenomena observed in plant growth are likely related to the water-cellulose interaction. In this section, however, the stress is primarily on the simplified system of isolated cellulose and water.

Notable contributions in this field have come from studies such as those by Goring and co-workers in Canada, by Forslind in Stockholm and by the researchers at the Swedish Forest Research Laboratory in Sweden. Before discussing these contributions, attention is called to results of Haase and Steinert (1959), discussed by the present author (Drost-Hansen, 1969b). Haase and Steinert observed notable anomalies in the permeability of treated cellulose membranes and in the apparent heat of transport across cellulosic membranes, near 32° and 45°C. The authors noted that no evidence of anomalies had been reported for the cellulose itself at these temperatures. [It should be noted, however, that in subsequent studies by Haase and de Greiff (1965), the effect was not reproduced. In this connection, see the discussion by Kerr (1970), by the present author (Drost-Hansen, 1969b), and in Section VI,J.]

Martin-Löf and Söremark (1969a,b,c) have studied thermal transitions

in cellulose utilizing various techniques. These authors have observed abrupt anomalies at a number of discrete temperatures (in some cases, near 0°, 35°, and 60°C); most of the studies were by some type of dilatometry. However, additional experiments were carried out, for instance, with D-glucose, where measurements were made of the optical rotation. This quantity showed a minimum near 28°C and a maximum near 47°C. These results should be compared with the study by Kendrew and Moelwyn-Hughes from 1940 (quoted by Drost-Hansen, 1967a): these authors studied the optical rotation of several different reducing sugars. In all of the available data, it appears that a thermal anomaly occurs in the vicinity of 15°C.

Finally, it is of interest also to note that the studies by Martin-Löf and Söremark also revealed anomalies near 30°C in the NMR linewidth in hemicellulose. The reader is referred for details to the extensive reports by these authors. It should be mentioned that the authors do not necessarily consider the anomalies as manifestations of higher-order (cooperative) phase transitions, but tentatively suggest the possibility (Martin-Löf and Söremark, 1969) that the anomalies may owe their existence to the sudden onset of rotational modes of individual water molecules.

Martin-Löf and Söremark note that Wahba has added both infrared spectroscopy evidence and optical refraction data to suggest the occurrence of transitions in the water-cellulose system; anomalies were also noted by Back who used a sonic pulse technique and by Kubat using a torsion pendulum.

The earlier studies by Ramiah and Goring (1965) were also concerned with water-cellulose interactions. Again, anomalous changes were observed in the expansion of water-swollen materials (cellulose, hemicellulose, lignin). The authors described the changes and perturbations in the water structure caused by the hydrophilic surfaces of the woody macromolecules. For some details and references, the reader is referred to the paper by the present author (Drost-Hansen, 1969b) or the original papers by Goring and co-workers.

Gary-Bobo and Solomon (1971) have studied transport across cellulose acetate membranes of varying porosity. The authors chose these membranes to "gain further insight into the nature of water-membrane interactions," and employed measurements of hydraulic conductivity and diffusion coefficients (using tritiated water) as a function of temperature. One of the aims of the study was to distinguish between influences due to geometry (such as tortuosity, etc.) and the effects of the water-membrane interactions. Previously, Solomon has advocated that viscous flow through membranes with even very small equivalent pore radii is essentially "classic"; Gary-Bobo and Solomon claim also that "all the experimental re-

sults can be accounted for in terms of known properties of free water and no anomalous behavior of water needs to be postulated." However, Gary-Bobo and Solomon do point to the importance in the diffusion process of the water-membrane interactions, even across membranes with large equivalent pore radii. The choice of cellulose acetate appears somewhat unfortunate for a study of water-membrane interactions for the very reason the authors stress as being of particular interest. The authors quote Franks (1965) stating that since "hydroxyl groups do not alter water structure much, if at all, the behavior of the water in the membrane might be expected to be similar to that of water in bulk." It would seem that, in order to study water-membrane interactions, it would have been better to choose a hydrophobic membrane or one of a less obvious hydrophilic nature. However, compare in this connection, also, the statement by Tait and Franks (1971) that the hydroxyl groups certainly are "sensed" by the aqueous environment, since water appears able to distinguish between  $\alpha$ - and  $\beta$ -methyl pyranosides. In spite of the hydrophilic nature of the membrane material, Gary-Bobo and Solomon demonstrated water-membrane interactions by the notable differences in the observed energies of activation for diffusion in the different membrane materials. Thus, in the membrane with the smallest pore radii, the apparent energy of activation 7.8 kcal/gram mole, compared to 4.8 kcal/gram mole for self-diffusion in water (the value at 20° obtained by Wang, 1965). Gary-Bobo and Solomon also stress the notable difference in the small-pored membranes between the apparent energies of activation for diffusion compared to viscous flow; they suggest that notably different mechanisms are involved and that, in this connection, "viscous flow is a relative motion of portions of a liquid, diffusion is a relative motion of its constituents." It is interesting to speculate that, were it possible to measure viscosity over a very wide range of shear rates, the "limiting value" for the apparent energy of activation for infinitely small shear rates might approach that observed for diffusion! As mentioned briefly in Section VI,J, it is not inconceivable that the viscosity of vicinal water may be shear-rate dependent. Forslind (1968) has previously claimed that water is non-Newtonian. At the same time, the studies by R. J. Miller (1968) failed to demonstrate the existence of a definite critical shear stress; thus, the water at least does not actually "gel" under the conditions of the studies by Miller and co-workers (who worked mostly with clay matrices).

### 11. *Diffusion Studies*

A vast amount of literature exists on the diffusion of various solutes (of both low and very high molecular weight) in water through various

porous materials. However, only a very limited number of studies have been reported where the particle size and the pore diameters do not differ greatly and the dimensions are still large enough to justify *a priori* the use of classic hydrodynamics (Poiseuille flow). Recently, Uzelac and Cussler (1970) have studied the diffusion of monodispersed spherical latex particles (diameter 910 Å) through Millipore filters. These filters had nominal pore diameters of 2200, 3000, 4500, and 12,000 Å. The results of the study are of obvious interest to biophysics as a model for the study, for instance, of red cells passing through vascular capillaries or large macromolecules passing through discrete pores in various types of membranes. Many of the results from this study are important with respect to a theoretical interpretation in terms of the movement of an inert sphere diffusing in a continuum liquid. The authors claim that the temperature dependence is that which would be expected on the basis of the simple expression used by the authors, although an inspection of the data may throw some doubt on the validity of the conclusion. The authors give the expression for the diffusion coefficient ( $D$ ) as:

$$D = \frac{kT}{6\pi\eta ak_1(a/R)} \quad (1)$$

where  $k_1(a/R)$  is a tabulated function. Admittedly, whereas the diffusion coefficients themselves differ by as much as 60%, the product  $a\eta/kT$  differs by only 10%. The ratio of this coefficient at 25° and 45°C, respectively, is less than 1 for the larger pore size (0.45 μ). Whether or not significance can be attached to this, the authors point out that the limiting value of the diffusion coefficient for the ratio  $a/R$  (particle diameter to pore diameter) = 0 is about 10 times larger than the Stokes-Einstein value. The authors claim that this result is not an experimental artifact although they are unable to give an interpretation for the observations. They also call attention to some other studies which have reported enhanced diffusion coefficients—much larger than those predicted by the Stokes-Einstein equation. It is in this connection that it is of interest to consider the possibility that part of the liquid structure in the pores of the Millipore matrix may be disordered, as proposed recently by the present author (Drost-Hansen, 1969b). Some rather tenuous evidence for this has already been noted, based on data for the diffusion coefficient of a number of gases in aqueous suspensions, although there is apparently no general agreement on this point. However, if, indeed, a disordered zone may also exist in reasonably small pores, a greatly enhanced diffusion coefficient might be expected if the viscosity of less structured liquid is lower than that of the ordinary bulk water (and the highly ordered, vicinal water).

## 12. Active Transport

Electrolyte pumps, required to explain active transport, have been discussed in inordinate detail over the past several decades. The differences in the electrolyte contents between interstitial fluid and cell fluid is remarkable. These different electrolyte solutions are separated by the cell membranes, and the various equilibria (or, rather, steady-state processes) are normally considered to require metabolically derived energy. Apparently, in all active transport processes, the source of energy for separating the different electrolytes is derived from ATP. To "explain" active transport in classic terms, various "carriers" are required; these are usually assumed to be proteins. In this section are discussed (if only briefly) some aspects of an alternative view of active transport. A more detailed discussion of this alternative explanation is presented in Section V,G, while some selected aspects of water structure, ATP, and the ATPase problem will be treated later in a separate paper (Drost-Hansen, 1971; in preparation).

Changes in the structure of the intracellular water is a possible cause of transmembrane movements against a "total stoichiometric" concentration gradient. Changes in "available" ions (i.e., changes in "effective concentrations") have been termed changes in "solubility." This, however, seems a poor terminology, since the total number of ions in solution may remain unchanged; instead, what is changed is the activity of the ions (and other solutes). An inspection of even the simplest forms of the Debye-Hückel expression for the activities of ions reveals that notable changes are effected through a change in the dielectric constant of the solvent.

All cells are considered capable of active transport. In this section, we restrict the discussion to plant cells, based on the extensive writings by Stadelmann with some comments in terms of the water structuring discussed in this chapter. In a recent review, Stadelmann (1970) calls attention to the criteria proposed by Sitte (1969) for active transport. Three criteria must be simultaneously obeyed to consider a movement of ions truly to be of the nature of an active transport: "(1) the process uses energy, (2) there is a stoichiometric relation between the amount of ATP used up and the amount of substance transported, (3) the energy supplied from ATP is used directly for the transport of the substance under consideration."

Difficulties have arisen in the past in attempts to demonstrate the reality of active transport for lack of suitable "trans-membrane carriers"; these "transport proteins" have been termed "permeases," "trans-locases," "transfer locases," etc. Such carriers should have some stoichiometric relationship to the number of ions transported. It is possible that one advantage of the alternate theory—that ions are transported merely in a

gradient of ion activity (due to the different aqueous environments)—is the fact that no stoichiometric relationship is necessarily required. The amount (and possibly the rate) of transport may instead be determined exclusively by the changes in activity of the ions and the water molecules (hence, changes in “apparent solubility,” in current terminology). Certainly, of the three criteria discussed above, requirement (2) may play the key role in settling the question of active transport versus a movement in an activity gradient. It is likely, in fact, almost certain, that both mechanisms will require energy [requirement (1)], and it will probably be difficult to prove or disprove any one-to-one correspondence between the energy used and the energy directly supplied from ATP for the transport process under consideration. It is also possible that the actual transport may depend on, or be facilitated by, one of the “transport proteins.” However, the transport proteins act merely as the vehicle for the transported solute. Effectively, this may reduce the energy barrier to be overcome in the process of moving the ion across the membrane, but it bears no relation to the energetics that determine why the ion is moved against the stoichiometric concentration gradient.

As discussed above, the alternative to active transport is the existence of solute activity gradients, caused by the different aqueous environments within the cells. Relaxation measurements may prove to be the most direct way of obtaining further information regarding the structural characteristics of the intracellular water. If the dielectric properties of the intracellular water were known, and particularly if it were possible to obtain information about the variation in effective dielectric constant as a function of electrolyte and nonelectrolyte contents and as a function of the proximity to the membranes and surfaces of the various organelles, it might be possible to calculate actual ion activities, using suitably modified, classic solution theory. In view of the above, the current work in the author's laboratory is now being directed toward measuring directly the dielectric properties of water near interfaces, especially in and adjacent to membranes, and eventually of intact biological systems. Thus, while it may be a decade or more before a detailed understanding will have been reached regarding the structural characteristics of vicinal water, empirical values for effective dielectric constants (and especially their dependence on distance from the interface—if such information can be obtained) may prove useful in estimating actual activity gradients in cellular systems.

#### E. NERVES

The functioning of nerves is little more and no less than the operation of membrane processes. We have chosen here to consider separately the

nerves as a possible site for the effect of vicinal water, i.e., a critical mode of exerting a notable influence on intact animals (as well as on isolated nerves). This choice is a logical one because of the discrete and readily delineated physiological role that the nervous system plays in animals. The uniqueness of the nervous system effects become most obvious in the discussion of the functional aspects of water structure with regard to the Pauling-Miller clathrate hydrate structure model for anesthesia (see Section VI,E,1). However, the present section is limited to a discussion of a few typical illustrations of the specific effects of temperature on various gross nerve processes without concern for the underlying membrane aspects, lipid transformations, or ionic transport effects. When reading the recent treatise on "Membranes, Ions and Impulses" by Cole (1968), it is interesting that although reference is made to temperature effects and temperature coefficients, most of the data referred to were characteristically obtained at 5°, 10°, and 20°C, or 6°, 12°, and 18°C. Thus, here, as in so many other fields of biochemistry, biophysics, and biology, temperature studies are often restricted to very limited numbers of observations. Temperature has truly been the Cinderella of physical parameters. Furthermore, where additional data are available, the inevitable tendency to draw smooth curves (or even merely straight lines) through all data points—rather than paying attention to individual trends—clearly prevails; as a result, a multitude of information is probably lost. Again, in the study of nerve processes, one should be aware of the unique properties of vicinal water and, specifically, the ability of such water to undergo higher-order phase transitions at discrete temperatures. This phenomenon no doubt influences, and often underlies anomalous, abrupt changes in biological functioning determined through the effects on the nervous system.

The effects of temperature on nerve properties are rather remarkable. Few physiological processes appear to be as dependent upon temperature as some of the processes associated with functioning of the nerve, especially, frequency of discharge of nerve impulses and conduction velocity. This may perhaps be the result of structuring of the water near the nerve interface in agreement with the fact that water structures can be further enhanced or modified by the presence of anesthetic agents of the clathrate-forming types.

A great deal of work has been carried out attempting to understand and describe quantitatively the functioning of nerves; a typical example is the study of the effects of temperature on the conduction velocity of the action potential. The nerve used in this study by R. A. Chapman (1967) was the giant squid axon. The purpose of the study was to test the accuracy of the

expression derived by Huxley for the conduction velocity of nerves. It is interesting that the agreement between theory and observed values is quite good in the range from approximately 5° to 25°C. However, above 25°C, a significant decrease in velocity occurs. At 30°C the conduction velocity reaches a maximum followed by a rather steep drop in the range from approximately 32° to 37°C. This drop is not predicted by Huxley's theory; it seems reasonable, therefore, to infer that this notable deviation is related to the structural changes in the vicinal water structure occurring at 30°C.

Even more remarkable anomalies were reported as the results of the studies by Lippold *et al.* (1960), the studies by Hensel *et al.* (1960), and by Hensel (1963). Somewhat similar anomalies were reported earlier by

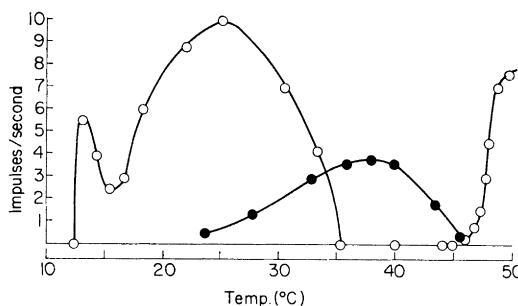


FIG. 19. Rate of nerve impulses as function of temperature in lingual nerve from cat. (Zotterman, 1959, with permission.)

Dodt and Zotterman (1952a,b) and even earlier studies by Laget and Lundberg (1949).

An exceedingly complex response of a nerve to temperature has been illustrated by Zotterman (1959). Figure 19 shows the data discussed by Zotterman for the rate of nerve impulses as a function of temperature in the lingual nerve from the cat. It is seen that the response exhibits several minima and maxima. There is little doubt that the minimum near 15°C is real as are the rapid decrease above 30°C and the rapid increase above 45°C. The latter is the so-called "anomalous cold response." It is certainly not possible at present to explain these extrema quantitatively in terms of changes in the vicinal water structure of the nerve. On the other hand, it would be truly remarkable if the similarity between the temperatures of the anomalous changes in the nerve discharge rate and the temperatures for changes in vicinal water structure was purely a matter of chance. It seems reasonable to seek a rationale for the observed complex behavior in

terms of the underlying water structure. It would certainly be hard to find another more traditional mechanism which predict *a priori* that pronounced extrema might occur at these temperatures.

Interesting effects of temperature on the nervous system were described in a review of the effects of temperature on insects (Clarke, 1967). As an example, Fig. 20 shows the drastic changes below 10° and above 35°C in the activity (impulses per unit time) of the tarsal nerve after the insect (*Periplaneta americana*) was acclimatized to 32°C (before being subjected to the various temperatures).

Yamashita and Sato (1965) have studied the effects of temperature on the individual taste units of the rat. In nearly all cases studied the re-

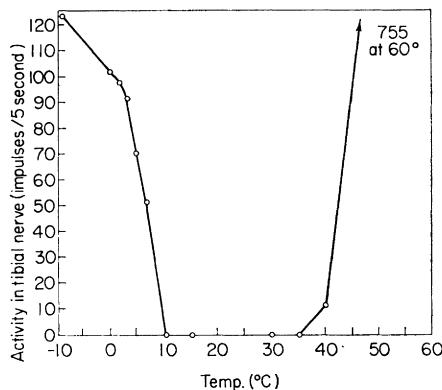


FIG. 20. Rate of nerve impulses in tarsal nerve of insect (cockroach). (Clarke, 1967, with permission.)

sponse to all kinds of stimuli (except 0.01 and 0.03 M sodium chloride) increased with temperature up to 30°C and decreased above this temperature. This effect was observed in the chorda tympani nerve of rats upon stimulation of the tongue with sodium chloride, potassium chloride, calcium chloride, hydrochloric acid, quinine, and sucrose. An inspection of the data reported by these authors clearly shows the notable maxima obtained in general (with the exception of sodium chloride as stated above). In the case of sodium chloride, the "normal" response was obtained also for the more concentrated solution (0.1 and 1.0 M). It is undoubtedly important that such vastly different solutes as various electrolytes and non-electrolytes all evoke a response with maxima near 30°C, regardless of the nature of the substance tasted. Thus, it must be concluded that the temperature response is a function of the nerve itself rather than of the substance that induces the response. The fact that rather distinct maxima are noted at 30°C may likely be interpreted as reflecting a structural change

in the internal aqueous environment of the nerve at the temperature of the thermal transition in vicinal water.

For an impressive survey of membrane behavior and nerve functioning, see the monograph by Cole (1968) referred to above. The author claims that this monograph is not a definitive work, yet, it covers the subject matter admirably. It is equally remarkable that in a book of 550 pages devoted to this topic, the subject index does not once contain the word "water," let alone water structure, or structure of water near interfaces.

