

# Effect of water and other solvents on the structure of biopolymers<sup>1</sup>

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AS A SOLVENT, water plays a unique role in biological systems and the structure of biopolymers. It stabilizes the double helical form of deoxyribonucleic acid (DNA) and certain conformations of proteins, and brings about protein aggregation and the formation of micelle-like structures such as the lipoprotein membranes of mitochondria. Certain other processes, such as the effect of radiation on living systems, are also influenced by water. For instance the photodimerization of thymines in DNA during ultraviolet irradiation is greatly enhanced in water (3, 4, 33, 35, 40). The action of water on biopolymers is usually reversed by other solvents.

The study of solvent-dependent interactions in biopolymers is expected to help explain not only phenomena at ordinary temperatures but those occurring in biopolymer solutions and living systems at the temperatures pertinent to cryobiology.

The interactions mentioned have one property in common. The more or less non-polar molecules or groups, each surrounded by water, tend to come together, reducing the number of water-solute contacts. This phenomenon has been known as hydrophobic bonding (19).

The cause and nature of hydrophobic bonding has been the subject of several investigations and reviews (19, 28, 31, 34, 42). The concepts underlying it have developed along two lines. The first was to assume strong solute-solute interaction arising mainly from van der Waals forces, or sometimes from hydrogen bonding or any ionic group interaction. It has been shown that solute-solute H-bond formation is almost negligible in water (5, 22) since water-solute hydrogen bonds, which have to be broken first, are of comparable magnitude. Klotz (22) has also shown that ionic group interactions are not the main ones involved in biopolymers. Solute-solute van der Waal forces are very important, as shown for example by the calculations of DeVoe and Tinoco (5) for the case of the bases in the DNA helix. However,

these cannot be considered alone in connection with macromolecule conformation because, in solution, interactions involving the solvent are at least as important. The conformation of a biopolymer in water is the result of the small free-energy difference between these large effects.

The second line of thought associates hydrophobic bonding with some change in the state of the water surrounding the non-polar groups. This change tends to drive the non-polar species together. The work of Frank and Evans (12) on the solubility of non-polar gases in water has supported this view. Frank and Evans compared the partial molal entropy of a non-polar gas going into water solution with that going into non-polar solvents. The decrease on going into water was greater by amounts over 10 e.u./mole of gas than that on going into non-polar solvents (1 e.u.  $\equiv$  1 cal/ $^{\circ}$ K). They attributed this to an increase in the structure of water and introduced the concept of an "iceberg" forming around the solute. In support of this was the fact that these gases formed stable, solid gas hydrates. In solid gas hydrates water forms cages of known shapes and sizes into which the solute fits (37). Mainly two kinds of structures are known, each with two types of cavities, although other structures have also been discovered lately (18). The important point is that water molecules still maintain a four-hydrogen-bonded structure in the cages. If a somewhat similar cage forms around the non-polar gas molecule in solution, it might explain the loss in entropy observed (in this view a shift toward more tetrahedrally bonded water).

Kauzmann (19) studied the solubility of some hydrocarbons in water. Considering only unitary entropy, i.e., the entropy contribution other than that coming from ideal mixing, he found a decrease of about 20 e.u. on transferring a mole of a solute such as benzene from a pure hydrocarbon liquid into water. This he attributed to some structural restrictions on the water surrounding the solute. The structural entropy decrease is also incorporated in the theoretical treatment of Némethy and Scheraga of the thermodynamic properties of aqueous

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solutions of hydrocarbons (27) extended to hydrophobic bonding in proteins (28).

The second line of approach thus attributes hydrophobic bonding to structural restrictions on the water molecules around the solute. If two such solutes came together in one cavity, there would be a decrease in the number of restricted water molecules around them. This action would result in a positive  $\Delta S$  contribution and thus a negative  $\Delta F$  for the association of the two non-polar species, which may be the driving force involved in hydrophobic bonding.

No doubt structural restrictions are involved, since water is a highly polar and structuring liquid. But there are other liquids more polar and quite structuring, such as formamide, which behave differently. Though water keeps the DNA helix stable, formamide denatures it.

Many free-energy contributions arise from the solvent when two biopolymer groups are brought together. These include solvation energies of associated and unassociated groups, reduction of electrostatic and dispersion forces, and entropies of mixing of both the native and the denatured forms of the biopolymer with the solvent and other effects. Structural changes in the solvent immediately surrounding the biopolymer constitute only one of these effects. The quantitative estimate of this last effect is difficult. As pointed out by Richards (31), the rigid orientation and environment of a non-polar group in a biopolymer are different from those in a non-polar liquid. The thermodynamic parameters derived from the transfer of a non-polar molecule from a non-polar liquid into water (19) include the properties of the non-polar liquid and may therefore not correspond to the properties of the water layer surrounding the biopolymer groups. The theory of Némethy and Scheraga (27, 28) considers in detail the statistical behavior of the water molecules surrounding a hydrocarbon or a hydrocarbon-like protein side chain. However it uses semi-empirical parameters rather than independently known molecular or macroscopic properties and would be difficult to extend to more general types of hydrophobic bonds (such as the ones in DNA involving the quite polar nucleotide bases) and to the action of other solvents.

In this paper we attempt to elucidate the special role of water—taken as a difference in degree—by a quantitative comparison of effects involved in the association of biopolymer side chains in different solvents. We specifically address ourselves to the following questions:

- 1) What are the free-energy terms that contribute to the free-energy difference between the folded or stacked form of a biopolymer and the extended (random coil, denatured) form? How does each of these vary from solvent to solvent?
- 2) Is there any one crucial physical property of the solvent involved in solvent forces that also gives water its unique role?
- 3) Are there forces in other solvents analogous to hydrophobic forces in water? What would be the relative strengths of these?

4) What are the thermodynamic properties of a water layer surrounding the side groups of a biopolymer?

5) Is hydrophobic bonding still operative if the side groups are not entirely non-polar but have sizable dipoles like the nucleotide bases of DNA?

In the next section of this paper we take the stability of the DNA double helix as our specific case. The more or less regular primary structure of a polynucleotide makes it a simpler case to be examined than that of proteins, in which side chains and their interactions are varied. The DNA case is a good one not only because the bases have dipoles and so represent a more general view of hydrophobic bonding (*question 5*), but also because systematic experiments have been made on its stability in different solvents with widely varying properties (2, 6, 17, 23, 36). The first part of the treatment considers the solvent mostly as a continuum with no structural changes in it surrounding the solute. Though it is difficult to make absolute calculations for a given solvent, the relative magnitudes of various free-energy effects in different solvents are obtained. We find that the dominant effect comes from the energy needed to create a cavity in a solvent before placing a bulky solute in it. This already explains the stability of the helix in water as compared with other solvents, though the effect may be fortified by water structure changes. The theory orders pure solvents into a sequence of decreasing "solvophobic force." The predicted sequence has recently been confirmed, though tentatively, by the experiments of Wacker and Lodemann (private communication; for additional data obtained while this paper was in press see (38)) on the ultraviolet photodimerization of thymine-sugar-phosphate-sugar-thymine (TpT) in different solvents (section on THYMINE PHOTODIMERIZATION).

In the section on *Change of Water Structure Near Solutes* the properties of water adjacent to solutes are studied quantitatively by examination of the simplest aqueous solutions, those of small non-polar gases (Ne, Ar, Kr, CH<sub>4</sub>, C<sub>2</sub>H<sub>6</sub>, etc.) on the one hand, and the properties of water—"oil" interfaces on the other. The theory of the aqueous solutions is based on a molecular theory of solid gas hydrates (26, 37) and contains no arbitrary parameters. The results, presented in *Change of Water Structure Near Solutes*, yield average structural entropy (and enthalpy) values per H<sub>2</sub>O molecule next to a solute and may be used in conjunction with the other effects (section on *Macroscopic Treatment*) contributing to a hydrophobic bond.

The implications of the theory for the temperatures of cryobiology are presented in the DISCUSSION.

#### STABILITY OF DNA HELIX IN WATER

The DNA helix is stable in water at room temperature, moderate ionic strength, and neutral pH. Extremes of pH or ionic strength and high temperature cause its denaturation or separation into two random strands. This process is usually followed by observable changes in the physical properties of the polymer solution like

viscosity, specific rotation, ultraviolet absorption, sedimentation coefficient, and the like. Many reviews have dealt with the denaturation behavior of DNA (21, 25, 34, 42).

Addition of organic solvents like alcohols, formamide, formaldehyde, urea, and others to aqueous DNA solutions at room temperature also cause denaturation (2, 6, 17, 23, 36). Water seems to be unique in stabilizing the double helix at room temperature whereas other solvents denature it despite widely varying properties in relation to water.

To see the solvent property involved in bringing solutes together in water, we have considered as quantitatively as possible all the free energies of the interactions involved in the two significant structures, the random coils and helix, arising from the presence of water and the other pure solvents. Some of the results on DNA in pure solvents have already been summarized (33). Here we give also some additional results.

We picked as our standard solution a dilute one of DNA at room temperature, 0.1 M NaCl, and pH 7. Under these conditions the helix is stable in water but is denatured in the other solvents; but for a free-energy calculation, either the helix or the coils may be a hypothetical state. The solvents studied were water, methanol, ethanol, *n*-propanol, *n*-butanol, formamide, glycerol, and glycol.

We considered the following reaction



Taking this as a simple reaction in thermochemistry and considering only the significant structures, the coils and the perfect helix, we have

$$\Delta F_{(H-C)} = \Delta F_{(H-C)}^{\circ} + T(Z_H - Z_{c_1} - Z_{c_2}) \quad (1)$$

where

- $\Delta F_{(H-C)}$  = the total free-energy difference between the helix and two random coils in a given solvent
- $\Delta F_{(H-C)}^{\circ}$  = the standard free energy for the same reaction. It includes no contribution from entropy of mixing
- $TZ_H$  = the contribution to the free energy arising from the partial molal entropy of mixing of helix with solvent. For a small molecule in dilute solution where Henry's law is obeyed, this term would be  $RT \ln X_H$ , where  $X$  is the mole fraction, but not for a polymer.  $TZ_{c_i}$  are similar terms for the two single strands

To calculate the actual value of  $\Delta F_{(H-C)}$  for a given solvent, we would need a good estimate of  $\Delta F_{(H-C)}^{\circ}$  in vacuum. DeVoe and Tinoco (5) estimated  $\Delta H_{(H-C)}^{\circ}$  in vacuum. They used point dipole approximation for the interaction of the bases in the helix. As they expected and as the work of Bradley and colleagues (1) and our unpublished work show, electrostatic interactions beyond the dipole term are important. In addition the  $\Delta S_{(H-C)}^{\circ}$  (vacuum) is not yet known. However for our purpose we need to calculate only the relative  $\Delta F$  from one solvent

to another, therefore only the solvent contributions to  $\Delta F_{(H-C)}$ . The magnitudes are rough estimates and should be considered only from a comparative point of view for water and the other solvents.

#### Macroscopic Treatment

Macroscopic treatment considers no microscopic change in the structure of water or any of the other solvents around the DNA helix or coils. It considers the solvent as a continuum and deals only with its bulk properties. The picture we have is of a helix in a cylindrical cavity and of a random coil with the bases pointing away from each other into the solvent with a cavity in the solvent formed around each (Fig. 1). The relation of the macroscopic model to the microscopic picture in which individual solvent molecules are considered is given elsewhere (32, 33).

*Calculation of  $\Delta F_{(H-C)}^{\circ}$  (solvent effect).* The contributions to  $\Delta F_{(H-C)}^{\circ}$  coming from the solvent are of three types:

- $\Delta F_{(H-C)}^{\circ}$  (solvent effect) =  $\Delta F_{(H-C)}^{\circ}(c) + \Delta F_{(H-C)}^{\circ}(bs) + \Delta F_{(H-C)}^{\circ}(R) \quad (2)$
- $\Delta F_{(H-C)}^{\circ}(c)$  = the difference in free energy of creating a cylindrical cavity for the helix and spherical cavities for the bases in the coil
- $\Delta F_{(H-C)}^{\circ}(bs)$  = the difference in free energy of interaction of the bases in the helix and the bases in coils with the solvent around them
- $\Delta F_{(H-C)}^{\circ}(R)$  = the reduction in base-base and phosphate-phosphate interactions in the helix due to the presence of the surrounding solvent

In this treatment we consider only the  $\Delta F$  contributions coming from the bases in the two environments. We assume the environment around the backbone to be almost the same in both the helix and coils. The vacuum value of  $\Delta F_{(H-C)}^{\circ}$  is assumed to be a constant and to cancel in comparing solvents. This assumption is quite justified if one considers the hindrance of the internal motions of polymer by solvent molecules.

The calculation of  $\Delta F_{(H-C)}^{\circ}(c)$  is approximated by the free energy needed to create the given surface area of the bubble or the cylindrical cavity with surface

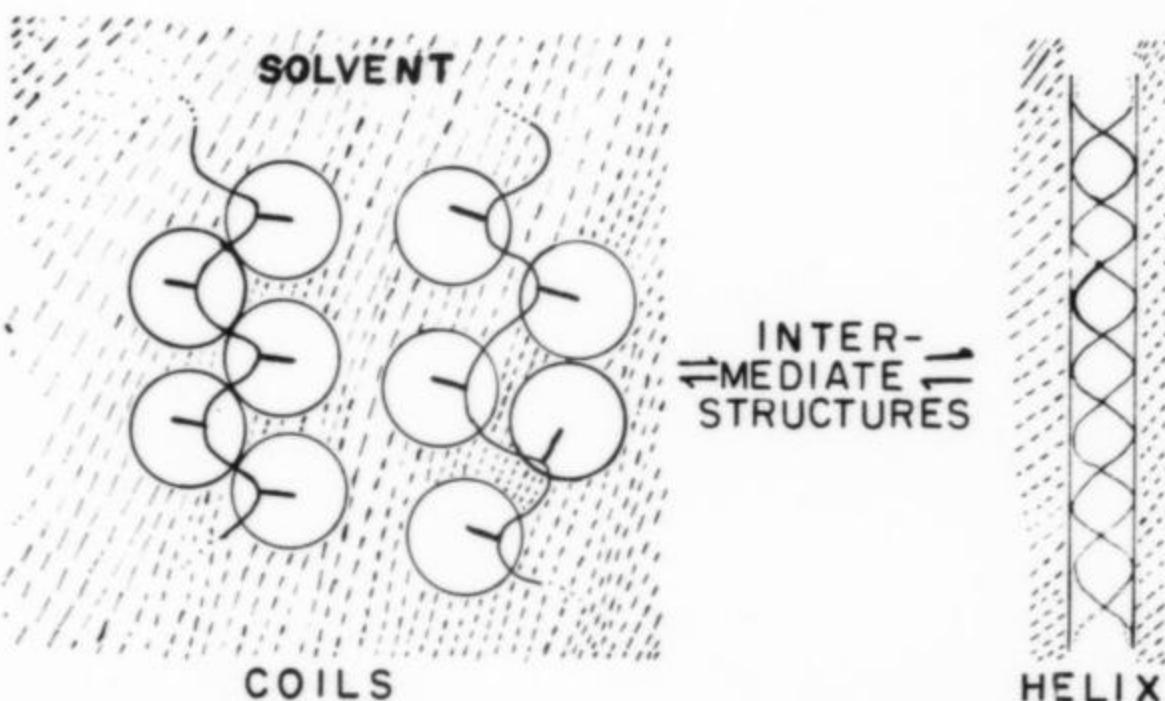


FIG. 1. Schematic representation of the helix and coil models in solution.