#### F. MUSCLE

Convincing evidence for ordered water in biological systems has come from the NMR studies by Hazelwood *et al.* (1969). These authors obtained the NMR spectra of water in muscle tissue and concluded that at least two different degrees of ordering exist in this system. One of these ordered forms is the result of specific interaction between the water molecules and the native macromolecules in the muscle cell. This water is characterized by restricted motional freedom, since it is more ordered than free water. One of the important observations is the fact that only one broad water peak is observed, which indicates that, on the average, all the water in the muscle experiences a significant restriction in motional freedom. Obviously, a number of uncertainties are present in interpretations of NMR spectra, but it appears that Hazelwood and co-workers have addressed themselves to these difficulties and, indeed, presented a very strong case against each of the possible objections.

Other results by Hazelwood and Nichols (1968, 1969) are the changes in sodium content of muscle as a function of age of the experimental tissue. The amount of ordered water appears to increase with age since gestation and this is accompanied by a reduction in sodium "solubility" in the intracellular fluid. This finding has been further substantiated through correlation with changes in membrane potentials.

Finally, Cope (1967a,b,c) has demonstrated that the sodium content in the interstitial water of actomyosin is markedly reduced compared to solubility in ordinary water, suggesting an organization into a more "rigid" space due to the proximity of the actomyosin molecules. In the more recent studies by Cope (1969), use was made of heavy water in a study of muscle membrane tissue. Again, many of the objections that can be raised to the interpretation of NMR spectra were critically discussed and eliminated, leaving only the possibility that the water in the tissue was greatly enhanced structurally.

The exclusion of intracellular sodium as an organism ages was discussed, for instance, by Hazlewood and co-workers who noted the correlation with an increased degree of ordering (determined by NMR measurements). This may be correlated with the decreased dielectric constant of vicinal water, mentioned by Derjaguin (see Section IV,A,6). The notion that sodium ions are excluded from the cells as the ordering of the water increases is imminently compatible with the idea that the dielectric constant of ordered water is far lower (by an order of magnitude) than bulk water. Of course, the dielectric properties of water in biological systems is still poorly understood and one may question the comparison of such water with that adjacent to a silica gel surface, although (as discussed in Section III,C,1,e, the "paradoxical effect") it appears that the chemical nature of the substrate may not play a dominant role in determining the type of water structure which becomes stabilized vicinal to an interface.

Considering the evidence which is accumulating for notable changes in the structure of water in cells, particularly vicinal to membranes and some macromolecules, it is regrettable that practically all studies in cellular physiology continue to be based on classic physical chemistry of aqueous solutions. To be sure, the use of classic methods was appropriate initially when no evidence was available for changed water structures. However, new approaches must now be sought, even though we presently do not know, for instance, how to cope with problems such as diffusion rates in an aqueous phase, the structure of which is different from bulk water. Very likely the activity of solutes in the cells (of both electrolytes and non-electrolytes) may be vastly different from the activities obtained from bulk measurements. It seems from these considerations that the next step forward in cellular physiology will depend on the availability of far more detailed information about the structure and properties of aqueous solutions in ordered vicinal water.

#### G. ASPECTS OF WATER STRUCTURE IN CELLULAR PHYSIOLOGY

We conclude this section with a brief review of more recent studies. Thus, some additional results obtained from NMR studies will be discussed together with some speculations regarding active transport across membranes. These topics, in turn, lead to a brief review of cooperative effects in biological systems. See also the discussion in Section V,D.

The idea that thermal anomalies of the type discussed in this chapter are manifestations of higher-order phase transitions was apparently first stressed by Schmidt and the present author (1961). Higher-order phase transitions are generally ascribed to cooperative processes. Independently, Ling (1962) introduced his "association-induction hypothesis," building

on a suggestion by Troschin and involving cooperative effects. Ling coined the expression "adsorbed polarized multilayers" for the ordered water structures, but this phraseology is perhaps somewhat unfortunate. However, the ideas from Ling's extensive studies have proved highly useful as conceptual models and recently as inspirational guidance to many NMR spectroscopists. Nevertheless, it should be recalled that ion-dipole and dipole-dipole interactions are not likely to be the cause of extensive structuring of water near interfaces and are anyway restricted to ionic or highly dipolar surfaces. Other possibilities for structuring do exist, however, including stabilization of clathrate cage-like entities. Furthermore, the "paradoxical effect" discussed in this chapter suggests that the detailed nature of the substrate may play only a minor role. The structures ultimately stabilized at a water/solid interface are most likely structures that are "almost stable" in the bulk liquid phase, were it not for the continuous disruption due to thermal fluctuations. Incidentally, one possible case of actual "polarized multilayer adsorption" may exist at the interface between an advancing ice surface and an unfrozen aqueous solution (or pure water); for a discussion of this aspect, see the paper by Drost-Hansen (1967b).

Recently, Cope (1971) and Hazlewood and co-workers (1971b) have discussed structuring of water, complex formation of sodium and potassium ions in biological systems, and the nature of cellular water and water/macromolecule interactions. The two papers review the general field of NMR studies of biological systems and contain excellent collections of references. It is interesting that it has been possible to make such notable advances based essentially upon the results of only this one type of measurement (although the more recent papers by Cope and by Hazlewood do contain references to other types of evidence, demonstrating structuring and cooperative phenomena in biological systems).

The paper by Hazlewood and co-workers (1971b) reviews the NMR measurements on water in the gastrocnemius muscle from rats. The interpretation of the results follows the ideas advocated by Ling, based on the "association-induction hypothesis" (discussed in Section IV,A,5). Among the suggestions emphasized by Hazlewood is the possible intracellular structuring of all the protoplasm, providing the driving force for a sodium transfer due to the changed solvent properties and thus eliminating the need for a specific sodium pump. The paper by Cope (1971) reiterates, and very lucidly discusses, the evidence for structuring of water in cells as based on the available NMR studies. Cope also (as discussed above) reiterates the evidence for the complexing of the sodium in the structured water of the cell. In addition to the experiments with gastrocnemius muscle of the rat, Hazlewood and co-workers (1971a) have also studied the

interaction of water molecules with the cells in cardiac muscle. It was again found that "dramatic shortening of relaxation times suggests that the water molecules within the myocardial tissue significantly interact with cellular macromolecules."

In summary, the recent NMR studies from many laboratories strongly suggest the existence of ordered water structures in biological systems. Thus, as emphasized, the studies by Hazlewood and co-workers have shown that a large fraction of intracellular water appears highly ordered and, furthermore, that in the immediate postnatal period, the intracellular sodium concentration decreases as the degree of ordering of the cell water increases. On this basis, it has been suggested that membrane transport may not require the operation of various active transport mechanisms, and issue has particularly been taken with the need for a sodium pump. At the present time, the evidence for ordering of water in cells appears conclusive—both as the result of the highly specific NMR results as well as the result of the type of general evidence reviewed in this chapter.

Considering the theorized need for specific solute transmembrane pumps, Tait and Franks (1971) observed that "the state of intracellular water seems crucial to these arguments. It has been found (reference to Kushmerick and Podolsky, 1969) that the diffusion coefficients of various solutes in an intracellular environment are a factor of two lower than in aqueous solutions, but the fact that the diffusivities of some cations, anions, and non-electrolytes are reduced to the same extent is in direct conflict with the concept (reference to Cope, 1966) of selective ion binding by the macromolecular cell components." I feel that the point made by Tait and Franks is well taken. Furthermore, if structuring of intracellular water is caused merely by proximity to an interface, as advocated by the present author, the difficulties encountered due to the "specific adsorption on polarized multilayers" is avoided. Thus, instead of "complexing" of the ions (for instance, the sodium ions) by the macromolecular solutes, nothing more may be needed than the formation of clathrate hydrate cages around the ions. See also the paper by Vaslow (1963), who discussed the possibility of small ions being found in clathrate cages, even in pure bulk solutions.

In connection with the problem of the nature of water in biological systems, and the standard approaches to solution chemistry in cell physiology, attention is called to the paper by DeHaven and Shapiro (1968). While these authors did not specifically discuss the nature of structural changes in vicinal water, they emphasized the probable changes in solute activities that must result from the effects of other solutes on the dielectric properties of the intracellular fluids. The authors outline how "classic" solution theory may be employed in the study of transport processes across

membranes if one takes into account the changes in dielectric properties of the intracellular water. I find this approach both valid and useful, but must emphasize that, in addition to the induced changes in "effective dielectric constant" of the intracellular water (as the result of the presence of other solutes), changes must also be expected due merely to the proximity of the water to the various macromolecular interfaces, resulting in different structures (and hence different dielectric properties) in the vicinal water.

The "polarized multilayer adsorption" envisioned by Ling, corresponds essentially to the structuring tentatively suggested by the present author for water adjacent to an ionic or highly polar interface. This is the type of interaction depicted in Fig. 8, page 32. However, as discussed in this chapter, and particularly in an earlier paper (Drost-Hansen, 1969b), a different and possibly more frequently occurring type of structure may be present. This type of vicinal structuring (see Fig. 9, page 33) is likely, for instance, to be most prevalent adjacent to nonpolar hydrocarbon side chains in proteins in solution or adsorbed on membrane surfaces, etc. However, in this connection, recall also the "paradoxical effect": the occurrence of thermal anomalies in the properties of water adjacent to both ionic surfaces, polar surfaces, and near such nonpolar surfaces as the air/oil interface and possibly even the air/water surface. This occurrence of the thermal anomalies—i.e., cooperative processes—near nonpolar surfaces argues against the suggestion by Ling that the cooperative effects are due to "polarized multilayers," organized by interactions with surface charges.

## VI. Functional Role of Water in Biological Systems

### A. INTRODUCTION

#### 1. Temperature Ranges of Life

In a universe where the temperature ranges from near 0°K to millions of degrees, life as we know it is restricted to a fantastically narrow interval—at most, about 100°C. That this is the range in which water (at 1 atm) remains liquid is hardly a coincidence. For plants, the range over which survival may occur may be as large as 90°C. However, the interval of temperature tolerance for most commercially valuable plants is usually limited to about 30°C or less (see Rose, 1967, p. 245).

It is likely that the study of organisms capable of living at temperatures

higher than present ambient temperatures\* will become more and more important if continued energy production (with its attendant thermal inefficiency) continues to increase the "average, ambient temperature" of the earth (presently about 12°) and/or if the combustion of carboniferous fuels continue to increase the carbon dioxide in the atmosphere, enhancing the "greenhouse effect" with its inevitable world-wide increase in temperature.

For an extensive review of temperature effects on biological systems (and particularly growth), see the monumental monograph "Temperature und Leben" by Precht *et al.* (1955) or the excellent and more accessible reviews in the treatise "Thermobiology" edited by Rose (1967).

In reviewing the literature on temperature effects on biological systems, particularly microorganisms, the reader is encouraged to look carefully for evidence of discrete effects near the temperatures discussed in the present chapter, namely, near 13° to 16°, 29° to 32°, 44° to 46°, and 60° to 62°C. These are the temperatures at which some vicinal water structures undergo abrupt changes. Let it be reemphasized that the phenomena that are not determined primarily by details of water structure may not reveal any unusual dependencies on temperature (compare the discussion of Glasel's studies, Section V,B,5). As a corollary to the first statement is the implied admonition that future work concerned with temperature effects in biological systems ought to be carried out at closely spaced temperature intervals, lest these studies miss what may possibly be important hints as to underlying mechanisms, that is, mechanisms which may reveal structural influences due to the unique properties of vicinal water.

It is worth while to recognize that it is impossible to separate temperature completely as an isolated independent variable. Other simultaneous dependent and independent variables will influence most of the systems of

\* A few organisms may live at temperatures higher than 100°C if the pressure is sufficient to prevent boiling, but such organisms (as well as others capable of living at what are ordinarily unphysiologically high temperatures, 60°-75°C, for instance) are of relatively little importance in this context. However, there is likely great virtue in focusing attention on the forms of life such as the thermophilic algae capable of living near 70°C (surviving to somewhere below the boiling point of water): the life processes in this temperature range, say, above 60°C, suggest that living systems may be able to choose selectively those constituent proteins, enzymes, membrane materials, and lipids that are compatible with far more disorganized water structures. However, an alternative, even if somewhat farfetched, is the possibility that these organisms have been able, through evolution, to select, enhance, and depend on those structured macromolecules which have the greatest ability to order vicinal water. In other words, the organisms have selectively produced those macromolecules that have the conformational stability required for physiological functioning, as well as the ability to influence strongly the vicinal water structure. For a review of "life at high temperatures," see the article by Brock (1967).

interest to biologists. Since few of the measurements made have any "equilibrium thermodynamic" meaning, time alone will be a parameter of significance. In addition to time (in an absolute sense) there are unexpected but apparently real natural fluctuations of cosmic origin [as studied by Piccardi (1962) and co-workers] as well as more obvious variations such as diurnal changes related to the circadian rhythm. An interesting example of the latter phenomenon is described by Damaschke and Becker (1964). The results from this study show that the oxygen consumption rates vary in discrete and abrupt manners with temperature, especially near 30°C, but that superimposed upon this intrinsically complex dependency, an additional effect of diurnal rhythm influences the results significantly.

## 2. *Role of Plant Alkaloids*

An inspired view of the possible role of water in plants has been presented by Tracey (1968) in an article, "Fitting the Environment by Modifying Water Structure." It is Tracey's contention that many of the low molecular weight solutes in the plants primarily serve the function of suitably modifying the cellular (and likely vicinal) water structure to enhance the possibility of survival of the plant. Tracey sees the controlling effect essentially as a distribution of availability of water between the components in the complex water-pore system, where, on the one hand, the water is required for the hydration of membrane constituents (primarily cellulosic, but including the lipoproteins) and, on the other hand, for hydration of the various solutes—the lower molecular weight solutes as well as nucleotides, proteins, etc.

Although a study of solute effects on the properties of dough may seem a very indirect way of probing for structural effects on water, it is interesting that in the hands of Tracey this approach has led to some intriguing results. Tracey has studied the effects on the rheological properties of dough of known central nervous system depressants compared to stimulants. Tracey notes that some 2000 plant alkaloids are known, which have pronounced effects on the central nervous systems of animals while they are apparently of little obvious, functional use to the plant. Since these alkaloids seem to serve no protective function so far as plant-animal interaction is concerned, the presence of the alkaloids in the plants is perhaps a device employed by the plants to optimize the use of the available water. It is interesting to note that apparently plants do not often suffer from a shortage of energy, but instead are subject to shortage of raw materials—primarily water, carbon dioxide, and combined nitrogen. Hence, Tracey speculates that "if some alkaloids enable a plant to 'im-

mobilize' water, then a response to water deficit might be expected to be increased alkaloid production." Alkaloid yields are, indeed, higher if plants are kept short of water. Tracey speculates further that the presence of the alkaloids may be the vestiges of a device which was effective in water control on the molecular level at a time when the availability of (fresh) water was the major hardship for the plant material, for instance, at the time when the plants began to colonize dry land.

## B. METABOLISM AND GROWTH

### 1. *Distribution of Optimum and Lethal Temperatures*

Metabolism and growth are complex physiological processes. Complexity is taken here to mean the simultaneous and consecutive involvement of a large array of individual chemical processes (reactions in the classic sense, diffusion, active transport, etc.). In 1956 the present author suggested that for such complex systems, temperature optima and minima might be predicted from a simple consideration of the structural changes in water.\* One may assume that many reactions which might potentially be rate determining in a complex biological system may undergo notable changes at the temperatures of thermal anomalies. Based on this assumption, it was proposed that during evolution, biological systems have tended to avoid the temperature regions near the sudden changes in the (vicinal) water structure and, hence (at least in the case of the mammals), have optimized body temperature as far away as possible from a lower and a higher thermal anomaly (at 30° and 45°C, respectively). Were the thermal anomalies to occur exactly at 30° and 45°C, the body temperature would then be expected to fall near 37.5°C. Figure 21 shows a histogram of frequency of occurrence of body temperatures for approximately 160 mammals. It is seen that this distribution does, indeed, center very closely around 38°C with a remarkably narrow distribution. (Note 37°C equals 98.6°F.) A small number of exceptions are indicated by the cross-hatched area under the curve, around 27° to 34°C. This group includes the anteater, the sloth, the echidna, the armadillo, and a few other species such as the duckbill platypus. It seems legitimate in a first approximation to neglect these exceptions because of the somewhat unusual nature of the animals compared to most other mammals.

The distribution of body temperatures of birds appears to be centered

\* At that time it was felt that evidence was available for the existence of thermal anomalies in all properties of water and aqueous systems. As pointed out elsewhere in this chapter (Section III,C,1,d), it now appears that the anomalies are most pronounced and possibly occur only in vicinal water rather than in bulk water.

around 41.5°C. It was proposed by Drost-Hansen (1965a) that the displacement (by 3° or 4°) toward higher temperatures might be a concession to the highest possible rate of energy production required for flight (approximate Arrhenius type of activation). It is interesting to note that both for all mammals and all birds studied, 45°C ( $\pm 1^\circ$ ) appears to be an absolute, upper thermal limit (lethal temperature). It is also interesting to note that those birds that do not fly, such as the ostrich, the kiwi, and the penguin, appear to have normal body temperatures around 38° to 39°C. This would tend to substantiate the proposed explanation for the higher body temperatures of birds. It is also well known that 30°C is a tempera-

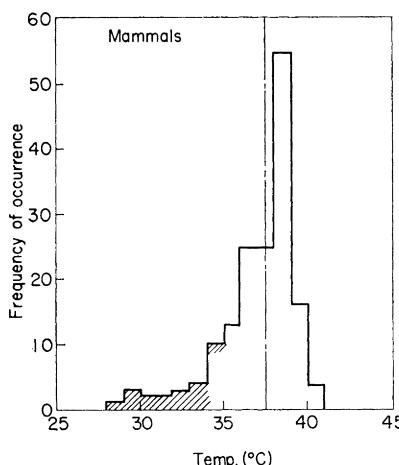


FIG. 21. Distribution of body temperatures of mammal (frequency distribution). (Drost-Hansen, 1965a, with permission from the New York Academy of Sciences.)

ture of considerable physiological importance in all mammals and birds. This will be further discussed in the section on hypothermia.

By analogy with the reasoning presented above, it has been suggested that an optimum might exist somewhere near the middle of the temperatures between 45° and 60°C. Indeed, a majority of thermophilic bacteria and thermophilic fungi are known to possess optima around 53° to 55°C. It is also well known that pasteurization temperatures usually tend to be 60° to 62°C; this suggests that the pasteurization temperature is a direct manifestation of the structural changes in the vicinal water near this temperature.

Finally, by the same type of argument it is proposed that optimum activity may be encountered for a group of organisms (plants and animals) between 15° and 30°C. A large number of different types of

organisms appear to have optimum activity between 22° and 26°C, including many insects (though not all), many fishes, and soil bacteria. Thirty degrees Centigrade is known to be an important temperature physiologically for both fishes and insects. For specific examples, see Drost-Hansen (1965a).