TABLE I.  $\Delta F^\circ_{(H-C)}$  (solvent effect) for the  $G - C$  sequence at 25°C

	$\begin{array}{c} \uparrow \\ G - C \\ \downarrow \end{array}$									
	kcal/Base Pair		Water	Formamide	Glycerol	Glycol	n-Butanol	n-Propanol	Ethanol	Methanol
$\Delta F^\circ$ (bs)			+44.5	+46.5	+49.9	+48.4	+41.1	+41.6	+42.0	+42.4
$\Delta F^\circ$ (R)			+4.1	+4.7	+4.7	+4.5	+4.5	+4.3	+4.2	+4.1
$\Delta F^\circ$ (c)			(-34.4)*					(-9.3)	(-9.7)	(-10.6)
			-37.7	-30.4	-33.1	-24.8	-12.7	-12.3	-11.5	-11.6
{Total $\Delta F^\circ_{(H-C)}$ (solvent effect)}			(+14.2)					(+36.6)	(+36.5)	(+35.9)
			+10.9	+20.8	+21.5	+28.1	+32.9	+33.6	+34.7	+34.9
{ $\Delta F^\circ_{(H-C)}$ (solvent effect)}		(o)						(+22.4)	(+22.3)	(+21.7)
{ $-\Delta F^\circ_{(H-C)}$ (solvent effect for water)}		o		+9.9	+10.6	+17.2	+22.0	+22.7	+23.8	+24.0

\* Brackets indicate values obtained with curvature-corrected surface tension.

tension values. It could also have been calculated microscopically from the number of base-solvent nearest neighbors and molecular interactions etc., but this would not be more accurate than the surface tension approximation (33).

We calculate  $\Delta F^\circ_{(H-C)}$  (bs) by considering a base interacting with the surrounding liquid by polarizing it with its dipole (29, 33, 39) and its van der Waals dispersion field (24, 33).

We obtained  $\Delta F^\circ_{(H-C)}$  (R) by using theories of effective dielectric constants for electrostatic interactions (20) and for dispersion forces (32) between bases immersed in a solvent (33). These calculations were summarized in a previous article which dealt also with the effect of solvents on the photochemistry of DNA (33).

Table I represents a sample calculation of the different contributions to  $\Delta F^\circ_{(H-C)}$  (solvent effect) for a polynucleotide with the sequence  $\begin{array}{c} \uparrow \\ G - C \\ \downarrow \end{array}$ . In estimating  $\Delta F^\circ_{(H-C)}$  (bs) part, parameters of  $3A^\circ$  and  $4A^\circ$  (suitable for reaction field model) for the radii of the two bases C and G, respectively, in the coil were used. The radii required in the Onsager reaction field model usually come out smaller than what would be expected from molecular dimensions (5). The helix-solvent interaction, based on a molecular hydration model built around the helix (32), was found negligible ( $\approx -0.3$  kcal per mole of base pair) and was assumed the same for all the solvents considered (33). For the  $\Delta F^\circ_{(H-C)}$  (c) calculation the radius of a base cavity in the coil was taken as  $5A^\circ$  and that of the helix cylinder as  $12.5A^\circ$ . These latter are dimensions expected from solute surface areas and mechanical models. These various cavity dimensions are the only parameters involved in the theory. Everything else is a property either of the bases or of the solvents known independently. Table I shows  $\Delta F^\circ_{(H-C)}$  (R) to be almost constant for all the solvents. The terms  $\Delta F^\circ_{(H-C)}$  (bs) and  $\Delta F^\circ_{(H-C)}$  (c) are dominant. The first is also quite constant and the  $\Delta F^\circ_{(H-C)}$  (c) is seen to be the main term responsible for the greater stability of the helix in water.

To see whether these conclusions depended on the cavity sizes chosen above, we investigated the dependence

of the various  $\Delta F$  contributions on the several cavity dimensions involved. The helix radius,  $12.5A^\circ$ , is not critical because the major terms in  $\Delta F_{(H-C)}$  (solvent effect) come from the random coils. Also  $\Delta F^\circ_{(H-C)}$  (R) is quite small and almost constant for all the solvents so it is necessary to look only at  $\widehat{\Delta F^\circ}_{(H-C)}$  (solvent effect)  $\equiv \Delta F^\circ_{(H-C)}$  (solvent effect)  $- \Delta F^\circ_{(H-C)}$  (R).

For the coils, two kinds of radii are involved: the effective reaction field radius,  $a$  (in  $\Delta F$  (bs)), of a given base and the actual solvent cavity radius,  $r$ . If the chosen  $a$  is kept fixed, and  $r$  is increased or decreased to the smallest reasonable value,  $r = a$  (a base has an actual radius  $> a$ ), the conclusions as to the relative denaturing ability of the solvents remain the same. This is also true if  $r$  is kept fixed, but  $a$  is increased. One may also set  $a = r$  for a given base and calculate  $\Delta F_{(H-C)}$  (solvent effect) as a function of this one parameter. The results are shown in Fig. 2. The actual magnitudes are of course quite strongly dependent on  $a$  but we see that the qualitative conclusion as to the relative effect of solvents in stabilizing the helix is valid down to the smallest ( $a = r \geq 3A^\circ$ ) reasonable base cavity.

In the previous article (33) the  $\Delta F^\circ_{(H-C)}$  (solvent effect) was split into its component  $\Delta H$  and  $\Delta S$  parts. It was shown that while the  $\Delta S$  part favored the helix in the other solvents compared with water, the  $\Delta H_{(H-C)}$  (c) was responsible for stabilizing the helix in water more than the other solvents.

We concluded that the great stabilizing power of water for the helix arises from the great energy needed to create a cavity in it. This stabilizes the polymer configuration in which the smallest total surface area of bases is exposed into the solvent—the helix in this case. This unique property of water, manifested in its high surface tension, is due to the strong cohesive energy of water molecules per unit volume. The association of non-polar solutes in water thus results from the great tendency of the large number of water molecules liberated from around the bases into the bulk liquid to stick together. Up to 80% of the  $H_2O$  molecules hydrating the bases in the single strands go into bulk water when the strands

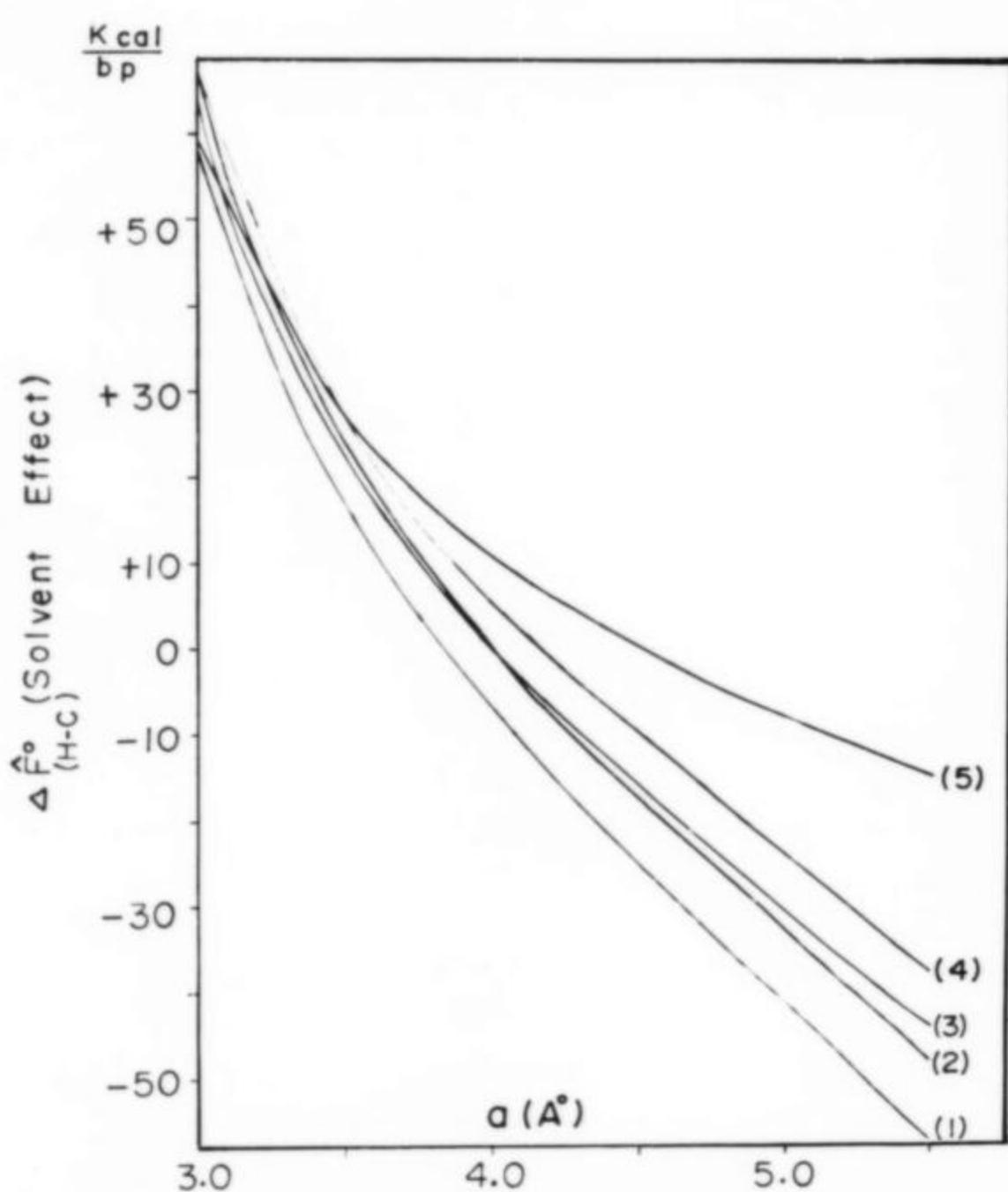


FIG. 2. Variation of  $\Delta F_{(H-C)}^{\circ}$  (solvent effect) with base cavity radius,  $a = r$ , in the DNA random coil at 25°C in different solvents. A cylindrical cavity of radius  $12.5 \text{ \AA}^{\circ}$  around the helix is used. Curves 1, 2, 3, 4, and 5 refer to water, glycerol, formamide, glycol, and *n*-butanol, respectively. Methanol, ethanol, and *n*-propanol curves lie above curve 5 within a range of 2.1 kcal at  $a = 5.5 \text{ \AA}^{\circ}$  to 2.5 kcal at  $a = 3.0 \text{ \AA}^{\circ}$ .

combine into a helix. The enthalpic part of surface tension is simply a measure of the energy difference between an  $\text{H}_2\text{O}$  on a cavity surface and one in the bulk.

Note that here the surface enthalpy refers to an empty cavity, not an interfacial value against a "base-like" liquid, because a single base unit sticking out into the solvent is surrounded by about 50 water molecules. Thus the solvent side is much closer to being in a macroscopic phase (as far as its enthalpy but not its entropy is concerned) than the solute side, which is a single molecule (31, 33).

The model given above can be pictured also in terms of another process by considering the free energy required in placing a DNA random coil from vacuum into water. This can be done in two steps: first, by creating all the empty cavities in water at the expense of some free energy ( $\Delta F(c)$ ); next, by placing the base units into each cavity. This recovers some of the free energy due to the attraction of a base to the walls ( $\Delta F(bs)$ ). A similar process gives the  $\Delta F$ 's for the helix (Fig. 1).

If one now tries to find another solvent with a surface tension like that of water or higher, one notices that perhaps the only such pure liquids are  $\text{H}_2\text{O}_2$  (liquid) and mercury! Thus water is indeed unique among the pertinent solvents. It is interesting that as pointed out to us (33) by Dr. A. D. Adler, this uniqueness of water with its unusually high surface tension and cohesive energy

density (heat of vaporization per milliliter) was noted as early as 1913 by L. J. Henderson (16). He was speculating about the unusual ability of water to rise in plants due to capillary action!

*Solvent - polymer entropy of mixing: calculation of  $T(Z_H - Z_{c_1} - Z_{c_2})$ .* In previous treatments on hydrophobic bonding (12, 19, 27) unitary free energy,  $\Delta F(u)$ , and unitary entropy,  $\Delta S(u)$ , were used. The unitary partial molal free energy of solute 2,  $F_2(u)$ , is defined by

$$\bar{F}_2 = \bar{F}_2(u) + RT \ln X_2 \quad (3)$$

The last term refers to the contribution coming from the partial molal entropy of mixing of the solute in the solution if the mixing is ideal. In the case of a polymer solute, the great difference in size between solvent and solute molecules prevents ideal mixing. Theories of entropy of mixing polymers with inert solvents have been developed in the field of polymer chemistry (10). We shall now consider a brief outline of the effect of the entropy of mixing on the free energies of native and denatured DNA forms in different solvents.

A DNA helix or coil in infinitely dilute solution tends to coil on itself, forming some sort of an effective sphere. Between such spheres are vast volumes of pure solvent. Two mixing terms appear here: mixing of the spheres as units with the solvent outside them, which we define as external mixing; and mixing of the segments of each polymer molecule in its "sphere" with the solvent inside the sphere, which we define as internal mixing.

The external entropy of mixing is estimated based on the treatment of Flory (11) for very dilute polymer solutions. In this type of treatment the solution is divided into cells each equal in volume to a solvent molecule making up a lattice. Now the (external) entropy of mixing between the large effective polymer spheres and the small solvent molecules is calculated by finding the number of ways the polymer spheres and solvent molecules can be placed on the lattice. Note again that the lattice is fine grained in the sense that all the cells are the size of a solvent molecule. Thus a polymer sphere placed somewhere in the solution will occupy several cells.

We compared the free energies of two hypothetical solutions, one in which all DNA is in helix form, the other in which all DNA is in single strands,  $c_1$  or  $c_2$  (eq. 1). Therefore we considered the entropy of mixing,  $-Z_H$ , of a helix with solvent and separately that of a single strand with solvent,  $-Z_{c_1}$  or  $-Z_{c_2}$ . Each of these has the external and internal mixing parts.

$$-Z_i = \Delta S_i(\text{ext}) + \Delta S_i(\text{int}) \quad (4)$$

The partial molal external entropy of mixing per "sphere," i.e., per polymer molecule,  $\Delta S_i(\text{ext})$  of the species  $i$  is given by

$$\Delta S_i(\text{ext}) = -R \left[ \ln \frac{N_i}{N_1 - 7N_i\rho} + \frac{1}{8} \ln \frac{N_1 - 7N_i\rho}{N_1 + N_i\rho} \right] \quad (5)$$

where

- $N_i$  = the number of "spheres" of species  $i$  in a total amount of solution
- $N_1$  = the number of solvent molecules outside the "spheres" in the solution
- $\rho_s = V_i/V_1$
- $V_i$  = volume of the "sphere" of the polymeric species  $i$
- $V_1$  = volume of a solvent molecule.

The above equation holds for each species of DNA by itself in solution, i.e., for  $i = H$  or  $c_1$  or  $c_2$ .  $N_i$  is calculated for helix or coils from the total DNA concentration in solution and the molecular weight of the DNA sample used, assuming all helix or all coils. To get  $N_1$  for one of the hypothetical solutions, the number of solvent molecules inside the spheres of that solution must be subtracted from the total number of solvent molecules. An effective polymer sphere  $V_i$  can be evaluated from measurements of viscosity or sedimentation coefficient of a solution in which the main species is  $i$ , or from light scattering (10). With such  $V_i$  estimated (see discussion below regarding internal mixing) the partial molal external entropy of mixing is found negligible for both helix and coils when expressed per base pair ( $T\Delta S \ll 1$  kcal/base pair).

Consider now the internal entropy of mixing. The mixing of the portions of a helix with its immediately surrounding solvent molecules (in  $V_H$ ) should be negligible because of the rigidity of the helical structure. In a single strand on the other hand, each segment, here a nucleotide unit, has considerable freedom of motion. Thus there will be a sizable amount of mixing of these units with the solvent molecules within the sphere, whose size is determined by the mean radius of gyration.

To get the  $\Delta S_i$  (int) one could use the concentrated polymer solution theory of Flory (10) if the segment and solvent molecule sizes were about the same. However for solvents of molecular volumes different from the nucleotide units—as it is the case for DNA in water—the theory gives unrealistic results. Therefore instead we assumed the nucleotide units to be distributed uniformly in the "effective sphere" of the coil. We then applied the dilute solution theory used above in the external mixing part. In the model coil used in the calculation, the average distance between two nucleotide units within the sphere is  $\sim 80A^\circ$  while a nucleotide unit has a radius of  $5A^\circ$ . Thus the dilute solution assumption is justified even within the sphere. With this theory we overestimate the internal entropy of mixing. The general conclusions drawn will thus be on the safe side.

In the external mixing part, the entropy of mixing of one polymer as a whole with a large amount of solvent is involved. The quantity per polymer is therefore the partial derivative of the total entropy of mixing with respect to solute concentration, i.e.,  $\Delta \bar{S}_i$ . In the entropy of internal mixing, on the other hand, the quantity per one polymer molecule is the total entropy in the sphere arising from the mixing of the more nearly comparable numbers of segments and solvent molecules. This entropy

is given therefore by the same formula which yielded equation 5, but before it is differentiated with respect to  $N_i$ , and with new meanings attached to the symbols. It is simply  $\Delta S = R \ln \Omega$ , with  $\Omega$  the number of ways of arranging random coil segments and  $n_1$  solvent molecules on a lattice making up the sphere of a coil. The formula is

$$\Delta S(\text{int})/\text{coil} = R \left[ \left( \frac{n_1 + m\rho_s}{8\rho_s} \right) \ln \left( \frac{n_1 + m\rho_s}{n_1 - 7m\rho_s} \right) + m \ln \left( \frac{n_1 - 7m\rho_s}{m} \right) \right] \quad (6)$$

where

- $m$  = the number of nucleotide units in the sphere, i.e., in one polymer molecule
- $n_1$  = the number of solvent molecules in the sphere
- $\rho_s = V_s/V_1$
- $V_s$  = the volume of a nucleotide unit
- $V_1$  = the volume of a solvent molecule

The  $m$  can be estimated from the molecular weights of the coil and the nucleotide unit.  $V_s$  is taken as a sphere of radius  $5A^\circ$ . The  $n_1$  depends on the difference between the volume of the sphere  $V_{c_1}$  or  $V_{c_2}$  and the volume occupied by the nucleotide units,  $mV_s$ . The quantity  $V_i$ , the volume of an effective polymer sphere in a given solvent, was involved also above in the  $\Delta \bar{S}_i$  (ext). The size of such a sphere will depend on the solvent used, the expansion of the mean size from the value in vacuum being expressed by a factor  $\alpha^3$ .

To get a reasonable idea about the relative magnitudes of  $\Delta S$  (int) in different solvents, we considered a poly U coil which has hardly any base stacking at 25°C (30). From the viscosity values of a sample of molecular weight  $1.9 \times 10^5$  of Richards and coworkers (30) a mean radius of  $216A^\circ$  was estimated. If we assume this same size sphere in all the solvents, equation 6 divided by  $m/2$  yields  $\Delta F_{(H-C)}(\text{int}) = -T\Delta S_{(H-C)}(\text{int}) \cong +T\Delta S_c(\text{int}) \cong T(Z_H - Z_{c_1} - Z_{c_2})$  given in Table 2 expressed per base pair. The  $V_1$  is obtained from the densities of the liquids at 25°C and  $m = 622$ .

It is seen that the variation is at the most 2 (kcal/mole base pair) from solvent to solvent. The trend is such that the mixing effects alone would make water destabilize the helix as compared with the other solvents. But these differences between water and the other solvents are too small compared with the differences in the main effects  $\Delta F_{(H-C)}^0$  (solvent effect) stabilizing the helix in water and which range from 10–25 kcal per mole of base pair as given previously in Table 1.

This conclusion is not dependent on the sample molecular weight or on the sphere radii that have been used in equation 6. If we change the volumes  $V_c = 4\pi/3 (216A^\circ)^3$  by a factor of 2.8 in either direction, keeping  $V_c$  still the same for all the solvents, the absolute magnitudes change between 7.1 and 11.6 (kcal per mole of base pair) but the variation between solvents is still

TABLE 2.  $\Delta F_{(H-C)}$  (internal mixing) at 25°C

Solvent	$V_1$ ( $\text{A}^3$ )	$\Delta F_{(H-C)}$ (int) (kcal/base pair)	Relative $\Delta F_{(H-C)}$ (int) ( $S-W$ ) (kcal/base pair)
Water	30.0	+10.3	0.0
Formamide	66.2	+9.4	-0.9
Glycerol	121.4	+8.6	-1.7
Glycol	92.4	+9.0	-1.3
n-Butanol	152.0	+8.4	-1.9
n-Propanol	124.7	+8.6	-1.7
Ethanol	97.4	+8.9	-1.4
Methanol	67.1	+9.4	-0.9

$V_1$  = Molecular volume of solvent.

$\Delta F_{(H-C)}$  (int) =  $\Delta F_{(H-C)}$  (internal mixing).

Relative  $\Delta F_{(H-C)}$  (int) ( $S-W$ ) =  $\Delta F_{(H-C)}$  (internal mixing for solvent).

-  $\Delta F_{(H-C)}$  (internal mixing for water).

within 2 kcal. The same applies if molecular weight is changed by a factor of 10. If the spheres vary in mean size within a factor  $1 \leq \alpha^3 \leq 8$  due to the expansion of a coil in going from one solvent to another, the differences between the  $\Delta F_{(H-C)}$  (int) values of different solvents will lie within 4.5 kcal/mole of base pair. This would still be small compared with the variation in  $\Delta F_{(H-C)}^\circ$  (solvent effect) between different solvents.

Thus the entropy of mixing effect does not invalidate our conclusions based on the previous  $\Delta F_{(H-C)}^\circ$  (solvent effect) alone. The energy gained on merging two cavities into one (with accompanied decrease in surface area) in water as compared with that in other solvents is sufficient to explain hydrophobic bonding (and the relative effectiveness of analogous "solvophobic" forces in the others). It is not the only effect involved in hydrophobic bonding, however, and our conclusions do not repudiate but supplement the previous concepts on hydrophobic bonding based on solvent structure.

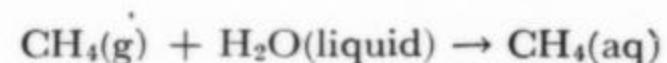
It is now worthwhile to investigate the effect of structural changes in water adjacent to various solute molecules and particularly next to helix and single strand DNA's and see its importance relative to the other effects treated.

#### Change of Water Structure Near Solutes

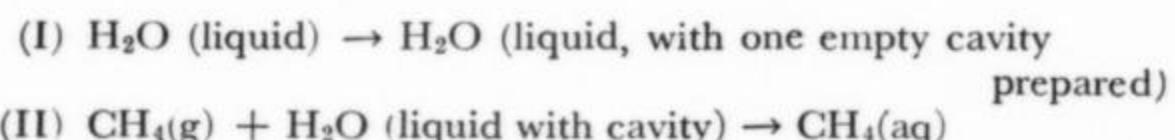
In investigating the nature of the structural restriction on the water molecules around the non-polar solute, we start by considering small non-polar solutes in solution. Non-polar gases like Ne, Ar, Kr, CH<sub>4</sub>, N<sub>2</sub>, O<sub>2</sub>, Xe, and C<sub>2</sub>H<sub>6</sub> form gas hydrates with water under pressure. The gas hydrate formed is of structure I (37) with two types of cavities, one of radius 3.95 Å° consisting of 20 water molecules, and the other of radius 4.3 Å° consisting of 24 water molecules. These hydrates have been studied theoretically by van der Waals and Platteeuw (37) and the theory extended to small polyatomic solutes like CH<sub>4</sub>, C<sub>2</sub>H<sub>6</sub>, and others by McKoy and Sinanoğlu (26). From the intermolecular potentials derived from gas properties the dissociation pressures of these hydrates are predicted.

In trying to determine whether such a gas-hydrate

type of cavity structure forms around a gas molecule in liquid solution, we can use the above theory. When a gas molecule, such as CH<sub>4</sub>, dissolves in water, we have



Again this can be written in two steps:



The unitary entropy and enthalpy of solution of the gas in water could therefore be written as

$$\Delta S_u = \Delta S(\text{I}) + \Delta S(\text{II})$$

$$\Delta H_u = \Delta H(\text{I}) + \Delta H(\text{II})$$

If we now assume that the cavity is the same as a gas-hydrate cavity we can calculate  $\Delta S(\text{II})$  and  $\Delta H(\text{II})$  a priori using the intermolecular potentials derived from the dissociation pressures of solid gas hydrates (26). Taking the difference with the experimental total values  $\Delta S_u$  and  $\Delta H_u$ , we obtain the "experimental"  $\Delta S(\text{I})$  and  $\Delta H(\text{I})$ , i.e., the empty cage properties. If the assumption about the cage is correct, then: a) the magnitudes of these should be independent of solute (for solutes which form the same β-ice gas-hydrate structure), and b) the values should agree with those derived for the solid hydrate empty β-lattice per cage.

Indeed the difference between  $\Delta S_{(\text{gas} \rightarrow \text{cage})}$  and the experimental standard, partial molal entropy of solution of the gas,  $\Delta \tilde{S}_2^\circ$ , comes out quite constant for all the above gases considered. We calculated  $\Delta \tilde{S}_2^\circ$  (unitary) assuming ideal mixing of gas and water molecules and a standard state of 1 atmospheric pressure in the gaseous state and a hypothetical solution of mole fraction equal to one. Results are shown in Table 3 for the case of a gas-hydrate cavity of radius 3.95 Å° comprised of 20 water molecules. We see  $\Delta S(\text{I})$  to be quite constant for both Lennard-Jones and Kihara potentials in all cases. It has a constant value of ca. - 0.65 e.u./mole of cage H<sub>2</sub>O. This compares very well with that from gas hydrates (37) for α(ordinary) ice → β (gas hydrate) ice (structure I) which is ca. - 0.61 e.u./mole H<sub>2</sub>O.

Glew (13) found experimentally through thermodynamic considerations that  $\Delta H_u$  values for CH<sub>4</sub> and other gas hydrate forming molecules at 0°C were equal to those for the reaction: gas + ice → gas hydrate. Since  $\Delta H$  for α-ice → gas hydrate ice (β) is approximately zero (37), the  $\Delta H$  for the above reaction is that of the gas molecule interacting with the cage. The correspondence with  $\Delta H_u$  value also supports the existence of such a cage in solution. Furthermore it shows the  $\Delta H$  for formation of such a cage to be approximately zero.

If one assumes that the local structure of water at temperatures closer to the freezing point than the boiling point is very similar to the ordinary α-ice structure, the above evidence might lead to the conclusion that on

TABLE 3. Entropy of water cages\* formed around solutes at 25°C

Gas	$\Delta S^\circ$ (solution)† (cal/mole °K)	$\Delta S$ (II) (cal/mole °K)‡	$\Delta S^\circ$ (I) (cal/mole °K)‡
Ne	-26.6 ± 0.4	-15.1 (L.J.)	-11.5 ± 0.4 (L.J.)
Ar	-30.7 ± 0.8	-17.1 (L.J.)	-13.6 ± 0.8 (L.J.)
Kr	-31.9 ± 0.4	-18.2 (L.J.)	-13.7 ± 0.4 (L.J.)
CH <sub>4</sub>	-31.66 ± 0.33	-18.63 (L.J.) -18.1 (K.)	-13.03 ± 0.31 (L.J.) -13.56 (K.)
N <sub>2</sub>	-31.27 ± 0.17	-17.96 (L.J.) -18.22 (K.)	-13.31 ± 0.17 (L.J.) -13.05 (K.)
O <sub>2</sub>	-31.02 ± 0.12	-17.27 (L.J.) -17.34 (K.)	-13.75 ± 0.12 (L.J.) -13.68 (K.)
Xe	-34.08 ± 0.63	-20.10 (L.J.)	-13.98 ± 0.63 (L.J.)
C <sub>2</sub> H <sub>6</sub>	-34.0 ± 0.4	-19.6 (L.J.) -17.3 (K.)	-14.4 ± 0.4 (L.J.) -16.7 (K.)

\* Gas-hydrate cage of radius 3.95 Å and 20 H<sub>2</sub>O's.  
 † Standard state is  $p = 1$  atm for gaseous phase, mole fraction = 1 in solution. Error in the experimental  $\Delta S^\circ$  (solution) values is given as average deviation among recent determinations: Butler, J. A. *Trans. Faraday Soc.* 33: 235, 1937 for Ne, Ar; Claussen, W. F., and M. F. Polglase. *J. Am. Chem. Soc.* 74: 4817, 1952 for CH<sub>4</sub>, C<sub>2</sub>, H<sub>6</sub>; reference (12) for Kr; Himmelblau, D. M. *J. Phys. Chem.* 63: 1803, 1959 for CH<sub>4</sub>, N<sub>2</sub>, O<sub>2</sub>, Xe; Morrison, J. T., and F. Billet. *J. Chem. Soc.* 3819, 1952 for CH<sub>4</sub>, N<sub>2</sub>, O<sub>2</sub>, C<sub>2</sub>H<sub>6</sub>; Morrison, J. T., and N. B. Johnstone. *J. Chem. Soc.* 3441, 1954 for Ne, Ar, Kr, Xe. ‡  $\Delta S^\circ$  (solution) =  $\Delta S$ (I) +  $\Delta S$ (II);  $\Delta S$ (I) = empty cage entropy.  $\Delta S^\circ$ (II) = entropy of solute in cage; L.J. using Lennard-Jones 12-6 potential; K. using Kihara potential (26).

introduction of the non-polar gas into water, the local  $\alpha$ -ice changes to gas-hydrate structure into which the gas molecule fits. This conclusion is being examined further at present with regard to  $\Delta\bar{V}_2$ ,  $\Delta C_p$  and  $\Delta\bar{S}_2^\circ$  of these gases at different temperatures.

If such a gas-hydrate cage forms around these non-polar small gases in solution, what sort of structure and structural restriction would we have around big molecules such as the bases of DNA? In trying to answer this we consider the interfacial entropy between water and some non-polar organic liquids. Comparing this with the sum of the surface entropies of water and the organic liquid we notice a decrease in entropy on forming the interface. This decrease amounts to ca. -0.7 e.u./mole of surface H<sub>2</sub>O, assuming a surface area of radius 1.4 Å for a water molecule. On the other hand with two normal liquids there is no such decrease. The mercury-benzene interfacial entropy shows it to be equal to the sum of the two surface entropies of both liquids.

Thus if the decrease in entropy is coming from the water, it corresponds well with the value from gas hydrates for  $\alpha$ -ice →  $\beta$ -ice and again with that for small solute solutions, ca. -0.65 e.u./H<sub>2</sub>O.

It appears that there is a short-range structure change

in the water adjacent to the solute molecules, but that this, rather than being like the "freezing" of liquid water, is more like  $\alpha$ -ice going into  $\beta$ -ice with a  $\Delta H$  per H<sub>2</sub>O about zero and  $\Delta S$  per H<sub>2</sub>O ca. -0.7 e.u. Such a structure change may well correspond to the partial cages around the large solute molecules as in the theory of Némethy and Scheraga (28), and in any case has roughly the same decrease in entropy per water molecule around the solute as in the case of an H<sub>2</sub>O molecule of a complete gas-hydrate cage.