Allowable ranges of temperatures for poikilotherms are often 15° to 16°C—truly a vanishingly small range out of the total span of temperatures in the universe. As will be discussed in another section, thermal adaptation may occur, but more frequently than not the adaptation is merely a slight change in temperature of, say, optimum activity (for instance, for growth or reproduction) rather than a notable change in the low-temperature tolerance limit or the upper lethal temperature. However, for unicellular organisms, a special form of adaptation may take place, namely, through the development of multiple optima for growth. Mitchell and Houlahan (1946) reported distinct binodal distributions for growth of a mutant of *Neurospora crassa*. Somewhat similar results were subsequently obtained by Oppenheimer and Drost-Hansen (1960) studying a sulfate-reducing bacterium. Later experiments (Schmidt and Drost-Hansen, 1961) tended to suggest similar behavior for *Escherichia coli*. More recently, Davey and Miller (1964) have also obtained very distinct multiple optima for growth of a number of microorganisms. Four different types of bacteria were used to cover the temperature range from 5° to 70°C; in all four cases, multiple growth optima were obtained. Oppenheimer and Drost-Hansen (1960) suggested that such organisms might be able to grow optimally in two different temperature intervals by utilizing different metabolic pathways. Some preliminary evidence was obtained for this proposition through a study of changes of pH of the medium on which *E. coli* was grown and from a qualitative study of the pigmentation in *Serratia marcescens* (Schmidt and Drost-Hansen, 1961).

## 2. Examples of Thermal Anomalies in Growth Processes

An interesting example of unusual temperature effects is described for the rate of mycelial growth of a fungus (*Waitea circinata*), studied by Agnihotri and Vaartaja (1969). These authors found that the mycelial growth of the fungus was strongly temperature dependent and, moreover, that exudate from pines (*Pinus cembroides*) further stimulated this mycelial growth. For both (without exudate as well as in the stimulated mode of growth), mycelial growth showed indications of an inflection point near 15°C and a relatively notable drop in rate of growth above 30°C. The same effect—a maximum in radial growth of the mycelium—was obtained in the presence of various nutrients such as aspartic acid,

malonic acid, glutamic acid, and arabinose, with notable drops in growth above 30°C.

Recently, Walker (1969) has studied the effects of temperature in 1° increments on the behavior of maize seedlings. The temperature range covered was from 12° to 36°C, and several anomalies were observed. Thus, significant irregularities in the concentration of many of the nutrients in the shoots of the plants occurred near 15°C. Minor but persistent anomalies were also noted in total leaf length of the seedlings, and irregularities occurred in growth rate at 29° and 30°C. The author was not convinced that all of the anomalies observed were real or whether some were caused by experimental artifacts. However, an inspection of the data of, for instance, dry weight of 23-day-old maize seedlings strongly suggests an anomaly near 29° to 30°C. Walker correlates his observations with the similar anomalous results by Davey and Miller (1964) at 15°C for the uptake of potassium by wheat. Walker, although aware of the claim for the existence of thermal anomalies in (vicinal) water, did not make any conclusive association between the water structure changes and the observed anomalies.

A good example of the abrupt change in growth at 30°C is shown in the data by Buetow (1962), referred to by Farrell and Rose (1967, p. 162, Fig. B). These results clearly show the dramatic change in the specific growth rate of *Euglena gracilis*: a sharp maximum occurs in the vicinity of 28° to 30°C.

Attention is called here to the monograph by Andrewartha and Birch (1954) (in particular Part III, Chapter 6). A large number of examples are discussed which clearly show that critical temperatures for many organisms frequently coincide with the thermal anomalies stressed in the present chapter. It is impossible to go over all the examples discussed by Andrewartha and Birch, but in particular the logistics curves are important (discussed in the section on "Weather: Temperature," Chapter 6, pp. 129–205). In this connection, the frequency with which excellent agreement can be achieved between some empirical or semitheoretical logistics curves over the interval from about 15° to 30°C is noteworthy, as is the frequent failure below 15°C and almost invariable failure above 30°C.\*

Levinson and Hyatt (1970) have studied the effects of temperature on the activation, germination, and outgrowth of spores of *Bacillus megaterium*. The study is particularly interesting as it was designed specifically to determine if there was evidence of thermal anomalies in these stages of bacterial spore growth. Measurements were made at closely spaced temperatures. The authors concluded that they "found no evidence of thermal

\* It is unfortunate that many authors have apparently chosen to study various organisms "exactly" between 15° and 30°C.

discontinuities, or 'kinks' in these biological processes, but we felt nevertheless that our data on the response of spores to small temperature increments had sufficient intrinsic value to warrant publication." The negative conclusions drawn by Levinson and Hyatt is quite astounding in view of the data reported. An inspection of their illustrations might equally well have suggested that anomalies do occur. Thus, Fig. 4 in the paper by Levinson and Hyatt suggests a distinct change in slope near 16° to 18°C for the germination temperature with a relatively abrupt peak or change in slope near 28° to 32°C. The authors felt these changes were not significant but offer little additional information to substantiate this conclusion. The authors further note "there was some suggestion of a sharp increase in germinability after heating at 56°C. However, as seen in the semi-log plot (Figure 1) activation appeared to be an exponential function of activation temperatures from 52 to 60°C." The authors do not point out, however, that above this temperature, the change in optical density is practically constant over the range from approximately 62° to 78°C; again revealing a rather notable change in the vicinity of 60° to 62°C. The point intended here is not that the study by Levinson and Hyatt provides strong evidence for the existence of thermal anomalies in biological systems. Instead, it is merely emphasized that the findings by these authors are not inconsistent with the notion of the occurrence of thermal anomalies and that no other current theory for germination and growth of spores is likely to predict the shape of the observed curves.

It is interesting that Levinson and Hyatt (1970) quote Thorley and Wolf (1961) as having observed three temperature optima (near 3°, 25°, and 41°C) for the germination of *Bacillus cereus* strain T. spores. Levinson and Hyatt go on to "explain" that the multiple optima were attributed by O'Conner and Halvorson (1961) to the use of a suboptimal concentration of L-alanine. In other words, in the presence of sufficient L-alanine there is no evidence of thermal anomalies in the response of the spores to temperatures of germination. It appears that Levinson and Hyatt have, indeed, missed the point: as stressed by Oppenheimer and Drost-Hansen (1960) and by Schmidt and Drost-Hansen (1961), it is on minimal substrates that the multiple optima are to be expected. The fact that the anomalies can be "swamped" by excess nutrient supply does not explain away the nature of the growth on minimal media. In the latter cases, limitations are imposed upon the organisms with respect to the available metabolic pathways and the choice is limited, therefore, with the result that the metabolites and/or appropriate enzymes are only those that are most compatible with the structure of the vicinal water in the respective temperature ranges.

Observations of interesting anomalies around 15° (to 20°C) have been

reported by Nishiyama. Because many of the papers by Nishiyama and co-workers as well as other Japanese authors are not available in English translation, we mention a number of these studies in some detail, based on a recent personal communication to the present author from Nishiyama.

Nishiyama has been concerned with the effects of relatively low temperatures on a number of plant phenomena (Nishiyama, 1969, 1970). In the most recent article, "What is Between 15° and 20°C?" Nishiyama suggests that general physiological (and pathological) changes occur in the temperature range between 15° and 20°C. An inspection of the illustrations in this article suggests to the present author that the rate of these changes is frequently the greatest around 15° to 17°C. Nishiyama (1969) specifically proposes that the changes may be due to "the phase transition point of water (crystal) in protoplasm." Further, Nishiyama (1970) has suggested "various crops are injured by low temperatures—below 15 to 20°C. One such example is a sterile type injury in rice plants (Figure 1-A in our report, Nishiyama, 1969)." Although Nishiyama clearly recognizes the importance of the role of water and the possibility that it may undergo some type of phase transition, he also draws attention to the fact that "it is to be noticed that the critical temperature varies with varieties and conditions of cultivation. We must consider the participation of protoplasmic substances, such as proteins and lipids, other than the water itself." Nishiyama goes on to mention cold injury to plants discussed by other Japanese researchers. Thus, injury to soybeans and red beans, and a type of delayed injury in rice plants, is observed for low temperatures, that is, temperatures below 15° (to 20°C).

Nishiyama has added several other examples in support of thermal anomalies in plant physiology and pathology. Thus, he states:

Dr. Yamashita *et al.* claim that there is a changeover temperature for day length requirement [in "Control of Plant Flowering" (Y. Goto, ed.), pp. 54–57. Yokendo, Tokyo, 1968 (in Japanese with an English summary)]. The temperature was estimated about 17.5°C in several plant species. Various fruits and vegetables after harvest are susceptible to cooling below and about 15°C. These include bananas [T. Murata, *Plant Physiol.* **22**, 401 (1969)], oranges and lemons [I. L. Eaks, *Plant Physiol.* **35**, 632 (1960)], apples [A. C. Hulme *et al.*, *J. Sci. Food Agr.* **15**, 303 (1964)], cucumbers [I. L. Eaks and L. L. Morris, *Plant Physiol.* **31**, 308 (1956)], cucumbers and pimentos [L. L. Morris and Platenius, *Proc. Amer. Soc. Hort. Sci.* **36**, 609 (1938)] and sweet potatoes [T. Minamikawa *et al.*, *Plant Physiol.* **2**, 301 (1961)].

### 3. Thermal Classifications of Microorganisms

The traditional classification of bacteria into cryophiles, mesophiles, and thermophiles may possibly be seen as a tendency for these groups of organisms to exhibit maximum activity (usually optimal growth) be-

tween various consecutive thermal anomalies in the vicinal water. It should be mentioned in this connection that one of the difficulties in making a clear-cut distinction results from the fact that multiple temperature optima are often encountered. Thus, growth curves over an extended temperature interval may show merely a broad and, at times, rather flat peak around 30°C! The studies by Schmidt and Drost-Hansen (1961) have suggested that this may result from considerable overlap of two growth peaks (each with optima near 23° to 25° and 37° to 39°C, respectively). Experimentally, we have noted that growth on "minimal media" tends to separate the overlapping peaks. Likewise, distinctly binodal growth curves are sometimes seen in very old cultures—long after the cessation of the logarithmic growth phase.

#### *4. Thermal Conduction in Biological Systems*

In connection with the problem of metabolic processes, the question arises as to how the cell dissipates the heat produced in the cellular processes. Naturally, the component of the cell water which is more or less bulk-like will have limited thermal conductivity (but rather high heat capacity). However, once a steady state has been reached, the heat evolved must be dissipated to maintain isothermal conditions in the homeothermic organisms and in the poikilotherm organisms in "equilibrium" with the surroundings. In this connection, recall that the heat conductivity of ice is almost an order of magnitude greater than the heat conductivity of bulk water. It seems eminently reasonable to suggest that the ordered water of the cell interface facilitates the conduction of heat from the interior of the cell to the surroundings. Heat conductivity studies of water between closely spaced mica plates have been carried out in Russia by Metsik and Aidanova (1966; also see Derjaguin, 1965). These studies demonstrated notably enhanced heat conductivity of vicinal water—as much as an increase by 50 or more (for thicknesses less than 0.1  $\mu$ ) (see, also, Section VI,F,2 on hyperthermia).

It is interesting to speculate that shivering may reduce the amount of ordered, structured water, somewhat similar to the breakdown, on agitation, of "set gels." In this fashion, the heat conductivity of the cellular water might be reduced and thus minimize heat flow to the environment upon cold exposure.

#### *5. Notes*

As suggested by Oppenheimer and Drost-Hansen (1960), temperature adaptation may, indeed, take place. Thus, some bacteria have definite bi-nodal distributions of growth rates as a function of temperature, with a

minimum near 30°C. As mentioned, similar results were obtained on a mutant of *Neurospora crassa*. The tentative proposal, by Oppenheimer and the present author, is that in different temperature intervals those different metabolic pathways are chosen which are best "suited" for the organism at that temperature interval. Adaptation then may, in part, be the proper choice of the substrate on which the organism is grown. Characteristically, for bacterial studies, one gets the qualitative feeling that thermal anomalies are enhanced when the organisms are maintained on a "minimal medium." This would imply that the organism does not have the normal "availability" of metabolic possibilities. It is also possible that genuine adaptation—resulting from modification of, say, the controlling protein structure—may be achieved through adaptation to a different water structure in a different temperature interval. However, this is not a likely possibility and is certainly not easily achieved. Hence, as will be discussed in the section on paleozoogeography, it is undoubtedly correct to say that the thermal anomalies at 15° and 30° (and perhaps 45°C) have, in the past, imposed a significant "barrier" leading to geographical zonation of multicellular organisms, dependent on the local average (or maximum) temperature. Thus, in a sense, throughout evolution the water structure changes have imposed inviolable, "invariant" constraints. Stehli, among others, has invoked this possibility in connection with paleozoogeographic studies (see Section VI,D,3).

As discussed in the present section, it appears that such phenomena as body temperature of mammals, optimal temperatures for many organisms, as well as maximum and lethal temperatures are determined by structural changes in water (Drost-Hansen, 1956). Later (Drost-Hansen, 1965a), it was more specifically noted that the interaction between the vicinal water structure and the nature and conformation of the underlying substrate is the result of mutual interactions:

The cooperative action between many water molecules in the water clusters of the solvent water may well be expected to influence drastically the rather large amount of water associated with the proteins or membrane material. In other words, the structural transitions in water may exert a direct and profound influence on the immediate environment of the macromolecules of the biologic systems; the effects of the transitions are not merely "solvent effects" manifested by minute changes in the solvent viscosity, dielectric properties or activity!

### C. GERMINATION

A vast amount of literature exists on the subject of germination (and vernalization). It is interesting that these studies have often considered

the effects of temperature in some detail. However, as in a number of other fields in biology, such as thermal adaptation, a vast number of complications occur due to other concomitant changes, such as changes in relative humidity (water activity), light, and pressure. Hence, with the exception of a relatively small number of studies, it is difficult or impossible to make significant systematic comparisons between the structure (and thermal anomalies) of vicinal water and the specific effects on the processes of germination and vernalization. Obviously, the study of the influence of water structure on these processes is further complicated by the fact that frequently the systems have not been studied as a function of temperature at closely spaced intervals, and the systems are, in addition, sensitive to various electrolytes and nonelectrolytes, which undoubtedly exert specific influences through direct chemical interaction, for instance, with singular functional groups in some controlling enzyme or at some membrane site.

A few examples of abrupt changes in germination rates with temperature were discussed by Langridge and McWilliam in Rose's monograph (1967, p. 244). The authors state "... the optimum temperatures for the germination of most seeds fall between 15° and 30°C, although higher optima (35° to 40°C) have been reported in tropical species, such as *Paspalum* and *Saccharum*." Also (Rose, 1967, p. 26), "... similarly, it has been shown that potato tubers immediately after harvest are able to sprout only within a narrow temperature range above 30°, which presumably protects them from premature sprouting in the autumn."

P. A. Thompson (1969, 1970a,b) has studied the germination of seeds in considerable detail by a variety of techniques. He has made use of a thermogradient bar—a polythermostat somewhat similar to the one used in the studies by Oppenheimer and Drost-Hansen (1960). In some cases, the results are in excellent qualitative agreement with similar results obtained for the rate of growth of a number of bacteria showing abrupt changes near 15° and 30°C, for instance, for *Silene tartarica* and *Silene coeli-rosea*. In other cases, vastly different temperature responses were obtained in the sense that critical temperatures occurred, for instance, near 25° or 36°C depending on the geographical origin (and, hence, climate) of the plants studied. There is little doubt that the study by Thompson will prove a most important contribution. He observed that a temperature difference as small as 0.5° may play a discriminating role in the germination of seeds. Thompson also introduced alternating temperatures in order to study an environment more nearly identical to that encountered in nature. Furthermore, multiple growth optima were also encountered on occasion. Thus, for *Fragaria vesca* Linn and, particularly, *Ajuga reptans* Linn, multiple growth optima were observed with minima near 30°C. At this point, attention is called in particular to Fig. 22 showing the percent-

age of germination curves as a function of temperature of two different species of *Fragaria vesca* Linn. The germination curves as a function of temperature clearly exhibit binodal character with relative minima between 26° and 29°C. This behavior strongly resembles the multiple temperature optima obtained for the growth of a number of bacteria studied by Drost-Hansen and co-workers (Oppenheimer and Drost-Hansen, 1960; Schmidt and Drost-Hansen, 1961).

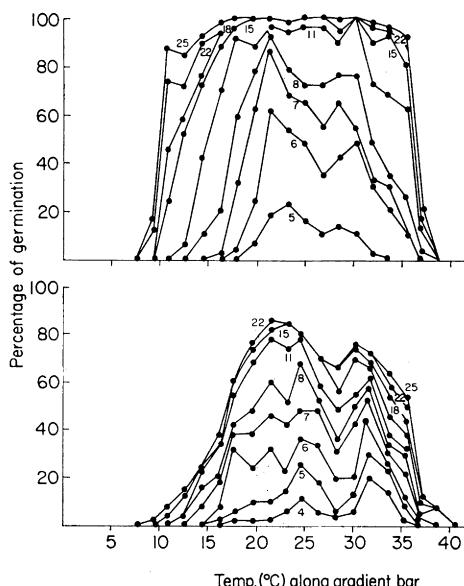


FIG. 22. Percent germination for two different species of *Fragaria vesca* Linn. (P. A. Thompson, 1970b, with permission.)

Finally, attention is called to some studies of the effects of gibberellins on the germination of some seeds. Thompson (1969) concludes

There would appear to be no requirement for a close relationship between responding species in taxonomic terms, nor is it easy to find any similarities from one to another, suggesting a common bond in terms of the conditions required for germination in normal circumstances. Gibberellins will substitute for light in dark-grown seed, for chilling treatments, and for fluctuating temperatures; they will procure germination at temperatures normally too high and also temperatures too low; and they will replace complex conditions for germination such as the combination of leaching and chilling required by the seed of *Mecognosi cambrica*, and the combination of light and fluctuating temperatures required by *Lycopus europaeus*.

The fact that the gibberellins may act in such a variety of ways and mimic such vastly different functions suggests to the present author that their effect is not based on a specific chemical reaction, such as interaction

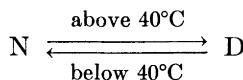
with one particular functional group in a controlling enzyme or substrate. It is suggested, therefore, as an alternative hypothesis, that the effect is due to some general influence and this most likely is through the action of the gibberellins on the structure of the water vicinal to the site of control of dormancy in the seed.

### 1. Vernalization

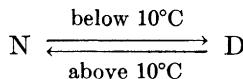
Vernalization is the induction of seeds to germinate after (often prolonged) exposure to low temperature. The subject is obviously of enormous practical importance. The need for prior cooling, before germination can take place, is the principal means whereby freshly discharged seeds from a plant are prevented from germinating upon release in the autumn which would expose the young plant to the cold of winter.