To what extent such a reduction in entropy of water around the DNA helix and coil retains the same value per H<sub>2</sub>O is not known. For one thing, as mentioned above, the bases are polar with dipoles ranging from 2.8 debyes in adenine to 8.0 in cytosine (5). In addition there is the effect of the negatively charged phosphates and the ionic atmosphere around and between them which might disrupt such an ordered structure.

The dipoles of the bases tend to orient the water dipoles around them and if strong enough this effect would interfere with the structuring of water around a benzene-like solute. However, the dipole effect is simply given by the  $T\Delta S$  part of the Onsager reaction field term (29, 39) in  $\Delta F_{(H-C)}$  (bs) given above, and amounts to about only 1 kcal/base pair (see Table IV of (33)). Since up to 80 H<sub>2</sub>O's are liberated into the bulk on forming the helix, this is a far smaller amount than the hydrophobic type structural effect  $T\Delta S_{\text{true}} \cong 17$  kcal/base pair as estimated from the -0.7 e.u./H<sub>2</sub>O figure above. Thus one still expects to find the two orientational effects additive ( $T\Delta S = T\Delta S_{\text{bs}} + T\Delta S_{\text{true}}$ ). The backbone-charge effect is more serious, but would seem to be about the same both for the helix and the coils.

To conclude this section we note that in the section on *Macroscopic Treatment* we found just the macroscopic model with the surface enthalpies of solvent cavities to account already for the tendency of water to keep macromolecular groups (whether somewhat polar or not) together as compared with other solvents. This also gave the relative order of the "solvophobic" forces in different solvents.

In addition, however, previous theories and further considerations above show that there may be structural changes (with no accompanying enthalpic change) in solvent immediately adjacent to the solutes. We estimated a very rough magnitude for the effect in water. In other solvents the magnitudes should be lower since even in formamide there would be fewer solvent molecules per unit surface area of solute. This structural entropic effect added on to the effects of *Macroscopic Treatment* does not change the conclusions there, but simply makes water even more favorable than other solvents for causing solvent forces that associate protein side chains and keep the DNA bases stacked.

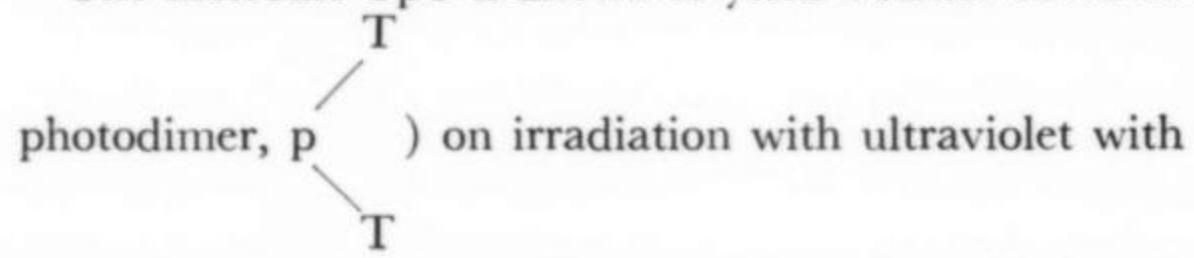
#### THYMINE PHOTODIMERIZATION IN DIFFERENT SOLVENTS

The results of the macroscopic model (*Macroscopic*

*Treatment*) enable a prediction that solvents can be ordered into a certain series (Table 1; see also (33) for  $\uparrow^A-T\downarrow$ ) depending on their tendency to bring polymeric groups such as the DNA bases together. These solvophobic interactions manifest themselves also in the photochemistry of DNA and model systems, so that the theory given above can be tested further by examining such phenomena.

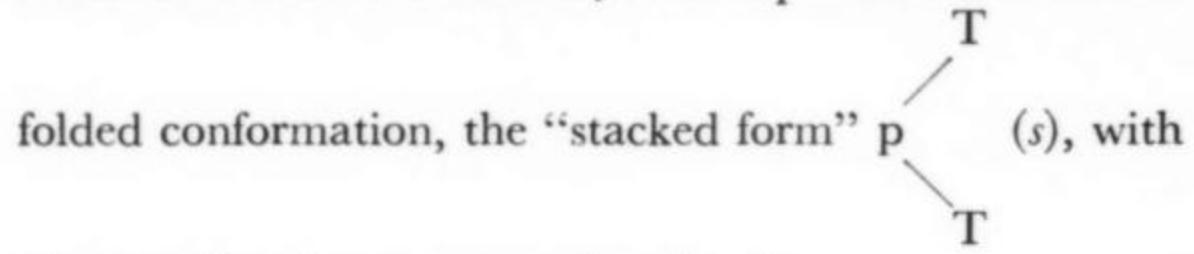
The relation between hydrophobic stacking of bases in single-strand DNA and the photodimerization of adjacent thymines by ultraviolet has been discussed previously (33). Here we shall mention new experiments (38) and calculations performed since then, which tentatively seem to support the theory of the solvent forces given above.

The molecule TpT is known to yield a stable covalent

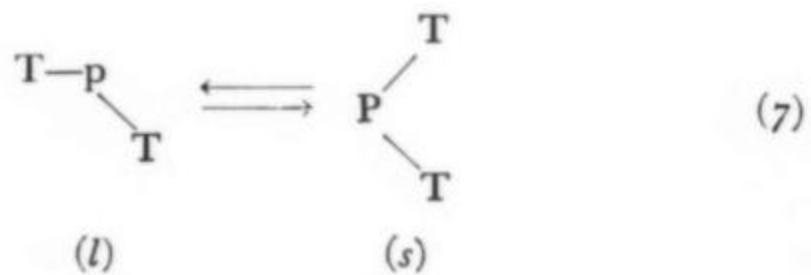


a cyclobutane ring formed between the 5-6 double bonds of the two thymines.

In an inert solvent the linear form of TpT, T—p—T(*l*), with the two thymines pointing away from each other into the solvent, is in equilibrium with the



the two thymines on top of each other.



The equilibrium concentration of (*s*) in different solvents will depend on the total free-energy change,  $\Delta F_{(s-l)}$ , in equation 7. The different effects that contribute to this free energy are analogous to those in equation (1). Here however the mixing effects are about the same for both forms of TpT. Moreover each can now be fairly well approximated by  $RT\ln X_i$ , so that the equilibrium concentration of TpT(*s*) will depend on  $\Delta F_{(s-l)}^\circ$  (compare eq. 1 and definitions there). Again, as in the DNA case treated in *Macroscopic Treatment*, we have

$$\Delta F_{(s-l)}^{\circ} = \Delta F_{(s-l)}^{\circ} (\text{vacuum}) + \Delta F_{(s-l)}^{\circ} (\text{solvent effect}) \quad (8)$$

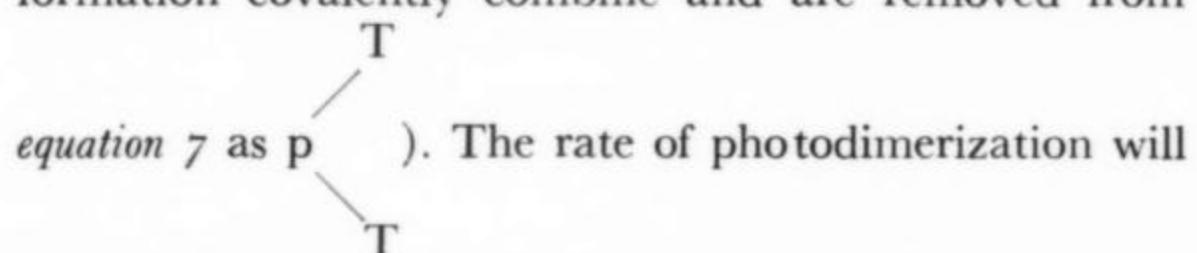
and

$$\Delta F_{(s-l)}^\circ \text{ (solvent effect)} = \Delta F_{(s-l)}^\circ (c) + \Delta F_{(s-l)}^\circ (bs) + \Delta F_{(s-l)}^\circ (R) \quad (g)$$

The definitions are similar to those in *equation 2* but apply to *equation 7*.

In comparing the extent of TpT folding in different solvents,  $\Delta F_{(s-l)}^\circ$  (vacuum) cancels out as a constant. The extent of folding in various solvents relative to a particular one, e.g., water, then depends only on  $\Delta F_{(s-l)}^\circ$  (solvent effect).

Now consider the photoreaction of TpT when ultraviolet light hits the equilibrium mixture of *equation 7* in a given solvent. The excited thymines (presumably triplet (Mantione and Pullman)) if found in the folded conformation covalently combine and are removed from



thus depend on the  $\Delta F_{(s-l)}^\circ$  (solvent effect) of equation 7, even if this rate is fast enough to upset the equilibrium above. On this basis the extent of photodimerization after a standard time and under standard conditions in different solvents should be greater the more negative the  $\Delta F_{(s-l)}^\circ$  (solvent effect) is in a given solvent. Thus, provided the dimer is the main photoproduct and there is no photoreaction with the solvent, photodimerization gives us a measure of the solvophobic force. Keto-enol tautomerism shifts in different solvents which would affect the electronic charge distribution around the 5-6 double bond in thymine (see (24a) for quantum mechanical calculations on TpT and photodimerization) does not seem to play a role in the extent of photodimerization in the different solvents.

The  $\Delta F_{(s-l)}^\circ$  (solvent effect) is calculated for the different solvents considered in *Macroscopic Treatment* and for *t*-butanol with parameters based on a molecular model. The model for the linear form,  $TpT(l)$ , considers a sphere around each thymine with a reaction field radius of  $a = 4.2A^\circ$  and an actual cavity radius  $r = 5A^\circ$ .

The model for the stacked form corresponds to structure I of Wulff and Fraenkel (41), where the two thymines and their methyl groups lie on top of each other. This structure has the greatest stacking and thus is favored most by the solvophobic forces. The stacked form has a resultant thymine dipole moment of 6.7 debye and is assumed in a spherical, reaction-field cavity of  $a = 5A^\circ$  and an actual cylindrical cavity of radius  $5A^\circ$  and height  $12A^\circ$ . We estimated  $\Delta F_{(s)}^\circ(R)$  by the same methods used in the DNA helix case with a  $5A^\circ$  cavity radius in the case of the dipole-dipole reduction.

Table 4 gives the total values of  $\Delta F_{(s-l)}^\circ$  (solvent effect) for the solvents considered. Note that the  $\Delta F_{(s-l)}^\circ$  (solvent effect) values order the pure solvents into a semi-quantitative sequence of decreasing solvophobic-force strength. The order of decreasing  $\Delta F_{(s-l)}^\circ$  (solvent effect) in the different solvents is the same as that in  $\Delta F_{(H-C)}^\circ$  (solvent effect) for  $\uparrow \begin{matrix} A-T \\ A-T \end{matrix} \downarrow$  polynucleotide and also for  $\uparrow \begin{matrix} G-C \\ G-C \end{matrix} \downarrow$  ((33) and Table 1) except for glycerol and formamide which interchange places.

Although on the sequence, e.g., water and glycerol,

TABLE 4.  $\Delta F_{(s-l)}^{\circ}$  (solvent effect) for TpT at 25°C

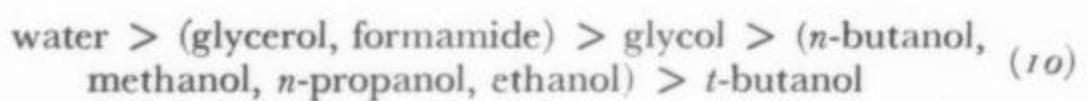
	kcal/Dimer	Water	Glycerol	Formamide	Glycol	n-Butanol	n-Propanol	Methanol	Ethanol	t-Butanol
$\Delta F_{(s-l)}^{\circ}$ (bs)		+3.2	+5.4	+3.8	+5.0	+3.3	+3.2	+3.0	+3.1	+3.3
$\Delta F_{(s-l)}^{\circ}$ (R)		+0.5	+0.9	+0.8	+0.8	+0.8	+0.7	+0.5	+0.6	+0.8
$\Delta F_{(s-l)}^{\circ}$ (c)		-26.0	-22.8	-20.9	-17.1	-8.8	-8.5	-8.0	-7.9	-7.0
Total $\Delta F_{(s-l)}^{\circ}$ (solvent effect)		-22.3	-16.5	-16.3	-11.3	-4.7	-4.6	-4.5	-4.2	-2.9
Relative $\Delta F_{(s-l)}^{\circ}$ (solvent effect)		0	+5.8	+6.0	+11.0	+17.6	+17.7	+17.8	+18.1	+19.4

Relative  $\Delta F_{(s-l)}^{\circ}$  (solvent effect)  $\equiv \Delta F_{(s-l)}^{\circ}$  (solvent effect) -  $\Delta F_{(s-l)}^{\circ}$  (solvent effect for water).

formamide and glycol, and glycerol and *n*-butanol, are widely separated, some solvents lie very close to each other. Glycerol and formamide differ only by 0.2 kcal/mole; *n*-butanol, methanol, *n*-propanol, and ethanol alcohols all lie within  $\leq 0.5$  kcal/mole of each other. Such differences may easily be overcome by changing the solute as in the TpT,  $\begin{smallmatrix} \uparrow A-T \\ A-T \downarrow \end{smallmatrix}$  case above. The differences are also small compared with the accuracy of the model. Thus the strength of the solvophobic force within each such group of solvents has to be considered essentially the same.

We may investigate this point further by examining the dependence of the solvent sequence on the TpT dimensional parameters chosen. If calculations are repeated after changing the *a* of TpT (*l*) from  $4.2A^{\circ}$  to  $3.4A^{\circ}$  (the value used in the  $\begin{smallmatrix} \uparrow A-T \\ A-T \downarrow \end{smallmatrix}$  case); after setting this same, *a* = *r* =  $4.6A^{\circ}$ ; and after replacing the cylindrical cavity of TpT(*s*) with a spherical one of *r* =  $5A^{\circ}$ , the solvophobic sequence remains the same. The solvents with  $\Delta F_{(s-l)}^{\circ}$  (solvent effect) values which differed from each other by  $\leq 0.5$  kcal/mole still differ within these amounts. Only within each such group, some solvents change places, showing again that these are about the same. The order for the solvents used in the experiments of Wacker and Lodemann (38) discussed below is unaffected.

We may now write the pure solvent decreasing solvophobic-force sequence as:



This predicted sequence is the same (33) for polynucleotides  $\begin{smallmatrix} \uparrow A-T \\ A-T \downarrow \end{smallmatrix}$ ,  $\begin{smallmatrix} \uparrow G-C \\ G-C \downarrow \end{smallmatrix}$ , hence any DNA, and also for TpT.

Tentative experimental results by Wacker and Lodemann (private communication; later data in (38)) give the per cent reductions in the extinction coefficient of TpT after irradiation with ultraviolet light in various pure solvents. Assuming that photoproducts between T and the solvents do not form, an assumption not valid in the case of UpU (14, 15), these can be taken as meas-

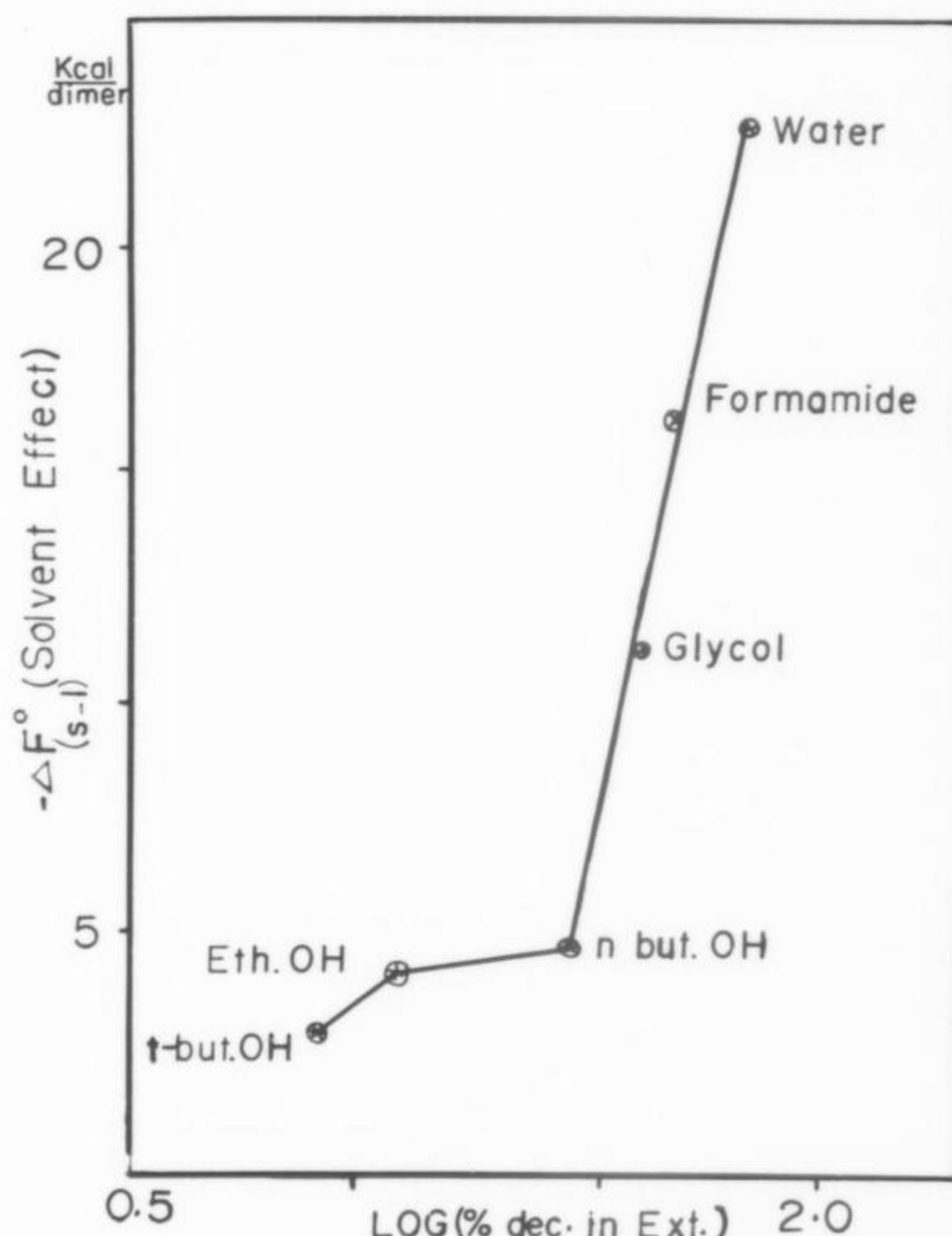


FIG. 3. Comparison of the theoretically predicted order of solvophobic forces (as given by  $-\Delta F_{(s-l)}^{\circ}$  (solvent effect) calculations on TpT) in different pure solvents with the extent of TpT ultraviolet photoproduct formed as measured by the per cent disappearance of thymine extinction after reaction (25°C). The photodimerization experiments are by Wacker and Lodemann (private communication; their additional data obtained while this paper was in press is in reference (38)).

uring the amount of  $\text{T} \begin{smallmatrix} \swarrow \\ \uparrow \\ \searrow \end{smallmatrix} \text{p} \begin{smallmatrix} \swarrow \\ \uparrow \\ \searrow \end{smallmatrix} \text{T}$ ) photodimer formed in the solvents considered. (On forming the photodimer the absorption of T disappears along with the double bond.) A plot of  $-\Delta F_{(s-l)}^{\circ}$  (solvent effect) versus log (per cent reduction in extinction), Fig. 3, shows the order of increasing photodimerization to be the same as that pre-

dicted by the calculated  $\Delta F_{(s-l)}^{\circ}$  (solvent effect) values of Table 4 and an earlier paper (33). No such correlation exists on the other hand between the increasing order of photodimerization and, for example, the dielectric constant of the solvents (38) which would affect the keto-enol tautomerism of thymine as well as some of the interactions.

Although further experiments and their quantitative analysis may be needed, the results tentatively lend some support to the theory of solvophobic forces. It may also be possible to extract quantitative values of the solvophobic-bond strengths directly from photodimerization experiments.

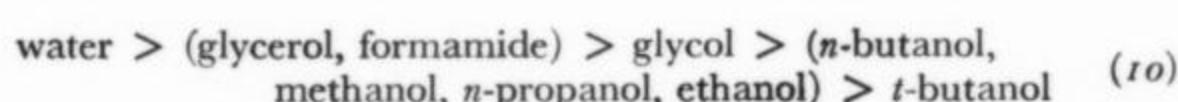
#### DISCUSSION AND CONCLUSION

In the sections above we considered the detailed nature of hydrophobic bonding in water. Analogous solvophobic bonding tendencies exist in other solvents. To put a large solute molecule or some biopolymer side chains into a solvent, some cavities must be created in the solvent first. This costs surface free energy although some of the free energy supplied to create the holes is regained when solutes interact with the walls. All solvents resist the creation of a cavity in them. Many of them may also be considered as structuring liquids. Thus on the basis of these two effects alone they all tend to favor the most compact configuration of a biopolymer with the more bulky groups turned in. What makes water unique is that in it these effects are by far the most pronounced. The energy needed to create a cavity in water is highest among the solvents because it has the highest cohesive energy density as measured by its heat of vaporization per cubic centimeter. This also makes the surface tension of water highest. Furthermore due to the small molecular size of water, there would be a greater number of "wall" molecules forming a cage surrounding a given non-polar solute in water than in the other solvents. Thus when two non-polar groups come together in water both the number of solvent molecules released into the bulk liquid and also the energy gained thereby are largest. This enthalpic change per  $H_2O$  is not very much affected by the detailed structure of the cage wall (section on *Change of Water Structure near Solutes*). But the entropy is affected and is found to be 0.7 e.u. per cage-wall  $H_2O$  lower than an  $H_2O$  in bulk water. Both this high value and the large number of  $H_2O$  molecules released in hydrophobic bonding make the structural entropy effect highest in water as compared with other solvents.

The cavity effect fortified by the structural effect gives water the greatest tendency to bring non-polar groups together. This tendency is not affected very much even when the groups are not entirely non-polar but have sizable dipoles, as in the case of the nucleotide bases of DNA.

From the free energy contributions arising from the effect of solvent on the association equilibrium of two groups, we are able to predict the relative strengths of solvophobic forces. The calculations reported in this and

the previous paper (33) predicted the relative strengths of the solvophobic bonds involving the association of nucleotide bases A, T, G, and C in different pure solvents. The sequence in order of decreasing solvophobic bond is



The solvents within parentheses give practically the same strength. The sequence applies to the stability of a DNA double helix of any base composition in different solvents, showing why only water keeps it intact. The sequence also applies to the extent of base stacking in single-strand DNA or polynucleotides (33), to the extent of folding in model compounds like TpT, and, to the extent of thymine photodimerization under ultraviolet dependent on this folding or stacking in different solvents.

Similar solvent sequences can be calculated for other biopolymer groups, e.g., different types of protein side chains. The above sequence may not be too different for these also. It is hoped that the theory will also be extended to mixed solvents.

The results given so far are not sufficiently quantitative to state an absolute stability in a given solvent. However, since hardly any adjustable parameters are involved in the theory, the relative strength of two different solvophobic or hydrophobic bonds, involving different types of side chains but in the same solvent, can be predicted.

The results above should hold not only at 25°C but also at the low temperatures pertinent in cryobiology. In the case of water the major constituent of cells, the hydrophobic bonding phenomenon, is expected to be even more pronounced at these low freezing temperatures.

Around a biopolymer and its side groups is a quite rigid first layer of hydration. It has been possible to study even the location of individual water molecules in this layer, for example around the DNA helix (7, 8, 9, and for the application of the results in theoretical calculations, 32) by techniques like infrared and nuclear magnetic resonance. This quite firmly bound first layer (roughly  $\beta$ -ice type) remains unchanged on freezing while the bulk water goes into ordinary  $\alpha$ -ice. Thus the structural effect is still operative (*Change of Water Structure Near Solutes*). On the other hand, the cohesive energy density of ice is somewhat more than that of liquid water ( $\Delta H$  of vaporization for water at 0°C is 596 cal/cm<sup>3</sup>;  $\Delta H$  of sublimation of ice at 0°C = 620 cal/cm<sup>3</sup>). This makes the creation of a cavity in ice somewhat more difficult than in water. On this basis extra aggregation of the more or less non-polar groups is expected on freezing.

This enhanced hydrophobic bonding may explain the phase separation that occurs in water-organic liquid mixtures on freezing. What happens in a cell at low temperatures depends on how much of the water is tied up in the first layers of biopolymers (and those caught up in a few more layers between, for example, nearby

proteins). If most of the water is in this state there will not be much  $\alpha$ -ice formation. The effect of organic solvents on the behavior of cells on freezing may also be related to this phenomena. Though the organic solvents would tend to denature biopolymers, they may nevertheless also act as further regions around which first layers of hydra-

tion are formed, decreasing the amount of water available for  $\alpha$ -ice.

We thank Professor A. Wacker and his co-workers for stimulating discussions and for making their experimental results available to us prior to publication.

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# Role of water structure in macromolecules<sup>1</sup>

IRVING M. KLOTZ

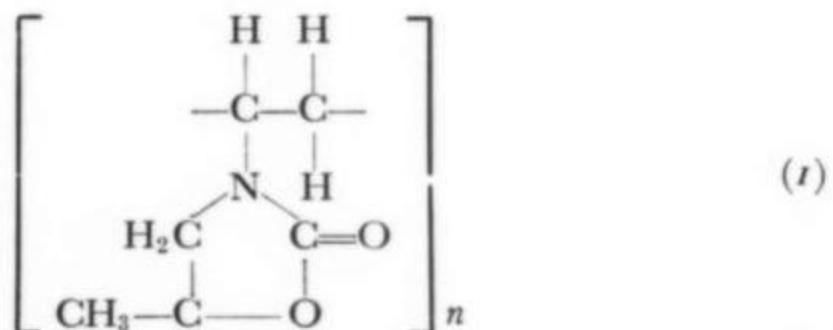
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AFTER REFLECTION, I have decided I will most nearly meet requirements of the title assigned to me if I address myself to three questions. First, what are some of the experimental observations that lead us to suspect that water may play a role in the establishment of configuration of biological macromolecules? Second, what are some of the current ideas about the manner in which the structure of water may be involved in these interactions with macromolecular solutes? And finally what experiments might be devised to try to clarify and choose between the theoretical interpretations of the observed behavior of macromolecules, particularly proteins and nucleic acids, in water? In short, then: what do we know? what do we think? what can we do?

## COMMON FEATURES OF MACROMOLECULAR INTERACTIONS WITH WATER

The macromolecular structures of biopolymers such as collagen, ribonuclease, and DNA are grossly different. Yet, as pointed out by von Hippel and Wong (56), the effects of certain simple compounds on the conformations of these macromolecules in aqueous solutions are strikingly similar. I should like to present some of the pertinent data for these biopolymers together with some unpublished observations on a synthetic polymer which show that there are remarkable common features in the interactions of all of these macromolecules, natural or synthetic, with water.

Let me start with the new, unpublished data. Some years ago we became interested in the properties of a newly available commercial polymer, polyvinylmethylloxazolidinone, because it exhibits some superficial resemblances to proteins. The repeating unit in this polymer is



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which, as you will note, cannot form intramacromolecular hydrogen bonds. One interesting characteristic of this polymer is that although it is very soluble in water at room temperature, it comes out of solution very sharply (58) as the temperature reaches 40°C, and is insoluble above this temperature. This behavior is very reminiscent of heat denaturation of proteins, which likewise tend to have a sharp "cloud point" if heated in aqueous solution.

We thought it would be interesting, therefore, to examine the effects of some protein denaturants on the cloud point of polyvinylmethylloxazolidinone. As the results in Fig. 1 show, the cloud point is raised by urea, especially at high concentrations. In other words urea solubilizes the polymer at temperatures it would otherwise not be soluble, just as urea solubilizes proteins. We might note in passing (reiterating an earlier conclusion (39)) that at least with the polymer the action of urea cannot involve breaking intramacromolecular hydrogen bonds, since this polymer cannot have any by itself.

Cloud points of proteins were also studied in detail some twenty years ago by Luck and his associates (3, 42) and among the compounds examined, sodium dodecyl sulfate was found to be particularly effective in raising the clouding temperature. Interestingly, sodium dodecyl sulfate increases the cloud point of polyvinylmethylloxazolidinone (Fig. 1).

In addition to these well-known denaturants, urea and sodium dodecyl sulfate, we have also looked at several neutral-salt denaturants such as NaSCN and LiBr. As the results in Fig. 1 show, these too raise the cloud point of the polymer.

Contrariwise, experience has long shown that some substances such as  $(\text{NH}_4)_2\text{SO}_4$  or sucrose stabilize proteins against denaturation. These very same substances added to polyvinylmethylloxazolidinone have now been observed to lower the cloud point (Fig. 1) of the polymer.

Let us now see how the effects of these small molecules on the synthetic polymer compare with their effects on a naturally occurring biopolymer. The thermal disorganization of ribonuclease in the presence of a variety of neutral salts and urea has been studied by von Hippel and Wong (56) and a few of their results are summarized in Fig. 2. Again the substances examined fall into two classes with respect to their influence on the transition temperature. Urea, LiBr, and KSCN lower the transi-

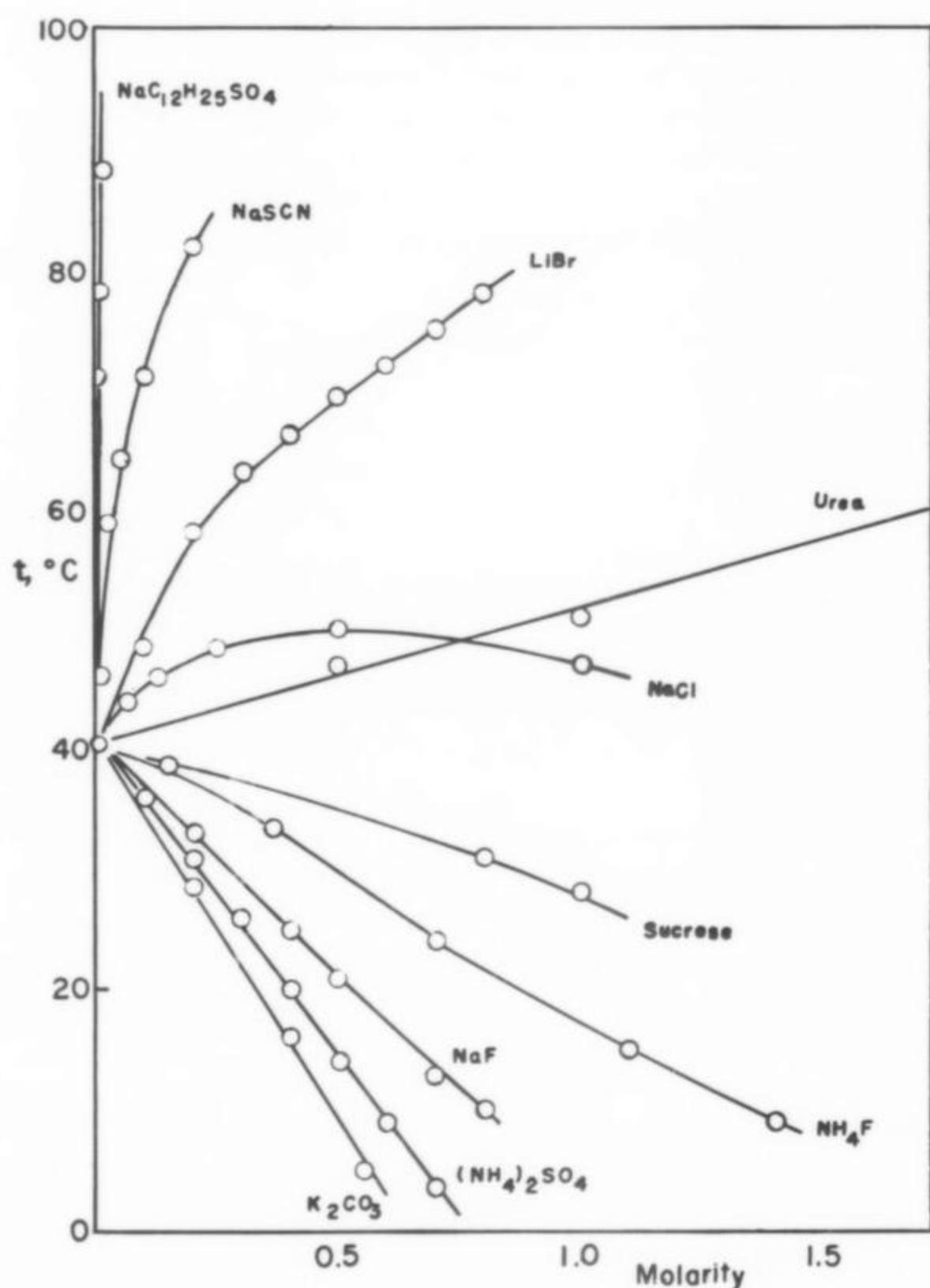


FIG. 1. Effects of various substances on cloud points of polyvinylmethoxazolidinone dissolved in water at a concentration of 0.2%.

tion temperature, that is, facilitate disorganization of the protein structure;  $(\text{NH}_4)_2\text{SO}_4$  raises the transition temperature, that is, stabilizes the structure of ribonuclease. Members of each class correspond with those in each of the two classes distinguished on the basis of their effects on polyvinylmethoxazolidinone.