It is proposed here that vernalization is the relatively slow restructuring (and probably the increased ordering) of water adjacent to some critical component in the seed, probably a membrane or a protein. Only after the vicinal water structure has changed to conform to the lower-temperature range is the seed latently capable of germinating. Recall in this connection that, whereas the substrate undoubtedly influences the nature of the vicinal water, the converse must also hold true, namely, that the structure of the vicinal water must influence the nature and conformation of the underlying macromolecular substrate. That the process is slow is perhaps related to the thermal memory effect discussed in Section VI,J, probably reflecting the difficulty in inducing order by merely removing available thermal energy (thermal energy [ $kT$ ] tends to disorder structures and, conversely, a lowering in temperature increases the ordering).

Recently, Levitt (1969) has surveyed the growth and survival of plants at extreme temperatures and presented a unified concept. Levitt proposes that temperature exerts a controlling role in the response of plants through the state of denaturation of the proteins. Specifically, he proposes the simple scheme shown below for interrelations between the native (N) and the denatured (D) forms of many enzymes:



and



In other words, Levitt proposes that only in a range, essentially  $10\text{--}40^{\circ}\text{C}$ , do the necessary enzymes occur in a native form, and that above and be-

low these temperatures, denaturation may occur. Denaturation by low temperature is the cold inactivation discussed in Section V,B,7,d. Levitt considers the ratio of hydrophobic to hydrophilic groups of the proteins. He specifically suggests that the properties will not only depend on conformation but also on the proportion of amino acids to hydrophobic groups of the nonpolar side chains in the proteins.

Levitt leans heavily on the previous studies referred to in this chapter by Brandts regarding the relationship between water structure and the nature of the protein. Levitt directly and explicitly invokes the role of the water structure in conformational stability of the proteins which, in turn, are seen as controlling factors in both cold and heat resistance of plants. In discussing chymotrypsinogen and chymotrypsin, Levitt notes that these proteins differ primarily only in the conformation in the vicinity of SS groups. He mentions that in one conformation the SS groups are "protected" (from the aqueous environment), whereas in the other conformation such protection does not occur. Levitt further discusses the role of water structure:

In other words, during the hardening period there must be a nearly complete turnover of proteins. This means that each newly formed protein chain must fold at a temperature which prevents the formation of hydrophobic bonds. They must all therefore fold with the hydrophilic, reactive groups within the folds and the hydrophobic, weakly reactive groups on the outside. All the newly formed proteins must therefore be resistant to aggregation and the plant can therefore undergo freezing dehydration without injury.

The present author is greatly impressed with the qualitative ideas embodied in the proposals by Levitt for the survival of plants at both low and high temperatures. However, as mentioned elsewhere in this chapter, he takes issue with the classic explanation for the existence of abruptness in various rate processes (those involving proteins). Indeed, Levitt illustrates the Arrhenius nonlinearity by quoting a study by Talma (1918) for the rate of growth of roots of *Lepidium sativum*. This process shows extremely sharp changes at exactly 15° and 30°C—changes in slope far more pronounced and far sharper than those which can be allowed for through the Arrhenius plot for, say, enzymically controlled (simultaneous and/or consecutive) reactions, as discussed by Brandts and others (see Section V,B,7,b).

#### D. GENETICS AND EVOLUTION

##### 1. *Adaptation to Thermal Changes*

The problem of thermal adaptation in organisms is of enormous importance: it plays a prominent role in ecology and paleozoogeography, in

thermal pollution, in the effects of fever on pyrogenic organisms, and in many other areas of immediate, biological interest. In the present chapter only one facet of thermal adaptation is discussed. Although thermal adaptation may, for instance, result in the ability to change the optimum temperature for various types of activity (growth, mobility, etc.) by several degrees, we stress here that frequently lethal temperatures are invariants. Thus, those lethal boundaries which are determined by changes in the associated water structure are probably time invariants, boundaries not likely to be transgressed easily—by any mechanism of thermal adaptation—without some very major changes in the biochemistry (see below).

Of interest to the biologist are the intact organisms; vastly different temperature effects may be expected at various points in the evolutionary states of the organism. Ushakov (1968) has pointed to the need to separate the temperature effects on the total organism from the temperature effects on the individual cellular level and, indeed, the role of temperature at the molecular biochemistry level. Considering the role of cells and proteins in the process of adaptation, one must take into account that any adaptation is a result of natural selection. The object of selection is the intact organism and, hence, adaptations are accomplished only at organismal and epiorganismal levels of organization. From this point of view, the term "cellular adaptation" may be applied only to unicellular or multicellular organisms at early stages of ontogenesis when the organisms pass through oligocellular stages of development.

It is the contention of the present writer that thermal adaptation can, indeed, occur, but that adaptation which significantly extends the range of biological functioning is very limited, if it exists at all. On the unicellular level, multiple growth optima are well known (discussed in Section VI,B,1). In higher organisms, however, the limiting crucial temperature-sensitive element (processes) may not be related to metabolism but rather to other phenomena, such as membrane transport and/or nerve activity. Hence, it is unlikely that intrinsic thermal boundaries may be significantly altered through the adaptation process, as this would probably require the emergence of organisms with completely different sets of proteins, enzymes, and lipids—with the ability to be compatible with a completely different vicinal water structure in higher (or lower) temperature intervals (that is, on either side of the boundary of the thermal transition ranges). However, one must allow for the possibility that a slightly higher thermal temperature limit may be achieved through suitable modification of the substrate, resulting in increased stability of the water structure "lattice." The stabilization may derive from slight modifications in the substrate conformation or change in (nonfunctional) nonpolar side groups or

through the introduction of low molecular solutes which may become available to stabilize the vicinal water structure.

Important contributions to the problem of adaptation of organisms to different environmental temperatures have been made by Hochachka and Somero (1968). These authors have studied the adaptation of enzymes to temperature; the enzymes were obtained from a number of different fish the natural habitat of which ranged from the Arctic (*Trematomus borchgrevinki*) to the Tropics [the South American lungfish (*Lepidosiren paradoxa*) adapted to living in the tropical swamp waters of Brazil]. The intermediate temperature ranges of fishes were represented by various brook and lake trouts (salmonids) and tuna.

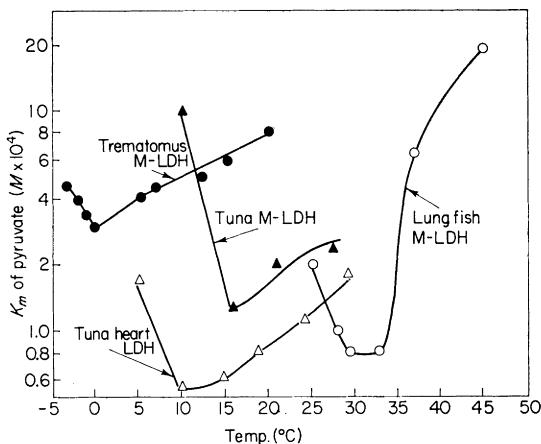


FIG. 23. Apparent Michaelis constant,  $K_m$ , of pyruvate lactate dehydrogenase (LDH) homopolymers from various fishes. (Hochachka and Somero, 1968, with permission.)

Figure 23 shows the effects of temperature on the apparent Michaelis constant,  $K_m$ , of pyruvate lactate dehydrogenase (LDH) homopolymers from the lungfish muscle, tuna heart and muscle, and muscle from *Trematomus*. In all cases notable minima in the Michaelis constant are exhibited near the temperature for optimum activity for the three fishes examined (corresponding to a maximum value for LDH activity). Hochachka and Somero (1968) discussed the possible mechanism for both long- and short-term adaptation to temperature. They conclude:

Our data on LDH from species facing a variety of thermal environments demonstrate that the above parameters (activation energies, thermal optima for maximum velocities, and heat denaturation) need not correlate with environmental temperature. Rather, in the adaptation of enzymes to temperature, the parameter most sensitive to selective pressure appears to be enzyme affinity

for ligands. Thus, the sensitivity of a given reaction to temperature can be minimized by compensatory changes in enzyme affinity for substrate, as in our study, or from modulators, as suggested by other work (Ingraham and Maaløe, 1967). In this connection, it is interesting that this mechanism apparently also operates in short-term acclimatization. At least two kinds of control processes are involved in acclimation (Hochachka, 1967; Smith, 1967): (1) control of the level of enzyme types already present in the cell, and (2) control of the level of enzyme variants (LDH isozymes, in our study) uniquely suited to function at particular environmental temperatures. Such enzyme variants, "induced" during short-term acclimation appear to be selected during evolutionary adaptation to temperature.

For another interesting example of enzyme kinetics and adaptation, see the recent article by Somero (1969). The Michaelis constant for two forms of pyruvate kinase was measured for, respectively, a cold- and a warm-adapted type of enzyme. The cold pyruvate kinase showed a sharp increase in the Michaelis constant at about 10° to 12°C, whereas the warm form of the enzyme showed a notable minimum around 12°C. As stressed elsewhere in the present chapter, a correlation is often found between the degree of abruptness in response to pH changes of the enzyme (or protein) and the degree to which these macromolecules exhibit thermal anomalies. In the examples studied by Somero, a remarkably sharp peak is observed in the activity of the enzyme between pH values of approximately 5.5 and 5.8—the resultant curve looking almost like a Dirac delta function.

The thermal properties suggest that the two types of the enzyme are created by an interconversion dependent on the temperature. This interconversion has adaptive significance: as the temperature is lowered, the warm enzyme is converted into the cold enzyme; the opposite situation obtains when the temperature is raised. Interestingly, Somero notes that "the two variants of the pyruvate kinase do not appear to be isoenzymes in the conventional sense. Electrophoretic and electrofocus analysis revealed only single peaks of activity." These findings may support the suggestion made earlier by Oppenheimer and the present author that adaptation (at least in the case of unicellular organisms) may consist of the selection of the proper metabolic routes, each consistent with the predominant water structure in the different temperature intervals. In the case discussed for the Alaskan king crab (Somero) and the trout (J. Baldwin and Hochachka (1970); see below), the adaptation may consist of the selection of the proper enzyme in the metabolic processes. Somero's observation that the isoenzymes are not "isoenzymes in the conventional sense" may mean only that their identical behavior in electrophoresis and electrofocus analysis reveal that differences exist only in the hydration structure rather than in the composition (or, in other words, rather than differences in specific functional groups).

Another interesting study of the temperature acclimatization of iso-enzymes was reported recently by J. Baldwin and Hochachka (1970). Two distinct forms of acetylcholinesterase was found in the brain of trout. One of these forms (the warm variant) was obtained in trout acclimatized at 17°C, whereas the cold variant occurred after acclimatization at 2°C. Acclimatization at intermediate temperatures resulted in the occurrence of both types of enzymes.

Baldwin and Hochachka found that the acetylcholinesterase from the trout acclimatized at 17°C showed a relatively sharp minimum in the Michaelis constant near 17°C, whereas the cold-adapted trout showed a broad minimum around 2°C—increasing rather rapidly above 10° to 12°C. The authors concluded, "In evolutionary terms, it appears that there is a strong selection for enzymes permitting large changes in activity in response to physiological changes in the substrate concentrations. This is reflected in the pattern of enzyme variants produced during acclimatization and those selected during evolutionary adaptation." The results of the study by Baldwin and Hochachka and the results by Somero are interesting when put in context. Thus, Somero (1969) has concluded that "the results suggests that the 'warm' pyruvate kinase and the 'cold' pyruvate kinase are formed by a temperature-dependent interconversion of one protein species."

In the introduction to this section on adaptation to thermal changes, it was contended that adaptation, which significantly extends the permissible range of the biological functioning, is very limited if it exists at all. The important and, indeed, valuable studies by Hochachka and Somero and co-workers suggest that acclimatization does occur, but the present author has not become convinced that this type of example of adaptation ultimately leads to the ability of the organism to live optimally over wide ranges of temperature. Although the trout or the king crab may develop slightly modified enzymes to cope with environmental temperatures from 2° to 17°C, it remains to be proved that this will allow these species to live and reproduce optimally over that wide a temperature range. The zoological stratification described, for instance, by Jones and quoted by the present author (Drost-Hansen, 1965a) suggests, as an example, the almost complete absence of commercially valuable fishes in the South Pacific below the 15° isotherm; see, also, the discussion of thermal pollution, Section VI,K, and particularly the review by DeSylva (1969). In conclusion, therefore, it appears that thermal boundaries set by the changes in the structure of vicinal water are not readily transgressed.

In connection with the study by Hochachka and Somero (1968) of the enzyme adaptations to various climatic environments, compare also the article by Cowey *et al.* (1969). These authors studied the LDH from cardiac and skeletal muscles of plaice (*Pleuronectes platessa*). They point

out that the behavior of the isoenzymes of cardiac and skeletal muscles is indistinguishable as determined by the effects of urea. However, it is possible to distinguish between avian and mammalian heart and muscle LDH on the basis of temperature stability as the heart enzyme is more thermally stable than the muscle enzyme. Compare this with the finding by Hochachka and Somero that the enzymes of the tuna heart appear to have a slightly lower temperature of maximum activity than the muscle enzyme.

It is proposed that the isozyme best suited to function at a particular environmental temperature is selected from those isozymes for which the changes in vicinal water structure is most compatible with the average

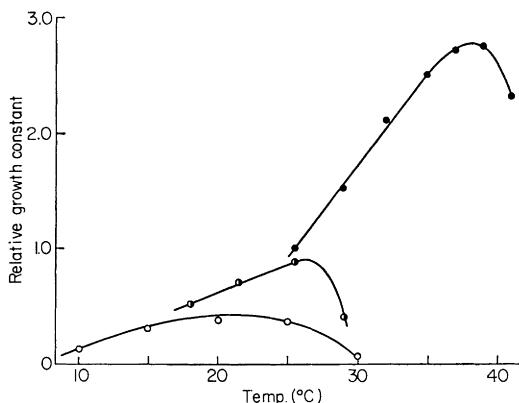


FIG. 24. Growth of three different strains of *Chlorella* as a function of temperature. (Fogg, 1969, with permission.)

environmental temperature. Very likely, only very minute changes in the specific nature of the amino acid sequences may determine the nature and degree of the vicinal water structure of the enzymes (as discussed in connection with the study by Glasel, Section V,B,5).

With respect to adaptation, Fogg (1969) has presented an interesting discussion (see Fig. 24). This illustration shows the growth of three different strains of *Chlorella* as a function of temperature. The two curves shown with maxima below and above 30°C are, respectively, two species of cold water strains—*Chlorella pyrenoidose* from Sweden and the Emerson strain of same organism. The organism with optimum for relative growth constant above 30°C is a high temperature strain, *Chlorella sorokiniana*. This example clearly shows that organisms of the same genus (*Chlorella*) may adapt to vastly different temperature optima. However, note that for the cold water strains the maximum (lethal) temperature oc-

curs near 30°C, whereas the high-temperature strain appears to decrease in activity above 37°C with a notable drop at 40°C.

For a far more general discussion of adaptation to temperature changes, see the monograph "Molecular Mechanisms of Temperature Adaptation," edited by Prosser (1967).

Finally, mention is made of an interesting study of thermal adaptation of enzymes, reported by Licht (1967). The study of Licht is impressive in that it provides a very careful study of enzyme activities observed at very closely spaced temperature intervals (the enzymes obtained from lizards). The temperature responses in the article, however, are not all readily interpreted in terms of the notion of water structure changes discussed in the present chapter.

An interesting discussion on the limits of microbial existence was presented by Skinner (1968). In addition to delineating in a descriptive fashion the extremes for limits of microbial existence, Skinner also discusses a few aspects of adaptation and mechanisms of tolerance. With regard to life at high temperatures, Skinner mentions two possible mechanisms, namely, that thermophilic organisms operate on biomolecules which are intrinsically thermally more stable than those of the mesophiles or that the protein of the thermophiles is somehow protected against denaturation. Finally, proteins of thermophiles may be denatured as readily as the proteins of the mesophiles, but the thermophilic organisms may be able to "repair the damage" more readily. At the other end of the spectrum, Skinner has described bacteria that may live in strongly saline pools in Antarctica at approximately -23°C, while a flagellate alga (*Dunaliella salina*) has been observed in brine pools at -15°C.

Skinner has also dealt briefly with barophiles (ZoBell's strain of a thermophilic, reducing bacteria, which did not grow at 85°C at atmospheric pressure, but was able to grow at 104°C under a pressure of 1000 atm), halophiles, osmophiles, and microorganisms with the ability to live in acids with pH values of around 0.5 (although the internal pH, even in these cells, is about 6 or 7). Skinner also mentions a blue-green alga able to grow at pH 13. Both the halophiles, osmophiles, and proto- and hydroxybacteria are interesting in the requirements for structural stability imposed on the cell membrane to retain a "reasonable" internal water environment.

## 2. Rate of Chromosome Aberration

A remarkable example of abrupt effects of temperature on a biological system was described by Wersuhn (1967) who studied the rate of chromosome aberration as a function of temperature during microsporogenesis.

The plant used for this study was the broadleaf bean, *Vicia faba*. Eleven different temperatures were employed between 19° and 35°C, and the results are shown in Fig. 25. It is seen that near 30°C the rate of chromosome disordering reaches a sharp peak. Wersuhn points out that each datum point represents 200 counts on each of five plants and that the notable anomaly at 30°C is statistically significant! Wersuhn also notes that this result agrees with the findings of Chira who apparently observed an increase in the disturbance of sporogenesis near 30°C for *Taxus*.

In connection with the spontaneous chromosome aberration discussed here the tentative analysis of the data in Section III,C regarding the properties of vicinal water near 30°C should also be noted. It was proposed specifically that below this temperature range, one form of vicinal water

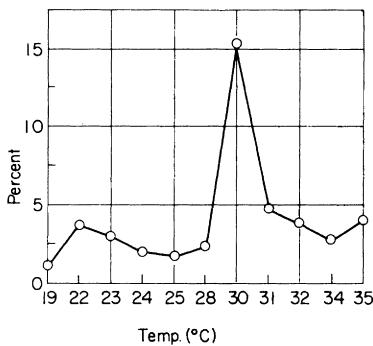


FIG. 25. Rate of chromosome aberrations as function of temperature during microsporogenesis. (Wersuhn, 1967, with permission.)

was stable, whereas above, say, 30° to 32°C, another form was preferred. In the transition, elements of both structures might coexist, together with a number of smaller, kinetic entities, possibly monomeric water molecules (associated with the large entropy of surface formation noted around 29° to 32°C). Comparing these results with the results by Wersuhn, it is proposed that near 30°C the vicinal water structure is highly disturbed and that in the process of cell division, the stabilizing influence of the vicinal water on the RNA-DNA genetic transfer system is disturbed. Thus, the genetic message in this temperature range becomes "scrambled." This problem will be referred to again briefly in Section VI,K dealing with thermal pollution effects.