Similar studies of the influence of simple molecules on the conformations of other biopolymers such as DNA (25), collagen (24), gelatin (4, 9) and myosin (55, 56) have been described. There is no need to show all of these data. The essence of the behavior of all of these polymers, natural and synthetic, can be illustrated schematically in Fig. 3. Some substances, e.g.,  $(\text{NH}_4)_2\text{SO}_4$  and sucrose, stabilize these molecules; other substances, e.g., urea and LiBr, destabilize all of them. These macromolecules are markedly different even in their primary structures, let alone their higher order stereoatomic arrangements. Nevertheless, the stabilizing or destabilizing effects of simple molecules are remarkably similar on all of these polymers. As has been pointed out previously in regard to urea effects (39) and more recently in connection with neutral salts (56), the specific chemical or conformational features of the particular macromolecule, being so different from case to case, can hardly play a dominant role in these interactions. It is

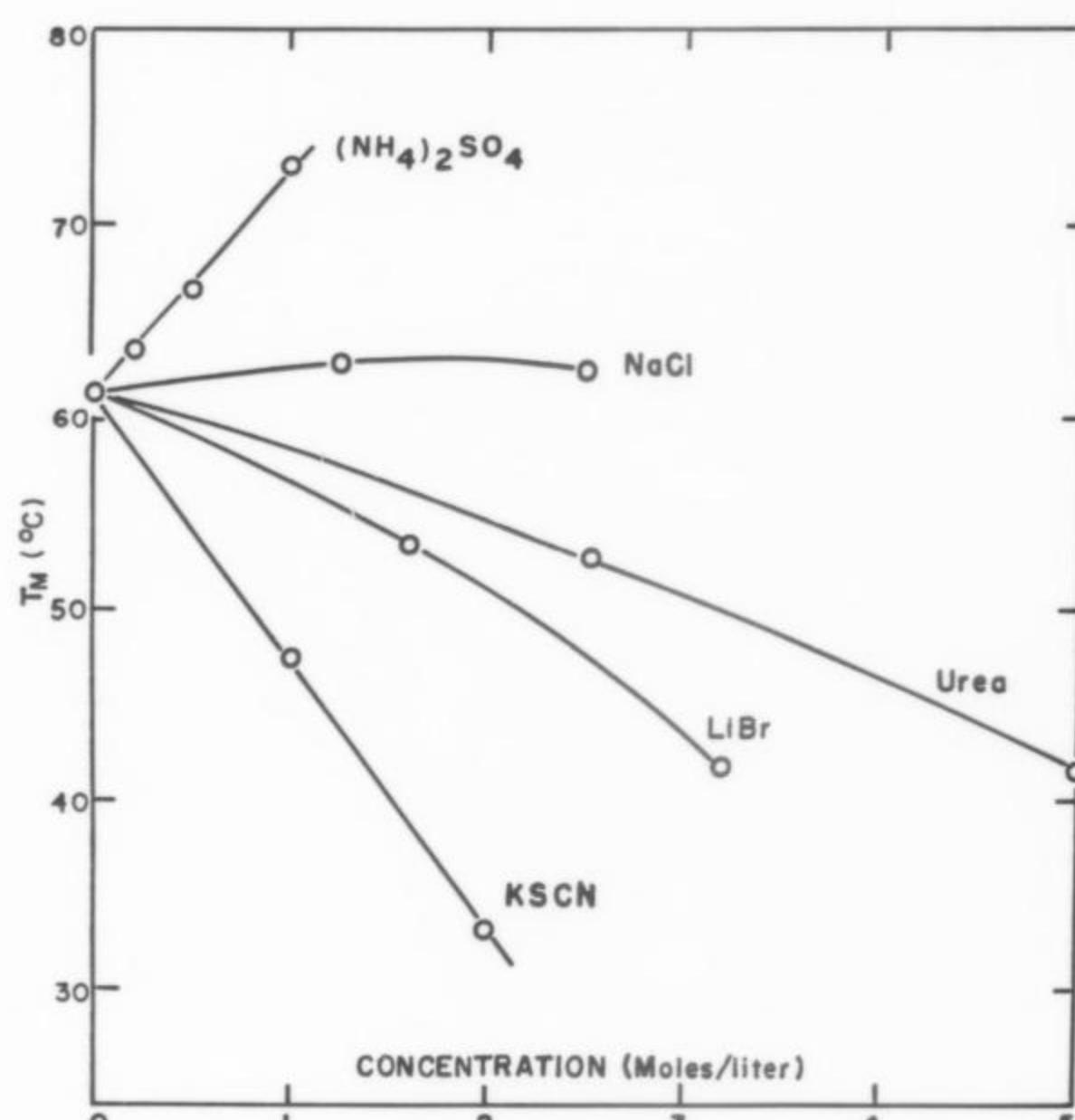


FIG. 2. Effects of various substances on transition temperatures of ribonuclease. (Data taken from von Hippel and Wong (56).)

NATIVE			
$(\text{NH}_4)_2\text{SO}_4$ , Sucrose			
PVOM (Ppt. temp.)	RNase (Trans. temp.)	COLLAGEN (Shrink. temp.)	DNA (Trans. temp.)
Urea, S.D.S., LiBr, KSCN			

#### DENATURED

FIG. 3. Comparison of response of various polymers to small molecules added to aqueous solvent.

difficult to avoid the conclusion, therefore, that these small molecules generate their effects by modifying the structure of this solvent. Such modifications could in turn perturb interactions between solvent and macromolecule and thus indirectly change the conformation and behavior of the large molecule. Local solvent structure in the neighborhood of the macromolecule thus seems to play a most significant role.

#### MOLECULAR INTERPRETATIONS OF INTERACTIONS BETWEEN WATER AND BIOMACROMOLECULES

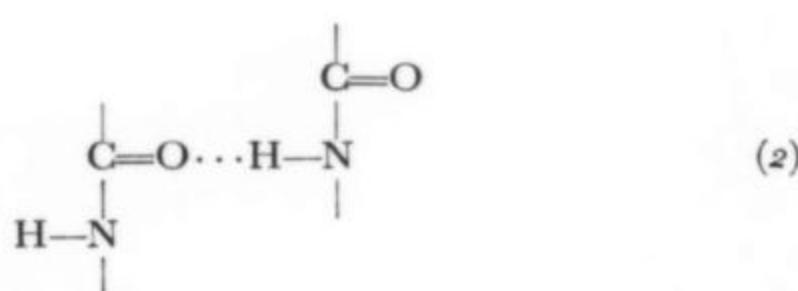
In essence water may influence the conformation of macromolecules if it has an effect on any of the non-covalent bonds which stabilize the conformation of the large molecule. In general such noncovalent bonds

TABLE I. Thermodynamics of  $C=O \cdots H-N$  hydrogen bond formation in *n*-methylacetamide at 25°C

Solvent	Association Constant	$\Delta F^\circ$ Kcal/Mole	$\Delta H^\circ$ Kcal/Mole	$\Delta S^\circ$ Gibbs/Mole
Carbon tetrachloride	4.7	-0.92	-4.2	-11
<i>n</i> -Methyl morpholine	1.9	-0.38		
Acetonitrile	0.75	0.17	-0.7	-3
Dioxane	0.52	0.39	-0.8	-4
Water	0.005	3.1	0.0	-10

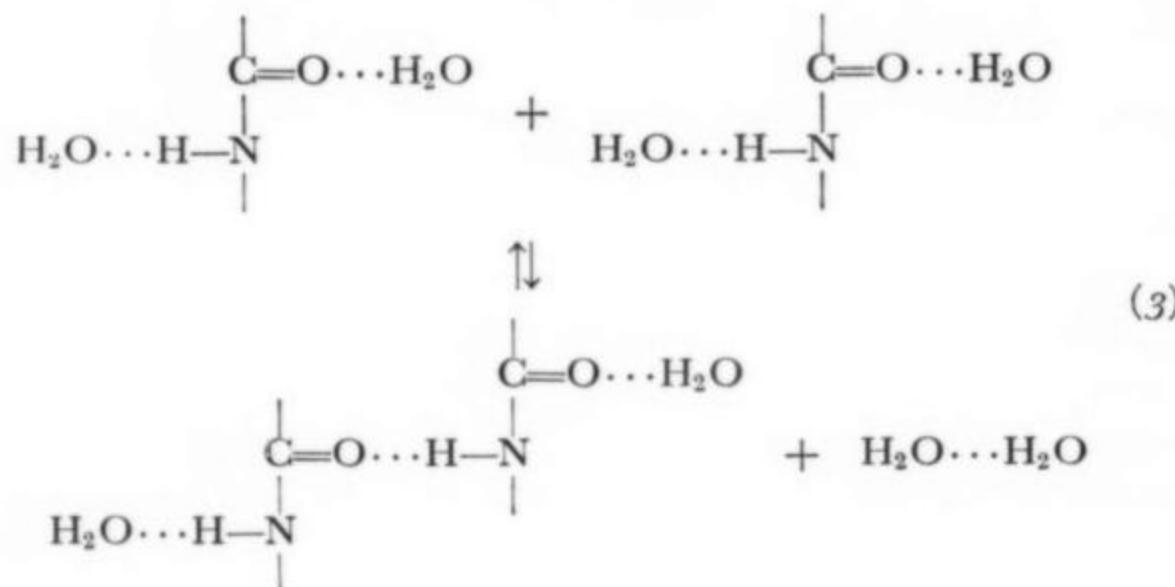
belong to one of three categories: 1) hydrogen bonds, 2) ionic bonds, and 3) apolar bonds. Evaluations of the strengths of such bonds and the role of water structure therein have been concentrated largely on proteins. The following discussion will be devoted, therefore, almost entirely to polypeptides and related model systems.

There have been several indirect attempts to evaluate the thermodynamic stability of interamide hydrogen bonds.



In our opinion the only reliable value for  $\Delta H^\circ$  of this bond is that obtained from equilibrium measurements with *n*-methylacetamide on the basis of changes in near infrared spectra (37). These results in several solvents are summarized in Table I. It is clear that the greater the hydrogen-bonding ability of the solvent, the weaker the  $C=O \cdots H-N$  bond. In aqueous solvent the heat of formation or of disruption of this bond is zero. In essence then an  $N-H \cdots O=C$  hydrogen bond cannot provide stabilization in water solutions.

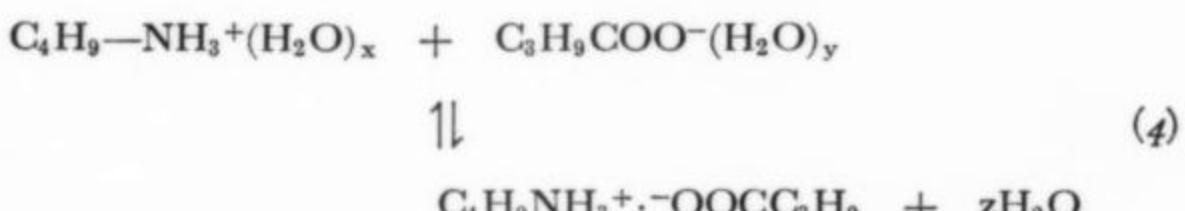
In a sense this should have been expected in water; the formation of hydrogen-bonded dimers of *n*-methylacetamide must involve the following details:



In other words since both the  $C=O$  and  $H-N$  groups in the monomer are hydrogen bonded to water, it is necessary to break these bonds before a  $C=O \cdots H-N$  bond can be formed. The competitive hydrogen bonding by  $H_2O$  thus weakens the thermodynamic tendency toward formation of interamide hydrogen bonds. Water should thus tend to disrupt peptide hydrogen bonds and

in this way interfere with any ordered macromolecular structure dependent on such bonds.

Similar conclusions have been reached in regard to ionic groups similar to those in side chains of proteins. For example an estimate (14) of the strength of the bond between butylammonium and isobutyrate ions, (4),



indicates an association constant near 0.03. A comparison with values in Table I shows that such an association is also weak. Evidently again the strong ion-dipole interactions between water and the constituent cationic amine and anionic carboxyl disrupt the ionic bond.

It has thus become generally agreed upon that apolar bonds are the forces largely responsible for stabilizing the conformations of protein molecules, and probably other macromolecules also. However, the role that the structure of water plays in these interactions is the subject of some divergence of opinion.

One view, christened by Dr. Kauzmann (34) as "hydrophobic bonding" may be described on a molecular scale as follows. Apolar groups, such as hydrocarbons, tend to stay out of water and in an apolar solvent, that is, the free energy of transfer of an apolar group from a hydrocarbonlike solvent to water is positive and unfavorable. However thermodynamic analysis shows that this unfavorable  $\Delta F$  is not due to an unfavorable change in internal energy  $\Delta E$  (or in  $\Delta H$ ). Actually the apolar group has a lower energy in a water environment than in chemically similar surroundings. Nevertheless, it does not go into water because  $\Delta S$  is unfavorable, that is, there is a large entropy loss when the apolar group dissolves in water. This entropy loss is attributed to a change in the structure of the solvent water. In particular the "iceberg" concept of Frank and Evans (22) is introduced. It is postulated, on good evidence such as Dr. Frank has discussed, that water molecules become more ordered around an apolar solute. The loss in entropy in this ordering when an apolar group is introduced into the aqueous phase is what contributes the unfavorable  $-T\Delta S$  to the observed positive  $\Delta F$ .

It follows then that two separated apolar groups in an aqueous environment would tend to come out of the water and associate with each other (Fig. 4), for this would release some of the more ordered water molecules around the individual groups, increase  $\Delta S$ , and make  $\Delta F$  negative. Such a tendency of apolar groups to get out of their aqueous environment and to join together is thus aptly described as a hydrophobic bond.