Regarding the rate of mutation, see the example quoted by Farrell and Rose (1967, p. 197, Fig. 17). The results (quoted from Ogur *et al.*, 1960) clearly show a very dramatic increase of rate of mutation above approximately 30°C. Indeed, the increase is approximately fifty-fold over the average (relatively constant) value attained between 15° and 30°C.

### 3. *Paleozoogeography*

No doubt the characteristic properties of vicinal water are truly invariant properties with respect to time. Thus, in the past, as now, changes must have occurred in the structure of vicinal water at the temperatures where thermal anomalies now are observed. Hence, it is probably correct to conclude that in the processs of evolution, the temperatures of thermal anomalies have presented "invariant boundaries" which in general it has not been possible for multicellular organisms to transgress. It is known, of course, that thermal adaptations can occur. As discussed in Section VI,D,1, however, these changes are often matters of only a few degrees or even less; whereas the occurrence of a multiple temperature optima suggests that, at least in lower forms of life—especially for unicellular organisms—alternative ways have been developed to cope with temperature variations over more extensive ranges.

Stehli (1957) has used the time invariant property of the thermal anomalies in vicinal water in a study of paleozoogeographic stratification, arguing that present-day species with well-delineated upper and lower lethal temperatures are probably not significantly different from earlier forms of the same organisms. When certain fossil species from the same geological period occur in various geographical localities, it is likely that this reveals that the same average thermal environment must have prevailed there as that which is presently conducive to these particular organisms. This has been further elaborated upon in Ager's monograph "Principles of Paleoecology" (1963).

Ager discusses the distribution of various faunas in the past geological periods. Thus, as Stehli has suggested, Ager notes that the 15° winter isotherm in Permian times corresponds roughly with the 55° northern latitude and goes on to point out that this agrees qualitatively very well with other faunal distributions. Incidentally, both Stehli and Ager seem to agree that the distributions suggest a climatic zonation parallel to the present equator and thus do not support hypotheses of polar wandering. Ager also discusses the 30°C anomaly. An interesting point in this connection is raised by Ager, namely, the fact that the temperature of the open sea presently rarely exceeds 30° to 35°C. In the past a "hypertropical belt" with a distinctive fauna might have existed. This warm belt may conceivably have disappeared as the climate toward the beginning af the Tertiary period began to turn cold and this, in turn, account for the extinction of a number of groups of organisms. Ager quotes Cailleux who calls attention to the fact that the absence of Paleozoic reef corals in the tropics may be due to their inability to tolerate unusually high temperatures.

## E. NARCOSIS

### 1. *Pauling and Miller's Theories of Anesthesia*

The theories of anesthesia by Pauling (1961) and by S. L. Miller (1961) imply that hydrates form in the water of the neurons (and around the neuron network)—both in the cells of the neuron and in the synaptic regions. These structures represent obstacles in some of the electrically charged chains. Thus, they interfere “as sand grains in small gears” with the movement of ions in the synapsis and in the neurons. This, in turn, enhances the impedance. It is probably worth pointing out that the picture is somewhat crude. However, as we already discussed, one need not invoke the existence of stable solid crystalline forms of material (of the kind most to the liking of the solid crystallographer), but rather merely partially induced and stabilized “fluctuating” structures. On the average, it is probably necessary only to stabilize the half-life of such structured units by an order of magnitude (or perhaps a few orders of magnitude) rather than attempt to invoke any microcrystalline entities. However, this is probably mostly a matter of semantics, although it is far from a trivial point.\* For instance, chloroform hydrates may be formed readily from an emulsion of chloroform in water, but this hydrate is stable only below 1.5°C. Thus, “hydrate crystals” are very likely not present in the brain at 37°C.

S. L. Miller (1968) observed that upon lowering temperature to, say, 27°C, “...there might be sufficient icelike water present owing to the protein side chains to make the hydrate lattice stable in the absence of the anesthetic gas. This could be responsible for the anesthesia observed on lowering the temperature of warm-blooded animals (hypothermia).”

The idea that the gaseous, low molecular weight, clathrate hydrate formers may be simulated in function by the side chains of various amino acids in proteins was discussed by Klotz (1965). Thus, Klotz compares the functioning of methane to alanine, propane to valine, isobutane to leucine, and mercaptan to cystine, etc. Compare mixed gas hydrates and the facilitated formation of such hydrates in the presence of a “help gas” (Section IV,C,4).

For a lively discussion of molecular aspects of the pharmacology of anesthesia, see Federation Proceedings, “Symposium on Molecular Pharmacology of Anesthesia” (1968). The likely role of water in anesthesia was particularly well covered in this vigorous discussion.

It is interesting to note that, if, indeed, the theories of Pauling and Miller are correct, they may provide a simple means for explaining the in-

\* For a discussion of the idea of structure, see Section II,A,2 and Eisenberg and Kauzmann (1969).

creased intellectual acuity experienced by persons subject to a helium-rich atmosphere, such as a 20:80 oxygen-helium mixture. Presumably, intellectual activity can be sustained in such an atmosphere for as long as 20 hours per day. Ascribing some ability on the part of the nitrogen molecule to form hydrates (dissociation pressure of the nitrogen hydrate at 0°C is 160 atm) and remembering that the helium atom is too small to form a clathrate hydrate, we see that the increase in nervous activity experienced in a helium-rich atmosphere may be interpreted as the removal from the brain of the clathrate-forming nitrogen by the helium. Thus, perhaps we all suffer from a slight case of "nitrogen stupor" in the normal atmospheric environment.

In this section some aspects of anesthesia have been discussed very briefly. These aspects were concerned with molecular facets of the functioning of the anesthetic agents on the neural level. Obviously, an important consideration in the administration of gaseous anesthetics is the transfer of the agents via the lungs into the bloodstream. It seems quite natural to expect that water structure plays an important role here also; the mass transport involved (on a detailed level of description) must still be concerned with transport properties across a water-air interface and the diffusion through a water-filled (semirigid) matrix. It is not the purpose of the present chapter to discuss the mechanistic aspects of gaseous anesthetics, but attention is called to the unusual interfacial tension phenomena which must exist in the lung, and these certainly are influenced by the presence of surface-active agents, the functioning of which must, in turn, depend on their interaction with the water at the air-water interface.

Schreiner (1968) has discussed a number of biological effects of the inert gases. One of the main points made by Schreiner is that notable biological effects are observed due to the inert gases (particularly those larger than neon) at all levels of biological activity ranging from whole body (man) to individual molecular phenomena (such as enzyme processes). Among the interesting results quoted by Schreiner is the correlation between the mycelial growth response of *Neurospora crassa* and the inert gases; the study shows a well-defined linear decrease in growth rates with the square root of the molecular weight of the gas. Schreiner notes, however, that such rather inert gases as SF<sub>6</sub> and N<sub>2</sub>O also affect the rate of mycelial growth in the mold but that the effect cannot be correlated with the square root of the molecular weight of these two gases. As a result, Schreiner considers the possibility that the inert gas behavior, including nitrogen, may be fortuitous. Schreiner has also discussed previous results (with Buchheit and Doeblner) suggesting that the polarizability of the inert gases correlates with the inert gas pressure required for 50% inhibition of the growth of *Neurospora crassa*. Again, it

is probably true that such studies by themselves do not present a choice between the Pauling and the Miller theories of anesthesia on the one hand, and the lipid solubility hypothesis for anesthesia (Meyer-Overton) on the other hand. However, the results do clearly show not only the notable physiological effects of inert gases, particularly argon, krypton, and xenon, but also that these effects appear to a first approximation to correlate well with the clathrate hydrate-forming capabilities of the gases.

Unfortunately, most studies of the effects of inert gases on biological systems which have been reported in the past have all been done at one, or at most, a few different temperatures (see, however, Catchpool, 1966). It would be interesting, for instance, to study the rate of growth of plants

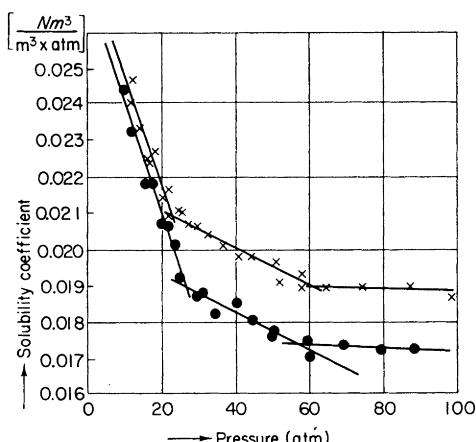


FIG. 26. Solubility of hydrogen in water as a function of pressure. (Schröder, 1969a, with permission.)

or fungi as a function of temperature at closely spaced intervals in the presence of fixed, relative pressures of oxygen and carbon dioxide for various clathrate-forming gases, including the inert gases. Thus, it is interesting to speculate whether an organism which exhibits multiple temperature optima (for a culture grown on a minimal medium) when grown under nitrogen (anaerobic) or under nitrogen-air mixtures (aerobic) might show a different temperature response in the presence of helium only (or, for the aerobic case, in a helium-oxygen atmosphere). Schreiner points out that "While it is therefore quite likely that helium group gases are not essential to life (1), we still cannot exclude with certainty the possibility that rigorous deprivation of an animal organism of a suitable non-metabolized inert gas, such as nitrogen, may not result in the eventual development of physiological abnormalities."

## 2. Schröder's Pressure Anomalies

Recently, Schröder (1968, 1969a) has described some unexpected anomalies in the solubility of gases in liquids. It appears that these results are destined to play a great role in our understanding of the effects of low pressures on biological systems. The results will probably be particularly interesting in connection with hyperbaric surgery, diving physiology, and

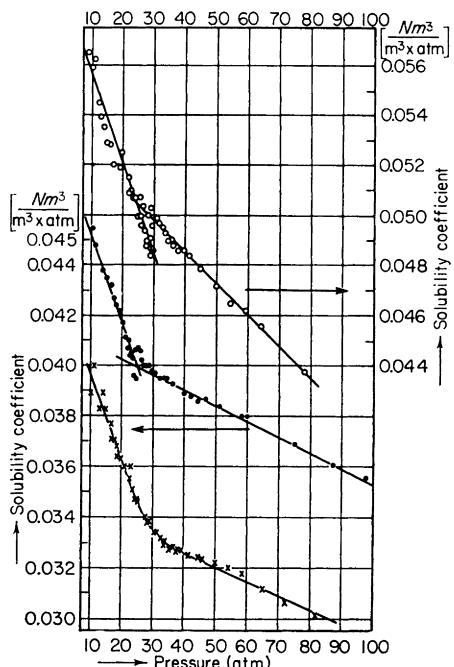


FIG. 27. Solubility of argon in water at three different temperatures ( $0^\circ$ ,  $10^\circ$ , and  $20^\circ\text{C}$ ) as a function of pressure. (Schröder, 1969a, with permission.)

the physiology of those marine organisms possessing a swim bladder (or other internal, gaseous phases).

Schröder determined very precisely the solubility of a number of gases (hydrogen, methane, nitrogen, oxygen, and argon) in various liquids, particularly water and aqueous solutions. Schröder (1969b) used these results for some intriguing speculations on the void volume in liquids. It suffices here to stress the empirical results and postpone a discussion of the possible mechanism to a subsequent paper. The main finding by Schröder is that the solubility coefficient [Bunsen's solubility coefficient,  $\text{Nm}^3/(\text{m}^3 \times \text{atm})$ ], when plotted as a function of pressure, displays abrupt

changes in slope ("kinks" or "knees"). For hydrogen in water at 20° and 40°C, the changes in slope occur at close to 22 to 24 atm and near  $60 \pm 2$  atm. Figures 26, 27, and 28 show typical examples of this effect. Attention is called particularly to Fig. 27, which shows the solubility of argon in water, determined at 0°, 10°, and 20°C. It is seen here that the solubility coefficient possesses a sharp change in slope between two rectilinear asymptotic curve segments in the vicinity of 24 atm (at the lower two

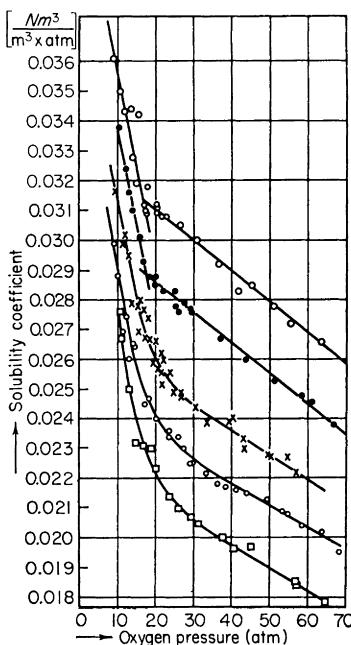


FIG. 28. Solubility of oxygen (at 25°, 30°, 40°, 50°, and 70°C) as a function of pressure. (Schröder, 1969a, with permission.)

temperatures), whereas at 20°C the curve has assumed a smooth, continuous shape. Somewhat similar results are shown in Fig. 28 for the solubility of oxygen at 25°, 30°, 40°, 50°, and 70°C, respectively. Again, a sharp knee is observed near 18 atm at lower temperatures, whereas no such anomaly is present at 40°C (or above).

Table XI shows the temperatures above which the sharp change in solubility has disappeared. This temperature is dependent upon the chemical nature of the gas. However, the pressure at which the anomaly is observed is independent of the nature of the gas. This is also evident in Table XII which shows the critical pressures for a number of gases in various solvents.

The fact that anomalies are observed with liquids as different as water and benzene tends to suggest that the phenomenon is independent of the structure of water. This is, indeed, possible as the effect may owe its origin merely to a general attribute of any liquid structure, namely, the possible existence of voids such as discrete, physical, actual "fluidized vacancies" or free volume.

It is seen in Fig. 27 that metastable conditions may exist, giving rise to two separate curve segments in the range from approximately 24 to 30

TABLE XI  
CHARACTERISTIC TEMPERATURES FOR GAS SOLUBILITY ANOMALIES

Gas	Temperature (°C)
CH <sub>4</sub>	75
N <sub>2</sub>	45
O <sub>2</sub>	35
Ar	10

TABLE XII  
SOLUBILITY ANOMALIES IN VARIOUS SOLVENT SYSTEMS

Solvent	Boiling point at 760 torr (°C)	Gas	Temperature of experiment (°C)	$\pi$ (atm)
Methanol	64.7	H <sub>2</sub>	50	19
Benzene	80	N <sub>2</sub>	10	23
Water	100	H <sub>2</sub> a.o.	20 a.o.	ca. 24 or 65
<i>n</i> -Butanol	117.5	H <sub>2</sub>	50	20
Propylenecarbonate	242	H <sub>2</sub>	50	45

atm. A metastable liquid structure is synonymous with a "memory effect" in structural properties\*—a fact which undoubtedly will prove over the years to be of great significance to biological systems. Other studies have recently been carried out along the lines thermal memory effect, particularly by Bach (1971; see Section VI,J). The effect observed in the case of the gas solubility may possibly not be a thermodynamic equilibrium effect, but rather a kinetic effect.

\* It should be observed that the total amount of gas dissolved in any of the systems studied may be relatively small compared to the amount of water which may have occurred as a residual impurity in the organic solvents used and, thus the anomalies may still be related to the structural characteristics of water. However, unfortunately, Schröder has not discussed in sufficient detail his experimental procedures to allow an estimate of the degree to which he was, indeed, dealing with water-free solvents.

A tempting explanation of the results obtained on aqueous systems is that the results imply the existence of discrete sites or voids for solute molecules. This would be imminently compatible with a theory of water structure based on the clathrate hydrate model. It would be particularly interesting in this connection to carry out solubility measurements as a function of pressure and temperature on solutions and suspensions of biologically interesting macromolecules and colloids. However, even in the absence of such information it is obvious that the phenomena must be of biological interest since pressures of the order of 18 to 25 atm are likely to be encountered by marine organisms such as fishes which easily reach depths far below the corresponding 180 to 250 meters. Furthermore, dives are presently being carried out to depths greater than 200 meters. In this connection, it is to be expected that both the kinetics and the equilibrium aspects of gas solubilities in blood and other body fluids may play a crucial role in decompression aspects of diving physiology. Should the hysteresis phenomenon shown in Fig. 27 turn out to be of general importance, and if it occurs with either nitrogen or oxygen, the kinetics of decompression may need to be seriously reconsidered.

Phenomena of the type discussed here show clearly the possibility of elucidating complex molecular mechanisms through simple physicochemical measurements. It is not possible to understand such changes without considering in detail the molecular structure of the liquid. Certainly, it would be difficult to understand observations of this type in terms of a continuum model for liquids; we are thus again impressed with the possibility that a clathrate hydrate model for water is essentially correct as described in this chapter to explain at least part of the observed structuring of water near biologically interesting interfaces. The fact that the critical pressures, at which the solubility regime changes from one domain to another, are independent of the chemistry of the dissolved gas lends further credence to this. At the same time, the types of lattice structure being induced or supported by the presence of the guest molecules no doubt depends on the "chemistry" of the dissolved gas, possibly through no more complicated parameter than size.

### *3. Deep Diving Pressure Effects*

In connection with deep diving narcosis, it is of interest to speculate that clathrate hydrate formation may occur due to the presence of argon as well as nitrogen. At high total air pressures, the partial pressure of argon is also notable. Since this gas certainly is known to form clathrate compounds with a fair degree of ease, it might be conjectured that the diving narcosis phenomenon is due, in part, to argon. Hence, it would be

of interest to determine if the narcosis phenomenon is as pronounced in dives where pure oxygen-nitrogen mixtures are used as in dives employing compressed air at the same total pressure.

Although deep diving physiology is currently of great interest, the amount of systematic work available that can throw light on some of the problems involved here is relatively limited. A large amount of work has been done; however, much of it has been rather sporadic. Apparently, among those concerned with deep diving narcosis, several authors appear to prefer the Meyer-Overton theory of anesthetic action, implicating lipid solubility rather than vicinal clathrate formation. With respect to the possible existence of an anomaly at approximately 20 atm, as implied in the study by Schröder, E. B. Smith (1969) mentions some effects of high pressures on mice in which tremors occur at about 50 atm. He also observed, "these tremors may be of similar origin to those observed with men in deep diving routine below 600 feet of seawater." The present author has not located any references regarding the possibility that the partial pressure of argon in compressed air (under sufficiently high total pressure) may reach levels where the anesthetic effect becomes notable. The reader is referred to the chapter by Bennett (see E. B. Smith, 1969) who, incidentally, strongly favors the Meyer-Overton hypothesis of the anesthetic action of inert gases.

## F. HYPOTHERMIA

### 1. *Physiological Responses to Low Temperature*

Hypothermia as used in the present context is the state of reduced body temperature in mammals.