The concept of a hydrophobic bond seems very reasonable also because it agrees with our common experience on a macroscopic scale that oil droplets dispersed in water tend to coalesce with each other and

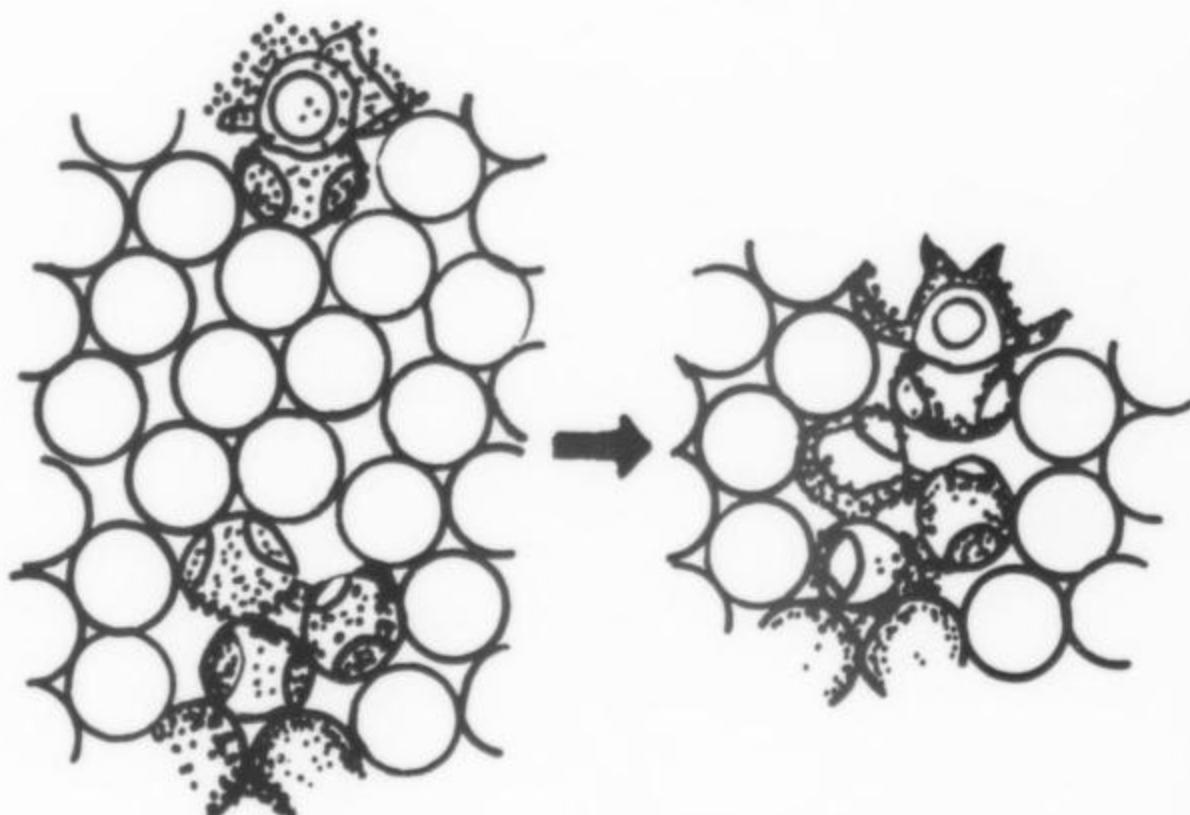


FIG. 4. Schematic representation of formation of hydrophobic bond between two apolar groups. Water represented by open circles.

"push out" the water between them, i.e., they are hydrophobic.

There are, nevertheless other properties of apolar molecules in water that are not common knowledge, but at the molecular level may have definite pertinence to interactions between apolar groups and the role of water structure.

If the concentration of an apolar molecule such as  $\text{CH}_4$  is increased in water (in practice by increasing the pressure of the supernatant gas to tens of atmospheres), a very interesting phenomenon is observed: out of the solution comes a crystalline substance. On analysis this substance is found to consist mostly of water and to have the stoichiometric formula  $\text{CH}_4(\text{H}_2\text{O})_{7.67}$ . Despite its unconventional formula this substance is a true chemical compound and in fact its detailed structure has been worked out in recent years by X-ray analysis (10, 11, 47, 57).

The fundamental unit in the structure of methane hydrate and other related apolar hydrates is the pentagonal dodecahedron (Fig. 5). The water molecules in these hydrates (or strictly speaking, the oxygen atoms) are at each of the 20 vertices of the dodecahedron. Each oxygen atom has four tetrahedral bonds, three hydrogen bonds along the three edges on the surface of the polyhedron radiating from the oxygen atom, and the fourth sticking out away from the polyhedron. The formula of such a dodecahedron composed of water molecules may be written  $\text{H}_{40}\text{O}_{20}$ . The dodecahedron has 12 faces and 20 vertices and according to the rules (faces + vertices = edges + 2) for Platonic solids (or by empirical counting) has 30 edges. Each of these edges is a hydrogen bond. Of the 40 hydrogens in  $\text{H}_{40}\text{O}_{20}$ , 30 would therefore be placed along the edges and 10 would remain for (half of) the bonds sticking out from the polyhedron. Since in each dodecahedron half of its vertex oxygen atoms can donate an H atom to a hydrogen bond and the other half are anxious to be acceptors in a hydrogen bond, it is not surprising that these dodecahedra tend to come together in a cooperative fashion until a superstructure

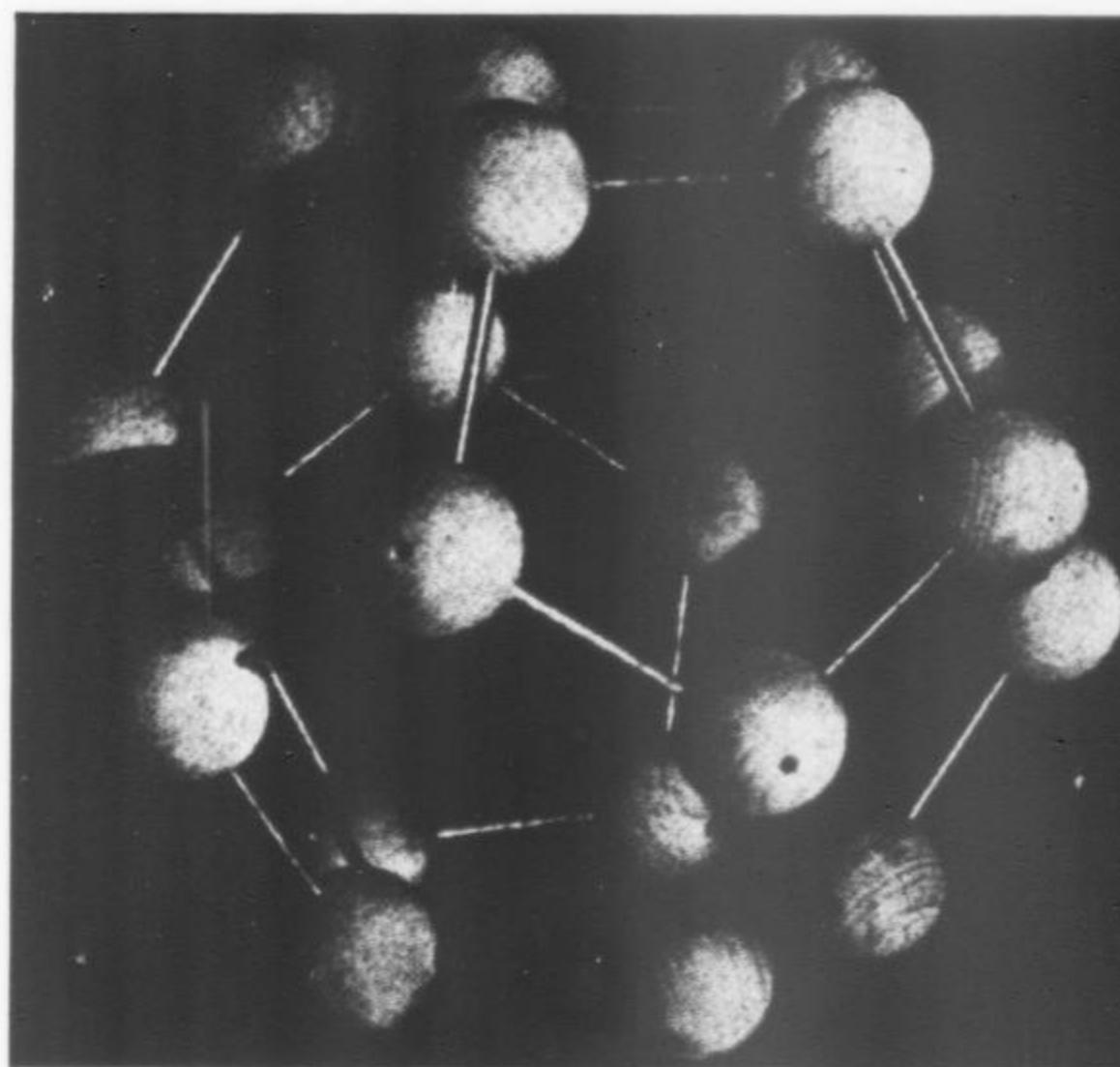


FIG. 5. Model of arrangement of water molecules in crystalline hydrates of apolar molecules. Each ball represents one water molecule. The molecules are arranged in pentagonal planes, 12 of which enclose a dodecahedral region. The hole inside this polyhedron is about 5 Å in diameter.

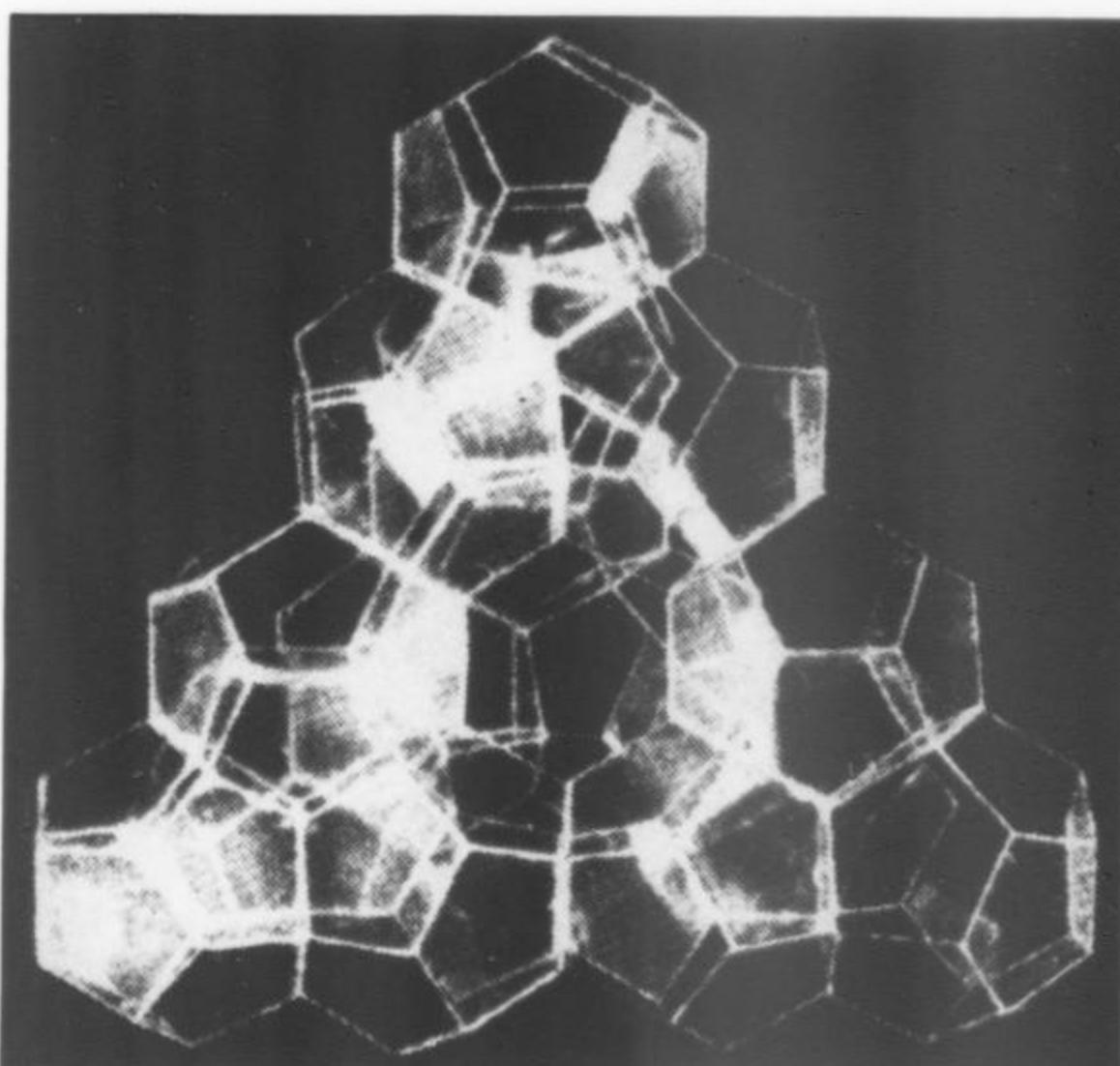


FIG. 6. Polyhedrons of type of Fig. 5 arranged to form a large lattice, as found in crystalline apolar hydrates. Compartments of 5, 6, and 7 Å are available for enclosure of apolar guest molecules within water structure.

like that shown in Fig. 6 is built up, which may contain 12-, 14-, 15- or 16-faced polyhedra.

These pentagonal polyhedra of  $\text{H}_2\text{O}$  are inherently unstable in themselves and would spontaneously change to normal hexagonal ice (Fig. 7) below 0°C and to liquid water above. However, as is apparent from Fig. 5 and 6,

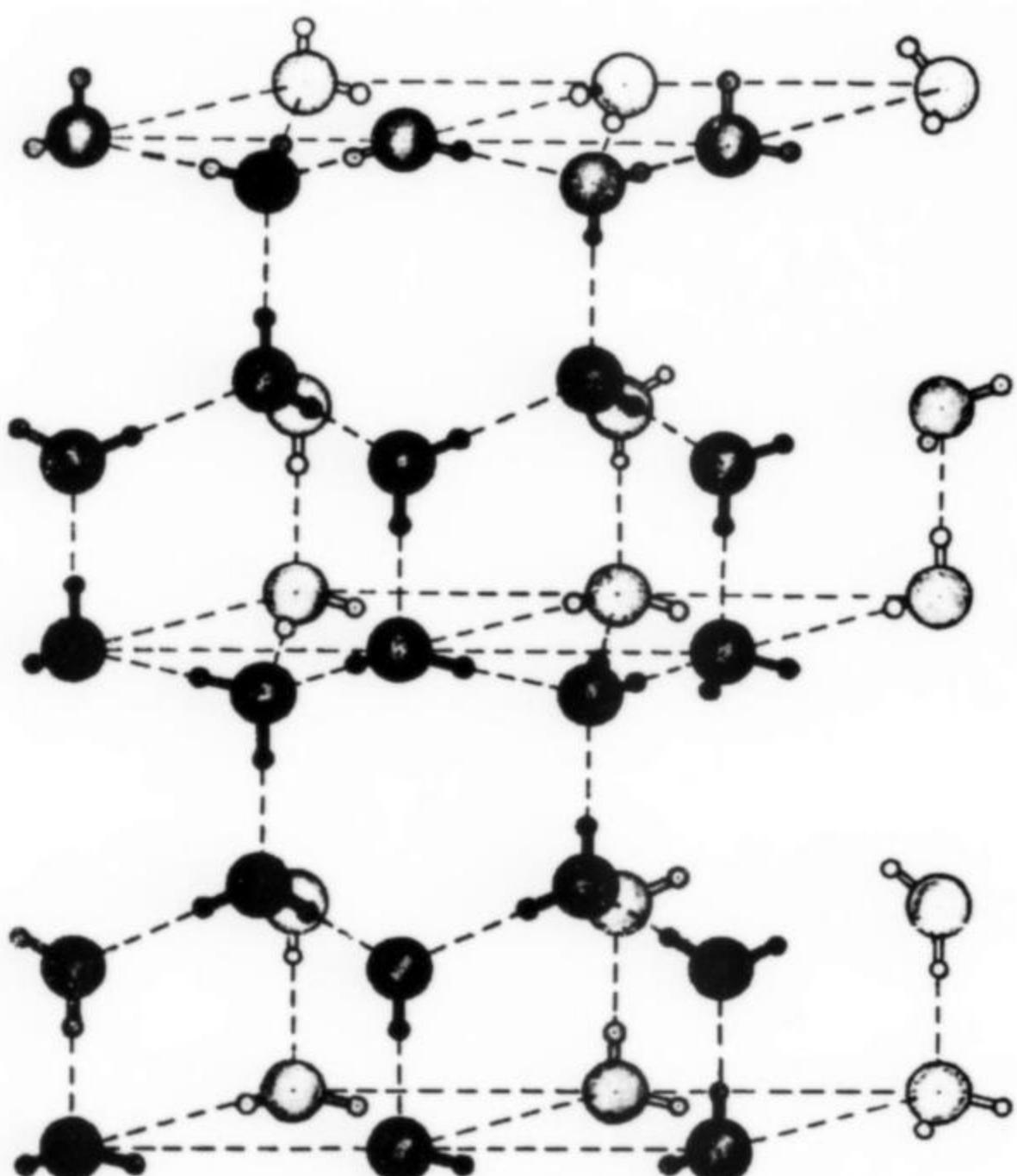
FIG. 7. Arrangement of  $\text{H}_2\text{O}$  molecules in normal ice.