Hypothermia, as a result of exposure to cold, has obviously been seen clinically since the beginnings of medical science; only fairly recently, however, has the artificial reduction of body temperature attracted some clinical interest. Thus, major cardiovascular surgery is occasionally performed on the hypothermic patient. In addition, hypothermia has been used earlier (with apparently relatively poor success) in the treatment of cancer: the rate of proliferation of malignant cells is reduced at low temperatures (as are all other growth processes), but apparently cancer cells are only slightly more susceptible to low temperature than normal tissue. Unfortunately, the difference in susceptibility is not sufficient to allow clinical use of this sensitivity. Mild hypothermia has apparently been used in the treatment of severe eclampsia, postoperative tetany, and some mental disorders as well as toxemia, septicemia, peritonitis, and hemorrhage. It is

notable that mild hypothermia usually does not involve reduction of body temperature to lower than 33° (to 35°C). Pronounced effects are observed as soon as the temperature of a mammal is reduced below 30°C (see Drost-Hansen, 1965a). Among the effects particularly seen around 30° to 32°C are loss of consciousness, rapid and drastic reduction in metabolism of various tissues, and loss of ability to restore body temperature to normal levels (when placed in a cold environment). Above approximately 33°C, physiological changes attending hypothermia are not pronounced, and control over the clinical state is good. It is interesting that the muscular activity in most animals in the form of shivering is at a maximum at temperatures of 28° to 30°C.

The effect of cold on the nervous system was particularly well delineated in a symposium "The Problem of Acute Hypothermia," edited by Starkov (1960). Table XIII, from this reference (Starkov, 1960, p. 38) shows the effects of reduced temperatures on conditioned reflexes.

The marked, abrupt changes at 30°C were further emphasized by a number of authors in the symposium on acute hypothermia. Thus, near 30°C, drastic changes in excitability of nerves, muscles, and motor centers in rabbits were described by Karpovich (1960, p. 69ff). Similar changes at 30°C were described for vasomotor centers by Klykov (1960).

J. A. Miller (1957) observed the critical nature of temperatures around 28° to 32°C and emphasized the low-temperature effects on various organs. For instance, heart action appears to be particularly sensitive to temperatures below 29°C. As mentioned in connection with the studies by Hazelwood and co-workers (see Section V,F), Miller also notes the difference between neonatal and mature organisms.

Elsewhere the present author (Drost-Hansen, 1965a) discussed the studies by Kuznetsova on gas exchange in and metabolism of hypothermic rabbits. In "deep hypothermia," more pronounced, often dramatic, and sometimes irreversible changes occur around 14° to 16°C (also see the article by Starkov, 1960). That 15°C is a critical temperature in hypothermia is also clearly emphasized by Andjus (1969) who reviewed some possible mechanisms for mammalian tolerance of low body temperature. Andjus notes (p. 361) that, in the rat, breathing and heartbeat are arrested shortly after cooling to a body temperature of 15°C. Deeper hypothermia is, indeed, possible; however, 15°C appears to be the limit for unassisted survival. Furthermore, cooling below 15°C induces changes characteristic of anaerobic conditions, whereas cooling to temperatures above 15°C is not associated with hypoxic changes, as shown by metabolic parameter measurements. Andjus shows a number of interesting graphs of various physiological responses as a function of temperature, some of which also suggest the criticality of 15°C.

The effects of cold upon the central nervous system and muscle activity are probably the direct and proximate cause of other significant changes seen near the temperatures of thermal anomalies (particularly at 30°C and, to a lesser extent, at 15°C, i.e., deep hypothermia). Prokop'eva (1960) notes the effects on the rate of blood flow. In initial stages of hypo-

TABLE XIII  
EFFECT OF VARIOUS BODY TEMPERATURES ON CONDITIONED REFLEXES<sup>a</sup>

Experiment	Temperature at which the conditioned reflex disappeared during overcooling (°C)	Temperature at which the conditioned reflex appeared during warming (°C)
Bobik		
No. 1	27	30
No. 2	29	32
No. 3	30	(Only after repeated combinations with short intervals)
Kashtanka		
No. 1	29	32
No. 2	28	29
No. 3	30	—
Pestrukha		
No. 1	30	Overcooling to 25°C (conditioned reflex reaction not restored when temperature increased)
No. 2	30	
No. 3	30	
No. 4	30.5	(35°C only after repeated combinations with short intervals) 31.5 35
No. 5	29	Conditioned reflex could not be produced when temperature increased, even by combinations with short intervals
No. 6	30	
No. 7	29	35
Ryzhik		
No. 1	33	(Only after repeated combinations with short intervals)

<sup>a</sup> From Karpovich (1960).

thermia, the rate of blood flow increases when the body temperature is between 32° and 33°C, but the reduction of temperature by about 1° below 32°C results in a notable decrease in blood flow. Another example of the effects of cold is discussed by Kuznetsova (1960, p. 257ff); he notes a sharp maximum near 30°C in, for instance, the amount of carbon dioxide excreted or the rate of metabolism in the hypothermic rabbit.

Cardiac and cardiovascular surgery has been attempted frequently on

hypothermic patients. This procedure is associated with considerable danger of ventricular fibrillation. Intravenous glucose transfusions, administered to the hypothermic patient, have been found to lower the incidence of ventricular fibrillation considerably (Virtue and Burnett, 1958, personal communication). The glucose concentrations used have often been remarkably high. It is of interest to consider some possible mechanisms by which the glucose may prevent ventricular fibrillation. It has been shown (Angelakos *et al.*, 1957) that dogs rendered hypocalcemic by administration of sodium ethylenediaminetetraacetic acid (Na-EDTA) (which forms a complex with calcium ions) were able to tolerate cooling to 14 to 18°C without the occurrence of ventricular fibrillation. Conversely, increased calcium ion activity (effected by intravenous administration of calcium) resulted in fatal ventricular fibrillation, even at temperatures as high as 22 to 27°C. It also is of interest to note that Binet (1957) reported that an increase of calcium in the plasma of the rabbit from 11 mg% to 30–40 mg% results in hyperglycemia (for ambient temperatures of 6 or 28°C). This would suggest that the increased calcium ion concentration evokes an increase in glucose concentration; the effect may possibly be a specific protective mechanism.

The interplay between calcium ions and glucose in physiological functioning appears to be complex; it seems possible that the glucose may directly complex with the calcium ion (similar to the effect of EDTA). This possibility seems reasonable and it is of interest to note that solubility data for calcium hydroxide in aqueous glucose solutions show a notable increase in dissolved  $\text{Ca}(\text{OH})_2$  with increasing glucose concentration (data by Balezin; see Seidell and Linke, 1952). To investigate the possibility of calcium ions complexing with glucose, the present author initiated in 1966 some measurements of calcium ion activities in glucose solutions (Thorhaug, 1967) using the then available specific calcium ion electrodes [manufactured by Orion and by Corning]. Unfortunately, the functioning of these electrodes appeared to be very sensitive to the presence of glucose in solution and no definite results were obtained except to note that no direct evidence was obtained for strong complexing effects of the glucose. However, experiments of this type ought to be continued with the improved ion selective electrodes now available. Finally, it is possible that glucose may specifically influence only vicinal water structures, and that this effect, in turn, alters the ion activities, rather than directly affecting the calcium ion activities through complexing. In this connection, see the discussion of the paper by Apffel and Peters (1969); see also the "classic approach" in the paper by DeHaven and Shapiro (1968).

In summary, although very complex physiological activities and

processes are involved in the overall physiology of hypothermia, the abrupt changes occurring near 30° to 32°C indicate that a narrow temperature interval is extremely significant. It is proposed that the dramatic effects associated with this narrow temperature interval are manifestations of the sudden changes in the structure of the vicinal water of the biological systems, most likely through the effects on nerve conduction, membrane permeabilities, and enzymes kinetics.

## 2. *Hibernation*

There is no sharp distinction between the concept of hypothermia and hibernation (see, for instance, the discussion in Lyman and Dawe, 1960). Characteristic examples of the critical role of temperature on hibernation are also discussed in an article by Bullard *et al.* (1960); thus, in both the ground squirrel and the hamster the heart rate drops as the temperature is lowered from approximately 35° (or 37°) to about 33°C. Between 30° and 33°C, however, the heart rate increases, while the metabolism of both animals drops simultaneously until a rectal temperature of about 30°C is reached; the metabolic rate then levels off at a more-or-less constant value over a wide temperature range. (These experiments were carried out in an atmosphere of reduced oxygen tension: 5–6% oxygen in nitrogen.)

Hibernation is far more difficult to explain than is clinical hypothermia from the point of molecular physiology. The dependence upon ambient temperature in competition with homeostatic temperature control leads to exceedingly complex behavior. The processes are slow, yet at no time do the systems approach thermodynamic equilibria. The heat conduction problem, particularly in furry animals, makes a direct comparison between ambient temperature and body temperature highly time-dependent and of little analytical usefulness. Yet, in spite of all these difficulties, an inspection of the available literature on mammalian hibernation (see, in particular, Lyman and Dawe, 1960) again reveal the critical nature of the temperature range from approximately 30° to 32°C. Somewhat similar anomalies occur in deep hibernation for temperatures around the 15°C anomaly (see, for instance, the article by Eisenstraut, 1960, p. 31).

Entrance into the hibernating state occurs in the squirrel around 30°C. In an extensive review by Strumwasser (1960) of the physiological processes regulating the hibernation in squirrels, it is noted that "The induction into the hibernating state occurs . . . when the squirrel's brain temperature had reached 32°, since, as a general rule, once this temperature is reached, there never occurs a spontaneous turning back." Strumwasser also observed changes in the heart rate during entrance into and arousal from hibernation as a function of temperature. The heart rate, according to

Strumwasser, declines as the temperature is lowered from ordinary body temperature to near 34.2°C. Over the following interval of only 0.6° (from 34.2 to 33.6°C), the heart rate drops from 153 beats to 68 beats per minute.

Here, as in most of the examples discussed in this section, factors other than temperature obviously play an important role. However, the point of the present discussion is that, superimposed on the various other influences, is the omnipresent effect of the structural changes in the vicinal water of the systems under consideration. Whenever the controlling mechanism involves some aspect of the structure of vicinal water, thermal anomalies will be manifest in the behavior of the system under consideration.

Subsequent to the two monographs mentioned on the problem of hypothermia and hibernation, a symposium was held on dormancy and survival (see, in particular, the article by Lyman and O'Brien, 1969).

#### G. CRYOBIOLOGY

The structure of water as well as the structure of ice play an important role in cryobiology. Some more or less standard studies of the freezing of biological systems will be discussed very briefly in this section. The freezing of biological systems upon exposure to cold is obviously an example of a nonequilibrium and, in fact, a nonsteady state phenomenon. Hence, it is not surprising that factors such as the heat conductivity of both ice and water as well as of the protoplasm and membrane materials, specific heats, and the heat of fusion of ice enter into the problem.

In this section is reviewed briefly the process of freezing in biological systems, as discussed most recently by Mazur (1966, 1970). The treatment by Mazur is both detailed and informative, but as in practically all other current studies of cryobiology, it is based primarily on the implicit assumption that "fine details" of water structure changes near interfaces can be neglected. (One credible, notable exception mentioned by Mazur is the possibility that some macromolecules may protect cells from freezing damage through hydrogen bonding or clathrate stabilization.)

Mazur (1970) begins his discussion of cryobiology by pointing out that intracellular water generally is readily supercooled to -10° to -15°C, even when crystalline ice is present in the external medium (the "equilibrium" freezing point of the cytoplasm is about -1°C, corresponding to an osmolar concentration of about 0.5 M). Thus, the cell membrane apparently can prevent the initiation of growth of ice in the cytoplasm in the cell in spite of the "crystalline" environment. Mazur also points out that, by the same token, the interior of the cell contains no elements which make good nucleators of supercooled water.

Mazur (1970) next invokes (a) Raoult law to predict the difference in vapor pressure between the internal water of the cytoplasm and the external water and (b) an equation for the rate of flow of water out of the cell in response to the (vapor) pressure gradient established. [This approach assumes the cell is permeable only (or primarily) to water.] A study of the two equations is interesting, but since they are based on equilibrium thermodynamics they apply only to "equilibrium," i.e., they apply only to exceedingly slow cooling rates, say, less than  $1^{\circ}/\text{minute}$  (in studies of yeast and erythrocytes). For higher rates of cooling, the supercooling of the cytoplasm increases significantly. It should be noted that experimentally it is reasonably easy to obtain freezing rates varying between  $1^{\circ}$  and  $10,000^{\circ}/\text{minute}$ , although undoubtedly with rather poor control or significance other than for an "instantaneous value." When freezing does occur intracellularly, it is likely that the nucleation is the result of a "puncturing" of the cell membrane, leading to the nucleation of the interior solution by the external solid phase.

Two types of freezing damage occur to the cell: either the cell becomes dehydrated by the outflow of water (because of osmosis) or the cell may undergo intracellular freezing. A loss of water to the external environment may greatly increase the solute concentration and, thus, produce drastic changes within the cytoplasm. This type of damage is referred to as a "solution effect." As would be expected, rapid cooling produces small ice crystals. However, the smaller the crystal, the greater the tendency for the crystallites to grow to larger crystalline units as the temperature is slowly raised during rewarming; this gives rise to an optimum rate of freezing which will do the least damage, in the sense of ensuring the greatest rate of survival of the cell. Figure 29 shows some effects of freezing on the survival of vastly different types of cells. The difference between the different types of cells is impressive.

An inspection of Fig. 29 shows that both erythrocytes and yeast cells survive poorly at temperatures of  $-190^{\circ}\text{C}$  for a cooling rate of  $100^{\circ}/\text{minute}$ . However, the reason for this low survival is entirely different: in the case of the erythrocytes the cytoplasm does not freeze but loses water to the intercellular ice, whereas in yeast the damage is due to intracellular freezing.

Mazur also discussed the better known reports of protective agents for cryobiological purposes such as glycerol, dimethylsulfoxide, sugars, and polyvinylpyrrolidone (PVP). However, in spite of considerable study, it does not seem possible at this time to present a "molecular picture" of the protective action of these materials in general. Compare the cryoprotective agents glycerol, dimethylsulfoxide, and some polyhydroxy com-

pounds with their "typical nonaqueous" behavior discussed by Franks and mentioned in Section II,C,3.

In connection with the readily obtained supercooling of about 10° to 15°C, there is little doubt that it is the membrane surface which prevents the ice from permeating into and nucleating the water of the cytoplasm. This bears on the problem of the possible existence of water-filled pores (see, for instance, the studies by Solomon). Actually, very small pores (say, 5 Å, the order of magnitude discussed by Solomon, 1968) would most likely represent radii of curvature so small as to make the crystalline ice phase unstable with respect to the liquid phase. However, even if larger

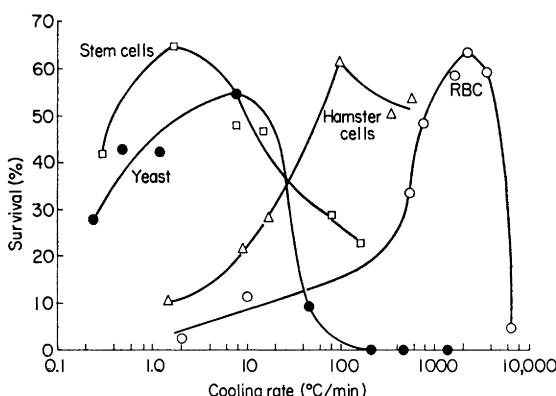


FIG. 29. Survival of various types of cells as a function of cooling rate. RBC—erythrocytes. (Mazur, 1970, with permission.)

pores were present, it is possible that freezing might not propagate through these pores because of the ordered nature of the water in the membrane.

Mazur (1970), finally, mentions the possibility that the cell water may not necessarily be treated as water or normal aqueous solutions. In fact, he calls attention to the possible existence of "bound water" and refers to a possible "ice-likeness." Observing that the water in cells and around macromolecules shows little ability to act as a nucleating agent for supercooled water, Mazur proposes that the ordered water, at least to this extent, fails to be "ice-like." Of course, as pointed out elsewhere in this chapter, the terminology "ice-like" is probably very unfortunate, as it is unlikely that water, either in bulk or as vicinal water, contains elements with actual Ice-Ih-like characteristics.

There is little doubt that the classic approach discussed so far has brought a certain amount of understanding to the problem of cryobiology,

although Mazur points to a great number of unresolved problems. The present author, however, takes issue with the statement that the equations used are consistent and "essentially picture a cell as a membranous bag of dilute solution, the solvent being water of normal properties." Mazur claims the model satisfactorily predicts which physical events will occur in cells during freezing. He also claims that the amount of heat absorbed by frozen cell suspensions during warming (as measured by differential thermal analysis) is in semiquantitative agreement with the view that the bulk frozen cytoplasm behaves like an ordinary frozen dilute solution. In view of the many questions which Mazur raises at the conclusion of his article of poorly understood or completely obscure phenomena associated with freezing, it is somewhat extravagant to claim that the model—based on the equations for the freezing process—accounts "satisfactorily" for many physical events which occur during freezing. It should also be noted that many different ice structures (ice polymorphs) possess lattice energies differing only relatively little from ordinary ice and, thus, may go undetected in a standard differential thermal analysis.

Meryman (1966a) has reviewed some of the evidence for ordering of water in biological systems and particularly calls attention to some studies by Hori (1960). Meryman has redrawn the original data obtained by Hori and concludes that for thin films of water:

It is interesting that the vapor pressure approaches zero at a greater film thickness between glass than quartz. Perhaps the lattice parameters of the quartz structure are too foreign to that of water to potentiate structure but, instead, propagate disorder. In any event, Hori's data show dramatically that, between glass at least, water films with a thickness of  $0.1 \mu$ , extremely thick by biological standards, neither freeze nor have a measurable vapor pressure. Although the circumstances in biological materials may differ, the point is well made that adjacent structure can dramatically affect water activity.

(Compare the more common notion that quartz is supposed to be responsible for extensive ordering of water by epitaxy because of the similarity between the ice and the quartz lattice.)

For general information about the field of low-temperature biology, the reader is again referred to the impressive monograph "Cryobiology," edited by Meryman (1966b). This volume probably contains the most extensive reviews of the state of the art of cryobiology at its time of publication; also see the recent article by Mazur (1970) referred to above.

For an excellent review of the effects of freezing on aqueous enzyme systems, see the article by Tappel in "Cryobiology" (1966).

## H. HYPERTHERMIA

### 1. *Upper Lethal Temperatures*

As discussed in Sections VI,B,1 and 2, rather abrupt, critical upper thermal limits are frequently encountered for various organisms. A direct application of this notion is discussed, for example, in the section on thermal pollution (Section VI,K). For mammals and birds, 44°–46°C appears to be an absolute, upper thermal limit for survival. It should be noted that Belding (1967) has suggested that a body temperature of 45°C may be sustained by birds over relatively long periods of time without injury, whereas a rise to about 47°C is lethal. In humans, body temperatures above 43°C (for instance, due to infectious diseases) is considered highly dangerous and the prognosis is generally poor. However, when the elevated body temperature is produced by external means, such as diathermy or hot baths, the probability of survival is significantly increased. This will be elaborated upon in the next section.

A rather detailed discussion of temperature effects on poikilotherms was presented by Fry (1967). The reader is referred to the article by Fry for many interesting details, especially regarding definitions and methods of determining thermal limits. Measurements are made by maintaining organisms for various lengths of times at constant temperatures and determining the time of death due to exposure to a particular temperature. Alternatively, the temperature may be recorded at which the animal dies (or suffers loss of motor ability) in an environment of constantly increasing temperature. The rate of temperature change now plays a crucial (and ill-defined) role. Each approach has its advantages and disadvantages. The problem is not amenable to a description in terms of physicochemical parameters because of its nonreversible, nonsteady state aspects.

In connection with structural changes at 45°C as the causative factor in death, it can be questioned if evidence exists for the operation of only one causative mechanism, or, if more than one direct cause is involved, what are the different possibilities. Specifically, What is the mechanism of thermal injury? Is it possible to determine the site or sites where water structure changes may be the direct cause of death of the organism? Unfortunately, there appears to be very little information available on which to base any judgment in this matter. Fry reports a few sets of data which tend to suggest the occurrence of two different, distinct mechanisms of death. Among possible mechanisms are failure of osmoregulation, increased production of lactic acid (an unlikely effect), or asphyxia and/or damage to the central nervous system. Fry mentions also that much of the

work by investigators such as Precht, Prosser, and Ushakov tends to suggest that the animal dies from the cessation of some regulatory activity, rather than from "collapse of its cells."

## 2. *Hyperthermia Therapy*

Recently, von Ardenne and co-workers (1965, 1966a,b) and Kirsch and Schmidt (1966) have actively pursued the treatment of cancer by hyperthermia. Initially, the treatment consisted merely in heating the patient, while more recently a "multi-step therapy" is employed, combining extreme hyperthermia (heating of the patient's body to near 43° to 44°C) with the prior, simultaneous, or subsequent administration of certain drugs (pharmacons). With the development of more effective pharmacons, the need for extreme hyperthermia has been reduced somewhat and present therapy uses temperatures as low as 42°C.

The treatment of cancer, both experimentally and clinically, by high temperature is by no means new. Cavaliere and co-workers (1967) have discussed the heat sensitivity of cancer cells and reported both some biochemical and some clinical studies. These authors mention that as early as 1866, Busch described the complete disappearance of a histologically proven sarcoma after the patient suffered two attacks of erysipelas (an acute infection of the skin by a Group A hemolytic streptococci, characterized by sharply delineated, red, swollen local areas with general fever and malaise; temperatures as high as 42°C are often observed). The article by Cavaliere and co-workers should be consulted for a number of examples and a rather careful review of previous studies. It is interesting and, in fact, impressive, that the previous studies as well as the work of Cavaliere and co-workers and von Ardenne and co-workers clearly demonstrate the increased heat sensitivity of malignant cells to temperatures ranging from 43° to 44°C. Although Cavaliere and co-workers were relatively successful in the clinical treatment of 22 cases of cancer of the limbs, they present their findings with considerable reservation—because, among other reasons, of the attendant clinical risk in any high-temperature treatment as well as other complications.

No molecular mechanism has yet been postulated for the role that temperature plays in that part of the original therapy which relied primarily on the effects of temperature alone. It was suggested (Drost-Hansen, 1966) that the effectiveness of the increased body temperature depends primarily on the structural transition near 45°C in water associated with the cells (rather than, for instance, merely increasing the activity of any administered pharmacon). Burk and Woods (1967), Olmstead (1966), and Szent-Györgyi (1965) have pointed out that cancer

cells generally have a much higher water content than normal cells. As an example, ordinary liver cells possess about 67% water compared to Rous sarcomas of chicken containing 93% water; in fact, there appears to be a good correlation between malignancy and the water content of cancer cells. It is now suggested that the molecular mechanism underlying the effectiveness of the hyperthermia therapy may, in fact, be due to one or a combination of several processes. The first of these possibilities involves the greater ratio of bulk-like water to structured water in the cancer cells as compared to normal cells. Since vicinal water (and solutions) appear to be stabilized near an interface (where it undergoes a transition at or near 44° to 46°C), the cancer cells may be more susceptible to temperatures in this vicinity than normal cells, merely because of the larger amount of bulk-like water.\* If the pharmacons administered tend to accumulate in the malignant cells, the effectiveness of these pharmacons may be due to their ability to alter the water structure in such a fashion that a lower transition temperature is obtained. Of course, the pharmacons (such as alkylating compounds) administered at the time of the treatment will exert a direct influence on the biochemical processes involved in the metabolism of the cancer cells. It is of interest to note that Burk and Woods have shown that at 43°C and above, 9- $\alpha$ -fluoroprednisolone accelerates loss of the Pasteur effect and metabolic death of the cells. In addition, the Pasteur effect in cells of mouse melanoma S91 remains essentially constant (for 1 to 2 hours) at any given temperature below 40°C. However, above 43°C the aerobic acid production (glucolysis) increased markedly more than the anaerobic glucolysis. [See in this connection the study by Haskins (1965) who investigated the effects of sterols on the temperature tolerance in a fungus of the genus *Pythium*.]

An alternative hypothesis is based on the assertion that cancer cells probably present a far more disordered interface to the cell fluid than do normal cells: since cancer cells generally are much less differentiated than normal cells, they do not present the intracellular water with the same degree of stabilization near the interface that is provided by normal cells (Szent-Györgyi, 1965). This effect would be particularly important near the point where an abrupt transition in the water structure occurs.

Both of the mechanisms suggested above may play a role simultaneously. In general, the effect of elevated temperatures is to produce greater instability in the water structures associated with the cells, and this effect may be further enhanced near the critical transition point due to the pharmacons administered at the time of treatment. The structural changes in water are suggested here as an important factor in the molecular process

\* (See, however, discussion of the paradox of relatively invariant thermal transition temperatures, Section III,C,1,2.)

underlying the phenomenon of enhanced lethal effects of high body temperatures, but this aspect is obviously only one factor in an exceedingly complex molecular system and many other factors may play equally important roles.

In connection with hyperthermia treatment, it is of interest to note that some of the phenylenediamines have been alleged to have a synergistic effect in the hyperthermia treatment of cancer. Apparently so does dopa. The phenylenediamines have the interesting property that their solubilities increase extremely rapidly over very narrow temperature ranges. Thus, conceivably, these compounds are highly "sensitive" to the structural details of the aqueous environment. This would again suggest that a more detailed understanding of the effects of various solutes on the structure of water and, particularly, the structure of vicinal water may aid in predicting the types of pharmacons which may be most useful in the treatment of cancer by multistep hyperthermia therapy.

It is interesting to speculate on a rather simple mechanism for the increased heat sensitivity of malignant cells over normal cells in terms of intracellular heat conductivity (in the vicinity of 44° to 45°C). If it is assumed that in hyperthermia treatment, malignant cells and normal cells are heated to the same temperature and if it is assumed that the metabolic rates in these cells are roughly equal (but obviously not identical in the two types of cells), it is seen that because of the greater amount of ordinary water in the malignant cells, the local, internal temperature and likely the "internal structural temperature" of the malignant cells may be notably higher than that of the normal cell. Recall that the malignant cell is generally characterized by a considerable increase in the amount of total water and this water is undoubtedly less structured (more bulklike) than the water in normal cells. If, indeed, the ordered water possesses higher thermal conductivities as suggested by Metsik and Aidanova (1966), the normal cell will then be able to dissipate (by thermal conduction) the energy produced, faster than the malignant cell. Thus, the malignant cell will be subject internally to a somewhat higher, local temperature. It may be by this mechanism that the heat sensitivity of the malignant cells is enhanced.

Finally, assuming again that the rate of energy production is approximately equal in normal and malignant cells, assume also the heat conductivities may differ by a factor of 70 between ordered water (in normal cells) and bulk water (in malignant cells), based on Metsik and Aidanova's data, rather large differences in internal temperatures may then be expected for the two types of cells. Spanner (1954) has considered the relation between the "heat of transfer" and the equivalent "osmotic" pressure of the cells. The present author does not necessarily accept the de-

velopment on which Spanner's estimates are made; however, if Spanner's calculations are correct to an order of magnitude, it is interesting that a temperature difference of  $1^{\circ}\text{C}$  produces an osmotic (thermomolecular pressure effect) pressure difference of about 130 atm, where  $\Delta P/\Delta T \approx -132 \text{ atm}/^{\circ}\text{C}$  (note the minus sign!). Thus, a difference in temperature as slight as  $0.01^{\circ}\text{C}$  may then be expected to cause changes in the rate of water permeation (driven by hydrostatic forces) equivalent to a pressure well over 1 atm. Considering that the differences in heat conductivity may be as large as one and even two orders of magnitude, temperature differentials of the order of  $0.01^{\circ}\text{C}$  are not at all unlikely within any given system of the cells presumed to be in isothermal (and isoosmolal) equilibrium.

In connection with the role of water in malignancy, attention is called to an article by Apffel and Peters (1969). These authors discussed the role of hydration of various macromolecules, in particular the glycoproteins, first noting that the glycoproteins may have distinctly varying capacities to bind water. The degree of hydration appears to depend directly on the specific nature of the monosaccharides in the saccharides of the glycoproteins and of the polysaccharides. They discuss the general phenomenon of tissue hydration in malignancy, noting the increase in amounts of water, for instance in liver, during carcinogenesis and tumor growth. The authors also consider, qualitatively, the conformational changes that may result from differences in hydration, leading to significant differences of interaction at the surface of a cell or a macromolecule. Thus, "Hydration shells are proper to microorganisms and cells coated with sialoglycoproteins, and to a much lesser degree, to solvated or dispersed macromolecules of that nature. Adsorption of sialoglycoproteins to the surface of cells produces a unique situation where all the oligosaccharide chains protrude into the medium in a single, committed direction. Because of the close association thus brought about, there is a tendency toward gelification. The resulting semi-rigid shell of hydration water is tantamount to a volume forbidden to many solutes, depending on their size, charge and shape." Finally, Apffel and Peters call attention to the note by Good (1967) who also stressed the importance of hydration phenomena superimposed on charge-charge interactions between cells or between cells and macromolecular solutes. It is particularly interesting, as noted by Apffel and Peters (1969) that Good suggests that "at interfaces, as between cells and medium, the hydration of charged ions is more stable because there is less thermal gyration and less mechanical disturbance [see Good, 1967]." It should be observed in this context, however, that the type of hydration discussed by Apffel and Peters (and by Good, 1967) is primarily the very direct (and energetically strong) interactions

between ionic sites and the water molecules (or strong dipole-dipole interactions). In this chapter, stress is placed instead on the (very likely) much weaker, but possibly far more extensive hydration phenomena involving energetically only slightly different states of water. However, the basic idea regarding the stabilization of water structure near an interface suggested by Good (and quoted by Appfel and Peters) is the same as the one advocated in this chapter, namely, the reduced "thermal gyration and less mechanical disturbance" which results from the "momentum sink effect." No doubt, the study of the hydration of cell surfaces and macromolecular solutes will prove an important requirement for further advance in a detailed molecular understanding of the role of serum proteins in cancer.

## I. CELL ADHESION

Adhesion in general and cell adhesion in particular are extremely complex phenomena. Attention is called here only to the types of interactions that do not depend on attractive forces deriving from functional groups of the membrane materials. Pethica (1961) has reviewed the type of forces which may exist between cell surfaces; these forces include attractive as well as repulsive forces. In addition to such forces as chemical bonds between the opposing surfaces, ion pairing, image forces, and van der Waals forces, Pethica mentioned a "hindrance" to attraction due to steric barriers such as "inert capsules and solvated layers." Pethica points out that the latter do not actually represent a force "except that the entropy effect due to the mutual disordering of adsorbed layers, as the surface is approached, might be regarded as a force. The effect of adsorbed inert layers may more usually be to increase in range between otherwise active groups, and to attenuate the attractions between the surfaces to the point where reversible collisions can take place."

As discussed in Section III,C,1,a, Peschel and Adlfinger (1967) have determined the disjoining pressure between surfaces (specifically, quartz surfaces), and the major feature of their results may probably be generalized to cell surfaces, at least to the extent that anomalous temperature dependencies may be expected in any quantitative data on cell adhesion.

Pethica has considered the role of image forces in the attraction between cells in solution. For this purpose, the cells were modeled as two thick planes of material with much lower dielectric constants. Using the expression (applicable to a structureless dielectric material of dielectric constant  $\epsilon$ ), Pethica calculates the osmotic pressure ( $\pi_1$ ) from the free energy ( $\Delta G$ ) expression:

$$\Delta G = \frac{3e^2}{2\epsilon_1} \quad (2)$$

Hence

$$\pi_l = \pi \exp \left\{ - \frac{3e^2}{2\epsilon k T l} \right\}. \quad (3)$$

where  $e$ ,  $k$ ,  $T$  have their usual meanings, and  $l$  is the separation between the cells.

From this one obtains the net attractive force (per unit area) :

$$\pi - \pi_l = \pi \left[ 1 - \exp \left\{ - \frac{3e^2}{2\epsilon k T l} \right\} \right] \quad (4)$$

and the work (per unit area) required to bring the two opposing surfaces together (from  $\infty$  to  $y = 2$ )

$$W = \pi \int_y^\infty \left[ 1 - \exp \left\{ - \frac{3e^2}{\epsilon k T y} \right\} \right] dy \quad (5)$$

Although the present author does not take issue with the use of a very low value for the dielectric constant for the wall material, assuming it, for instance, to be a lipid (with  $\epsilon_w = 2$  or 3), it should be pointed out that  $\epsilon$  in Eqs. (2) through (5) is the dielectric constant for vicinal water, and this value is likely to be different from that of bulk water. Vastly different results will be obtained from those arrived at by Pethica [by graphic integration of Eq. (5)], since the "true" value for  $\epsilon$  may possibly be an order of magnitude lower than the value for bulk water (see the discussion in Section IV,A,6 where Derjaguin quotes values for  $\epsilon$  of  $\approx 8$  to 10 near interfaces). Again returning to Eqs. (4) and (5) and recalling the abrupt changes which have been observed in dielectric constants for vicinal water, it is not surprising that cell adhesion may show notable anomalies as a function of temperature. Pethica has suggested that only for separations of about 100 Å will the majority of forces considered, including the van der Waals forces, play a notable role. However, it is the contention of the present author that because the properties of water often show anomalies which appear to extend over as much as 1000 (to 10,000) Å, the structural changes in water may well play the dominant role in quantitative theories of cell adhesion. See also the article by Pethica and co-workers on possible ranges of structurally modified water near certain polymer surfaces (G. A. Johnson *et al.*, 1966). Finally see also the studies by Weiss (1967).

Abdullah (1967) has studied the aggregation of platelets *in vitro*. He notes that a distinction is usually made between platelet aggregation (interparticle association) and platelet adhesion (some standardized measure

of adsorption of platelets onto a standard glass surface). For both processes, Abdullah suggests that "some platelet aggregating substances act by increasing ice-likeness (ordered structure) of water around platelets" and that the active, initial process is followed by a "chain reaction" which results in the accretion of many layers of platelets onto the first-formed layer. Abdullah measured the effects of various nonelectrolytes on platelet suspensions, following the degree of aggregation optically. Very interesting results were obtained with a number of normal aliphatic alcohols (pentanol, hexanol, octanol, and decanol), two tetraalkylammonium salts, and argon and xenon (in oxygen-rich mixtures). From the data obtained, Abdullah suggested that water becomes ordered in the vicinity of an interface and this ordering acts as an "entropic trigger" which carries the system (i.e., the platelets or the platelets-glass interface) over a small potential energy barrier; in other words, the entropy change decreases the internal energy, allowing the net entropy of the system to increase.

Garvin (1968) has reported some very unusual temperature dependences for cell adhesion, specifically the "recovery" from adsorption onto solid surfaces of polymorphonuclear neutrophiles in human blood. Garvin noted that above 45°C the percent recovery increases almost linearly from 0 to 100% over less than 4°C. In other words, above 45°C there is a very rapid decrease in the tendency for the neutrophiles to adhere to the solid substrate. This suggests that the phenomenon of cell adhesion (and cell-cell interaction) may be influenced drastically by the structural change of the vicinal water around the cell (in this case, the neutrophile) surface at 45°C.

#### J. THERMAL HYSTERESIS EFFECTS

The role of time is one of the essential differences between the study of the thermodynamics of purely physicochemical systems (however complex they may be on the molecular level) and the study of biological systems. With biological systems, measurements as functions of temperature and pressure as independent variables are also invariably measurements of the same parameters as a function of time. At best, steady-state may attain; more often, growth or "decay" occur simultaneously. In principle, we can allow for the effects of time, but effects for which corrections cannot be made may occur when, for instance, both time and temperature change.

Memory effects in physicochemical systems have rarely attracted much attention as the presence of hysteresis invariably suggests lack of rapid approach to equilibrium and thus prevents true equilibrium thermody-

namic parameters to be measured. Yet, in kinetic studies, memory effects, or at least time-dependent behavior, is noted in some instances. Characteristically, practically all liquids, water in particular, may be significantly supercooled, whereas the ice lattice (or crystalline hydrates) apparently never superheats. Clay suspensions, once agitated, may "reset" at rest or at low shearing rates, whereas an initial disturbance can lead to the immediate disruption of the prevalent structure (which is causing the gel rigidity).

Recently, the present author and his co-workers have had ample opportunity to note strong time-dependent variations. Among these have been memory effects (or at least, time-dependent effects) in the properties of membranes. Thus, thermal anomalies have, from time to time, been observed in membrane properties when studied during slow heating—either through discrete increments of temperature or using continuously variable temperatures. Likewise, Kerr (1970), working in the author's laboratory, has observed thermal anomalies in the viscous damping of a water-filled vibrating quartz capillary. The anomalies are particularly pronounced during heating. The anomalies are sometimes completely absent (or notably displaced) upon repeating the measurements with decreasing temperatures. The most significant contribution, however, to the study of the possible existence of thermal hysteresis has come from the work by Bach (1971). Bach observed a memory effect in the structural properties of water deposited on a silver (or silver oxide) surface, using a differential thermal analyzer (DTA), and on glass, using a vapor phase osmometer in a differential manner.

It is not difficult to propose an explanation for time-dependent effects. As temperature is increased and thermal energy thus enhanced, any ordered matrix or array is readily disturbed into a more disordered state (i.e., a state of higher entropy). However, upon cooling, removal of a "like amount" of thermal energy does not readily cause a reordering of the system into the original order of the crystalline lattice. It is obviously far easier to disrupt and disorder a lattice than to perform the converse: to induce a specific order by merely lowering the available thermal energy fluctuation. Characteristically, thermal hysteresis phenomena have been observed especially with aqueous systems near interfaces (although supercooling does illustrate a bulk phenomenon of similar type). Near 15° and 30°C, for instance, water in biological systems (or at or near almost any aqueous interface) will undergo a phase transition, as discussed in previous sections. However, since an increase in temperature (for instance, from 28° to 33°C) will have resulted in a disruption of a structured matrix, it is possible, and sometimes likely, that a similar decrease in temperature may not reversibly lead to the "same" change in biological functions—at least,

not the same change at the same rate of change. Thus, thermal hysteresis must be expected in living systems also.

In summary, near a biological interface, water structures are stabilized which differ from the bulk structures. Disruption of these structures by increasing the temperature is readily achieved. However, structures that are stable at low temperature may not readily be reformed upon lowering the temperature; thus, the possibility exists for a significant lack of "symmetry" in the behavior of biological systems under temperature cycling. The attention of the experimental biologist to this possibility may likely prove rewarding. It is also of interest to note that the clathrate formed by hydroquinone and argon is "stable" (or, rather, may be kept in a bottle nearly indefinitely) once formed, although at room temperature its (equilibrium) vapor pressure is several atmospheres. The reason for this (meta) stability is the high energy of activation required to break a number of H-bonds in this hydroquinone lattice in order to release the trapped argon (see van der Waals and Platteeuw, 1959).

It is interesting that in many studies, particularly on biological system, the experimentally observed errors often tend to be larger in the vicinity of the temperatures of the thermal anomalies. Based on the studies by Bach (1969), Kerr (1970), and Thorhaug (1971) (all formerly working in the author's laboratory), it is suggested that this may be related to thermal hysteresis effects. Bach, in particular, has proposed that whether or not the system has been cooled or heated immediately prior to an experiment may influence the physical states attained. Thus, the possibility exists that a particular experiment near the temperature of one of the thermal anomalies may find the system in question in one of two states, corresponding, respectively, to either the structure which is stable above the transition temperature or the structure stable in the lower temperature range. Since these states will have different properties (for instance, reaction rates), it is not to be wondered at that the scatter in these cases occasionally are larger than toward the middle of each temperature interval.

#### K. THERMAL POLLUTION

The general question of thermal pollution is obviously not immediately related to the specific discussion of the structural and functional role of water near the cell surface (and in biological systems in general). Furthermore, the question of thermal pollution is an exceedingly complicated one, but not merely in the ordinary sense of complications as they are encountered in the study of any biological phenomenon, say, metabolism. In the case of thermal pollution, factors enter which are extraneous to a con-

ceptually homogeneous approach to the problem. Thus, as an example, the effects of elevated temperatures will influence the entire life cycle of any of the multitude of organisms making up the ecological network and include as well additional "external" factors such as environmental temperature fluctuations (frequency and amplitude of variations) and attendant changes due to additional stresses such as salinity fluctuations, chemical pollutants, and politicians. However, one specific aspect of thermal effects on the structure and properties of water near interfaces may play a singularly important role in determining the overall response of the entire ecosystem. What is implied here obviously is the abrupt and likely relatively invariant constraints imposed on any biological system due to the sudden changes in vicinal water structure at the temperatures of the thermal anomalies; hence, from this point (and this point only) is discussed the more obvious aspects of the possible existence of guidelines for allowable thermal pollution limits.

Fundamental to the problem of delineating permissible temperature intervals for biological organisms—a problem of crucial significance in any thermal pollution study—is the simple statement (Drost-Hansen, 1965a) that "If we are correct in assessing the importance of the structural changes in water for the behavior of biologic systems, it may be possible to delineate ranges of environmental temperatures that are conducive to life." It is, indeed, this idea which was elaborated upon subsequently in the paper by the present author (Drost-Hansen, 1969c) on thermal pollution limits.

In connection with the effects of temperature on biological systems in nature as distinct from laboratory studies, we must take into account the effects of varying temperature. This undoubtedly plays a crucial role, as is already known from both marine biological studies as well as physiological studies, for instance, on land plants. The overall dynamics are further complicated by variations in light intensity, availability of inorganic nutrients, etc. The question here is whether or not it is more appropriate to be concerned with the extremes of temperature rather than the average temperatures. Certainly, a "steady" temperature of 28°C could conceivably be compatible with growth and reproduction of an organism, but even relatively short-time excursions of  $\pm 4^\circ$  from this average (to temperatures between 24° and 32°C) might lead to catastrophic results in the narrow temperature range from, say, 30° to 32°C.

For marine fishes, the probably critical nature of temperatures around 30°C was carefully reviewed by DeSylva (1969).

Elsewhere the present author (Drost-Hansen, 1969c) has emphasized that in thermal pollution studies it is necessary to be concerned with the effects of temperature on each of the different stages of life development.

A simple example of the different requirements for optimal development is illustrated in the study by Calabrese (1969). In this study the effects of the salinity and temperature on some marine bivalves were studied in considerable detail. Specifically, Calabrese studied the effects of temperature and salinity on the development of embryos and larvae of *Mulinia lateralis* over wide ranges of the two variables. The percent of embryos developing normally shows a notable peak as a function of salinity at approximately 25 ppt. This sensitivity to electrolytes is paralleled with a notable maximum in the number of embryos which developed normally as a function of temperature. The survival of larvae and the number of eggs developing normally both showed maxima in the range between 15° and 30°C with precipitous decreases in normal development above 30°C. It is interesting also that the percent survival of the larvae as a function of the combined effects of salinity and temperature shows a rather wide range of survival: between temperatures of 7.5° and 27.5°C, and for salinities ranging as high as 35 ppt and as low as (10 to) 15 ppt. However, the percent increase in the mean length of these larvae as a function of the change in the same parameters showed a notably more restricted domain of optimum development, namely, between temperatures of 7.5° and 22.5°C and salinities between 20 and 35 ppt.

The abrupt changes in biological functioning, which have been mentioned in this chapter, near the temperatures of the thermal anomalies likely play a crucial role in thermal pollution. The literature provides a vast number of such examples of abrupt changes near 15° and 30°C. See, for instance, the cases discussed in the paper on thermal pollution by the present author (Drost-Hansen, 1969c). Figure 30 shows the percentage of normal development, compared to the development of major anomalies, in the frog (*Rana cyanophlyctis*). It is seen that, above approximately 33° and below 15°C, none of the progeny develops normally, whereas 100% normal development occurs between approximately 21° and 31°C. Undoubtedly this is not a unique example. Compare, for instance, the discussion in Section VI,D,2 on rates of mutation (chromosome aberrations). Furthermore, it should be stressed again that the effects of temperature in an ecological system must be fully concerned with the effects of temperature on *all* stages of the life cycle, ranging from the egg and sperm stages through the development of mature individuals (Drost-Hansen, 1969c): "obviously, even if only *one* life stage is sensitive to the temperature changes around the thermal anomalies, the ecological significance may be great."

In summary, then, it is suggested that significant and, in fact, possibly disastrous results may occur to the ecology of a particular area should the temperature for any length of time exceed the temperature of one of the

thermal anomalies (the anomaly which occurs above the range of optimum temperature for the majority of organisms in that locale). This suggests that it may be possible, on the basis of purely physicochemical observations, to propose rather clearly delineated limits for thermal pollution. This is particularly true in cases such as in subtropical and tropical climates where the temperature may already be close to 30°C.

## VII. Review and Conclusions

The status of our current understanding of the structure of water and aqueous solutions was reviewed in Section II of the present chapter. It is

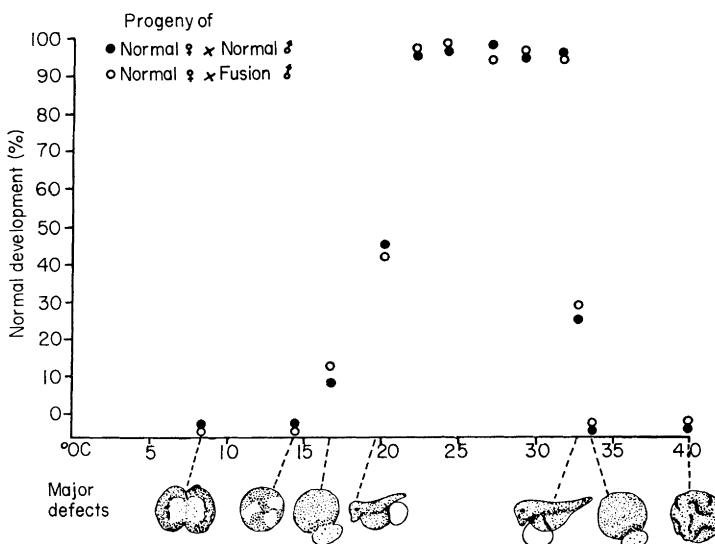


FIG. 30. Development of major anomalies in the frog (*Rana cyanophlyctis*) as a function of temperature. (Data by DasGupta and Grewal, 1968.)

still not possible to decide unequivocally if the structure of water can best be represented by a "continuum model" or a model invoking structural elements. The evidence in Section III suggests that vicinal water likely consists of or includes structured entities. For aqueous solutions, much of the available evidence, especially for solutions of nonaqueous electrolytes, also suggests that the water in such solutions may be structured. However, the brief review of the properties of aqueous alcohol solutions amply demonstrates that even such relatively "simple" systems are exceedingly

difficult to interpret in terms of structure. This, together with the general lack of a definitive theory for the structure of water itself, makes it difficult to speculate on the structure of water in biological systems. The present author contends—but certainly cannot prove—that Ice-Ih-like elements are not likely to exist in bulk water and aqueous solutions (nor in water near interfaces). On the other hand, some evidence does exist for clathrate formation in solution, especially near interfaces and likely in the water of the biological systems. Stress has been placed on the fact that “ordering” and “structuring” in aqueous systems implies the existence of some specified geometric arrangement of the water molecules, but it also (and perhaps in particular) implies enhanced temporal stability. Thus, structuring may simply mean an increase in average lifetime of a particular type of “flickering cluster” or other structured entity, by a few (or more) orders of magnitude (say, from  $10^{-11}$  to  $10^{-9}$  or  $10^{-8}$  second).

A number of examples of thermal anomalies in the properties of vicinal water were discussed. These anomalies likely reflect the existence of cooperative phenomena such as higher-order phase transitions. Since order-disorder require the existence of large structured entities in order to exhibit cooperative properties, the thermal anomalies thus reveal (notably) enhanced ordering in water adjacent to an interface.

The more-or-less abrupt thermal anomalies in the properties of vicinal water indicate that for large surface-to-volume ratio systems, great caution must be exercised in attempting interpretations of data in terms of, for instance, Arrhenius- or Eyring-type equations. This was discussed particularly in connection with the careful and closely spaced data reported for the diffusion coefficient of thiourea (Section III,C) and further illustrated in the discussion of Bangham's permeability studies (Section V,C,9).

Very tentative speculations were put forward regarding the nature of possible structured elements near interfaces. An apparent paradox was also stressed; namely, that it appears that thermal anomalies occur in the properties of vicinal water at almost identical temperature ranges regardless of the specific nature of the material in contact with the water. Specifically, in the case of water near various “solid interfaces” (ranging from some types of silica surfaces to certain macromolecules), the detailed chemical nature of the substrate appears to play, at most, a minor (but not negligible) role. This was interpreted as the result of the substrate acting as a momentum sink for thermal fluctuations which, in the case of bulk water, would have disrupted the particular structural arrangement present. The vicinal water thus gains stability merely by the proximity to the solid. Various aspects of the structure of water in biological systems were reviewed in Section IV. It was stressed that even in those cases where there

appears considerable evidence for order and structure, it must be kept in mind that a dynamic situation prevails.

A review was given in Section IV of Hechter's proposed "dualistic theory," invoking the existence of both transmembrane pumps (driven by metabolic energy) and the more-or-less complete ordering of the intracellular water. Possible structuring of intracellular water has also been proposed, for instance, by Szent-Györgyi, by Jacobson, and by Ling, and, more recently, by Hazlewood and co-workers, by Cope, and by Fritz and Swift (based on NMR studies). One particular facet is stressed by the present author, namely, the likely important role of mutual interactions between solvent and substrate. The point is made (and referred to in several places in this chapter) that within a certain temperature region primarily only one structure of vicinal water is stable. This structure may, in turn, impose restrictions on the substrate to which it is adjacent and, indeed, in some cases, possibly play the dominant role in determining the conformation (particularly of macromolecules in solution). Conversely, some specific structural characteristics of the substrate may affect the detailed, structural characteristics of the vicinal water. A brief review of clathrates was presented and the likely occurrence of these as structured elements of vicinal water was discussed. It was mentioned that in ordered systems of water (especially ice), some reaction rates may be enhanced, although it appears too early to make any definite conclusions as to the importance of this phenomenon in biological systems.

The last section of the chapter illustrates that vicinal water notably affects a great many functions of actual biological systems and the question, therefore, arises as to the possible "sites of action" of these water structure effects. A number of possibilities were considered, although far from exhausting the possible number of such "sensitive sites." Among the more likely sites of water structure effects, various solutes were mentioned [ranging from low molecular, organic nonelectrolytes, gel-forming materials, such as polysaccharides, to peptides and nucleotides (including RNA and DNA), proteins, and, specifically, enzymes]. Other possible sites discussed were the lipids and particularly the lipoproteins, especially in membranes. The evidence for structured elements of water in membranes was reviewed briefly, including some examples of thermal anomalies in membrane properties. The difficulties in distinguishing between ordered water near membranes and in the pores of membranes (if/or where such exist) was discussed, as were the effects of unstirred layers. Among other possible sites of water structure effects, the functioning of nerves and finally, muscles was mentioned. In connection with the discussion of water structure near various macromolecules—mimicking biomolecules—the study by Glasel was considered in some detail. His results are particularly

important as they suggest that some macromolecules may be able to order water structure vicinal to their surface, whereas other macromolecular solutes appear to lack this ability. Some of the materials that do possess relatively extensive water structures may exhibit this property only over a limited pH range. These findings are especially pertinent in connection with the discussion of anomalies in biological systems—although there is strong evidence that thermal anomalies in vicinal water occur and are reflected in the behavior of many biological systems, it is equally certain that other biological systems do not show evidence of thermal anomalies. If the “controlling factor” in the latter systems is determined by the type of biomolecules with little or no vicinal water structure, the absence of thermal anomalies in these systems is easily understood.

Both in the discussion of proteins and in the discussion of the hemolysis of the erythrocyte membrane, the enthalpy–entropy compensation phenomenon—most recently discussed in detail by Lumry and Rajender—was considered in some detail. It appears that the compensation phenomenon reveals the dominant role of the solvent structure and, presumably, the vicinal water structure in particular. This role was further emphasized and illustrated through examples of marked anomalies in the temperature effects on enzymic rates. It is contended by the present author, although not proven, that the anomalies observed are often far too abrupt to be accounted for by any reasonable extension of the idea of competing simultaneous and/or consecutive reactions. Cold inactivation (the tendency for cold denaturation of some proteins at low temperature) was also discussed and seen as a possible result of the effects of the stabilized vicinal water structure (for instance, below 15°C) imposing conformational restraints on the macromolecules in solution.

In the final part of this chapter, the functional role of water in actual biological systems was examined. A discussion was presented of optimal and lethal temperatures in terms of domains of stability of structured vicinal water and further examples of abrupt anomalies in biological systems were described. Here, and particularly in connection with the hyperthermia treatment of cancer, the role of the increased heat conductivity of ordered water (compared to bulk water) was stressed—based on the measurements of heat conductivity of vicinal water between mica plates (showing notable effects over distances of the order of 1000 Å). Control of germination and vernalization was also discussed in terms of nonspecific effects related to the general changes in vicinal water structure and the constraints thus imposed upon, for instance, some controlling enzyme or membrane system. Thermal adaptation and, particularly, the rate of genetic damage as a function of temperature were also discussed in terms of changes in vicinal water structure at the critical temperature. This in-

formation, in turn, was applied to paleozoogeographic studies, based on the assumption that the thermal anomalies likely represent truly "time invariant" boundaries.

The problem of narcosis, particularly the theories of Pauling and Miller, was reviewed briefly. A "pressure anomaly" in the solubility of gases was reported by Schröder, who noted the existence of abrupt changes in gas solubility at around 20 atm. This pressure corresponds to depth in the sea within easy reach of present-day deep diving technology. Hypothermia was discussed in general terms and attention called to the frequent occurrence of very drastic physiological changes around 29° to 32°C. These effects were observed also in the physiological processes of hibernating animals. Further cooling, eventually resulting in the freezing of cells and tissue, was discussed briefly in the section on cryobiology. Finally, hyperthermia was discussed in connection with the lethal effects of temperatures near 45°C (for mammals and birds) and the use of hyperthermia in the treatment of malignancies. The enhanced sensitivity to high temperatures of malignant cells does correlate extremely well with the structural change in water near 44° to 45°C, although only tentative mechanisms can be suggested for the specific selectivity. The problem of cell adhesion was mentioned briefly and again seen as a matter of interaction between neighboring vicinal water structures. Thermal hysteresis effects were also discussed and attention called to the likely biological importance of this generally ignored subject. The effects of structural changes on biological systems were finally related to thermal pollution problems, and it was proposed that it may be possible, on the basis of data gathered to date, to delineate allowable thermal pollution limits.

### VIII. Summary

The purpose of this chapter is (a) to call attention to the fact that water near interfaces frequently appears to undergo notable changes in properties and structure at a number of discrete temperature ranges (namely, near 13–16°, 29–32°, 44–46°, and 60–62°C) and (b) to attempt to correlate these changes with abrupt unexpected changes in the functioning of biological systems near those temperatures. The anomalies in the properties of vicinal water are most likely caused by higher-order phase transitions, due to cooperative processes. It appears that vicinal water structures, giving rise to thermal anomalies, may occur adjacent to highly dissimilar types of surfaces, including some (but not all) ionic, strongly dipolar, and completely nonpolar interfaces. The existence of at least four

thermal anomalies suggests the occurrence of a minimum of five different vicinal water structures. The cooperative processes probably reflect relatively long-range ordering of the water molecules vicinal to the interface. It appears that very small energy differences between different structural arrangements may play an important role. The effect of the vicinal structuring appears to influence the activities of both the ions and the water in cells; the implications of this phenomenon for the problem of active transport are briefly discussed. The likely "sites of action" of the structural changes of the vicinal water are discussed (for instance, in terms of water interactions with proteins, enzymes, membranes, etc.). Finally, a number of examples are reviewed where it appears that the vicinal water plays a dominant role in the functional behavior of the biological systems. Some difficulties in interpretations have been considered, such as the complexities caused by lipid transformations (known to occur in completely anhydrous lipid systems); however, in spite of obvious exceptions (and some possible alternate mechanisms in individual, specific cases) it appears inescapable that a cause-effect relationship exists between thermal anomalies in the properties of vicinal water (due to structural changes) and anomalies in a large number of vastly different biological systems.

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