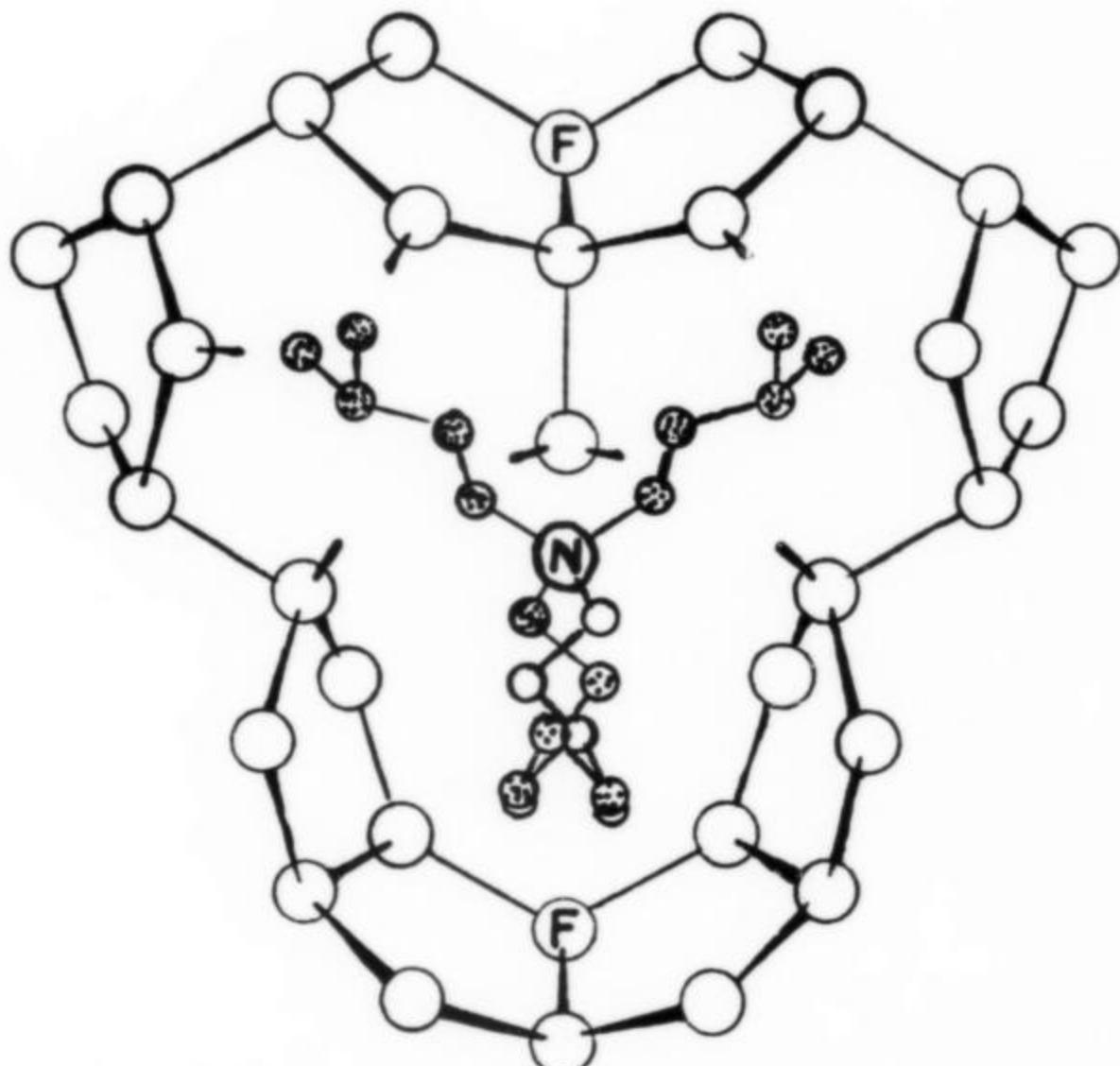
TABLE 2. Some molecules that form apolar polyhedral hydrates

	Class 1	Class 2	Class 3
Ar	$\text{CH}_4$	$\text{CHCl}_3$	$(n\text{-C}_4\text{H}_9)_4\text{N}^+\text{F}^-$
Kr	$\text{C}_2\text{H}_2$	$\text{CH}_3\text{CHCl}_2$	$(n\text{-C}_4\text{H}_9)_4\text{N}^+\text{O}_2\text{CC}_6\text{H}_5$
$\text{Cl}_2$	$\text{C}_2\text{H}_4$	$(\text{CH}_3)_2\text{O}$	$[(n\text{-C}_4\text{H}_9)_4\text{N}^+]_2\text{WO}_4^-$
$\text{H}_2\text{S}$	$\text{C}_2\text{H}_6$	$\text{C}_3\text{H}_8$	$(i\text{-C}_5\text{H}_{11})_4\text{N}^+\text{F}^-$
$\text{PH}_3$	$\text{CH}_3\text{Cl}$	$(\text{CH}_3)_3\text{CH}$	$(n\text{-C}_4\text{H}_9)_3\text{S}^+\text{F}^-$
$\text{SO}_2$	$\text{CH}_3\text{SH}$	$\text{C}_3\text{H}_7\text{Br}$	$(n\text{-C}_4\text{H}_9)_4\text{P}^+\text{Cl}^-$

these polyhedra contain large cavities, which in fact are 5–7 Å in diameter. If some of these polyhedra, and not necessarily all, are filled with an unreactive molecule such as  $\text{CH}_4$ , the superstructure becomes stabilized with respect to ordinary ice or even liquid water. In fact some of these hydrate ices do not melt until well above 30°C.

As has already been implied, many molecules other than  $\text{CH}_4$  form these stable apolar hydrates. A few of these are listed in Table 2. The compounds in class 1 fit into a lattice made up of a unit cell containing 46  $\text{H}_2\text{O}$  molecules with eight cavities. Six of these cavities are tetrakaidecahedra with an internal diameter of 5.9 Å, and two cavities are dodecahedra of 5.2 Å diameter. If only the larger cavities are filled by an inert guest molecule  $M$ , then the formula per unit cell is  $6M \cdot 46\text{H}_2\text{O}$  or stoichiometrically  $M(\text{H}_2\text{O})_{7.67}$ . If all the cavities are occupied by  $M$ , the corresponding formulas are  $8M \cdot 46\text{H}_2\text{O}$  and  $M(\text{H}_2\text{O})_{5.75}$ , respectively.

Class 2 compounds (Table 2) have a unit cell containing 136  $\text{H}_2\text{O}$  molecules and 24 cavities. Eight of these are hexakaidecahedra with a diameter of 6.9 Å and 16 cavities are dodecahedra of 4.8 Å diameter. The inert molecules  $M$  occupy the eight large holes. Thus the

FIG. 8. Projection of clathrate cavity containing tetraisoamylammonium ion within framework of water molecules. Note F<sup>-</sup> ions directly above oxygens. (From Feil and Jeffrey (18).)

formula per unit cell is  $8M \cdot 136\text{H}_2\text{O}$  or  $M(\text{H}_2\text{O})_{17}$  stoichiometrically. In this group of hydrates, mixed hydrates are also common in which a small molecule such as  $\text{H}_2\text{S}$  fills the 16 smaller cavities, in addition to eight  $M$ 's in the unit cell. These mixed hydrates thus have the stoichiometric formula  $M(\text{H}_2\text{S})_2(\text{H}_2\text{O})_{17}$ .

In both class 1 and class 2 hydrates, each guest molecule  $M$  is completely enclosed in one polyhedron, with 12–16 faces. This is not a necessary condition for a stable apolar hydrate, however, as the substances in class 3 demonstrate. There is no single unit cell for all of the substances in class 3. We shall look at a few of the typical lattices found in this series.

Figure 8 shows a projection sketch of the structure of  $(i\text{-C}_5\text{H}_{11})_4\text{N}^+\text{F}^- \cdot 38\text{H}_2\text{O}$ , determined by Feil and Jeffrey (18). The four isoamyl groups project out tetrahedrally into four cavities, two 14- and two 15-polyhedra. Only three polyhedra can be seen in the projection in Fig. 8, the two at the bottom center being essentially superimposed, as the contrasting shaded and open circles of the carbon atoms of the two isoamyl groups indicate. The common vertex of the four polyhedra is occupied by the  $\text{N}^+$  atom instead of  $\text{H}_2\text{O}$ , so that strictly speaking each polyhedron is not fully completed by  $\text{H}_2\text{O}$  molecules. Nevertheless this is a stable hydrate. Furthermore one should notice that  $\text{F}^-$  atoms are incorporated in the lattice, each one taking the place of one  $\text{H}_2\text{O}$  molecule. Ordinarily ions tend to disrupt water structures, but in this case the stabilizing effects of the hydrate lattice more than compensate for any ionic effect.

A similar structure inserted to demonstrate another point is that of  $(n\text{-C}_4\text{H}_9)_4\text{N}^+\text{C}_6\text{H}_5\text{COO}^- \cdot 39\frac{1}{2}\text{H}_2\text{O}$  determined by Bonamico, Jeffrey and McMullan (7). A projection formula of this structure is shown in Fig.

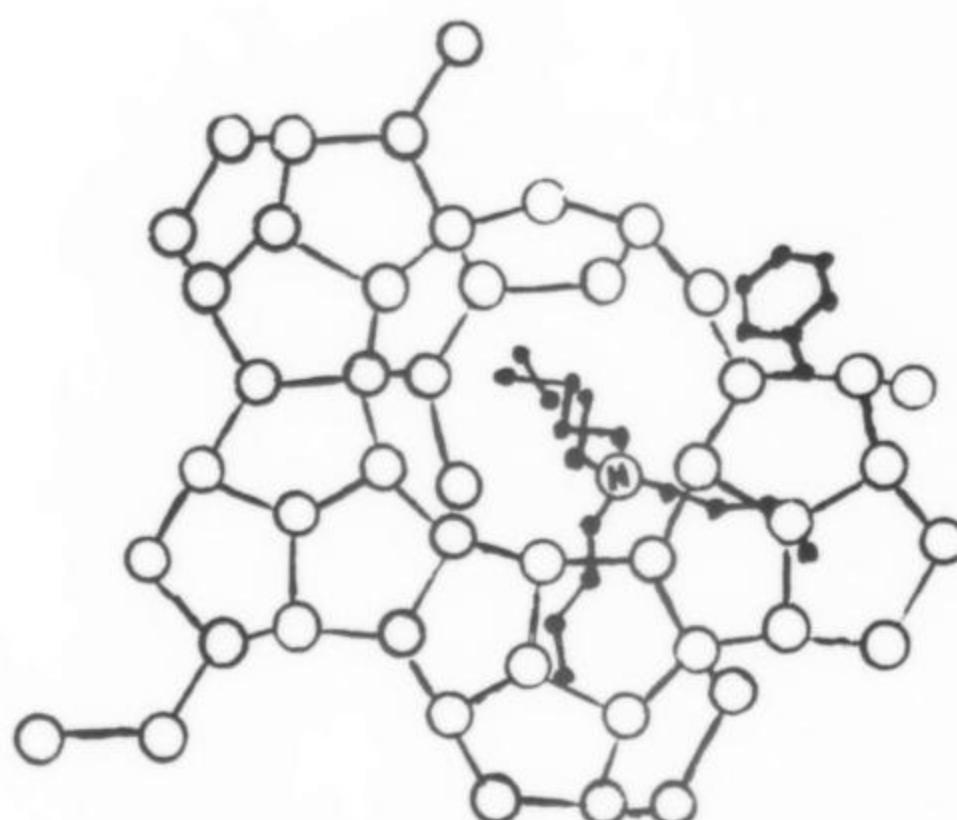


FIG. 9. Projection of clathrate cavities containing tetrabutylammonium and benzoate ions within framework of water molecules. Note position of benzoate ion at right-hand side. (From Bonamico, Jeffrey and McMullan (7).)

9. In regard to the tetrabutyl ammonium group, the arrangement is essentially as in the isoamyl compound, each hydrocarbon chain projecting into a polyhedral cavity. A new aspect of the benzoate compound is the position of this anion. In this case (upper right of Fig. 9) the benzene ring is enclosed in another polyhedron and the  $\text{COO}^-$  group fits into one of the pentagonal faces taking the places of two  $\text{H}_2\text{O}$  molecules.

Finally a third structure (6) in this series, illustrating some other points, is that of  $(n\text{-C}_4\text{H}_9)_3\text{S}^+\text{F}^- \cdot 23 \text{ H}_2\text{O}$  shown in Fig. 10. In this structure we find departures from the relatively simple 12- to 16-faced polyhedra characteristic of the other hydrates. The cages in Fig. 10 are much more unsymmetrical and complex and are bounded by quadrilateral rhombuses (see center of Fig. 10) as well as by pentagonal and hexagonal faces. The cavities are both large and irregular. Furthermore it is of interest that a pair of  $(n\text{-C}_4\text{H}_9)_3\text{S}^+$  units<sup>2</sup> occur in "back-to-back" juxtaposition within a single major cavity; the  $\text{S}^+ \cdots \text{S}^+$  distance is 3.49 Å, essentially a normal van der Waals distance. Particularly striking in this connection is the fact that the two triplets of apolar butyl groups of two molecules face away from each; they tend to be far apart and embedded in (three pairs of minor) water cages rather than adjacent to each other and excluding intervening water molecules, as expected from the hydrophobic viewpoint. In this arrangement the alkyl groups also almost completely shield the  $\text{S}^+$  atoms from an aqueous environment. In essence then the guest species is the entire double molecule,  $(\text{C}_4\text{H}_9)_3\text{S}^+ \cdots \text{S}(\text{C}_4\text{H}_9)_3$ . The complete water cage, only half of which is shown in Fig. 10, is made up of 48 pentagonal faces, 10 hexagons and two quadrilaterals. It has a volume of 1550 Å<sup>3</sup>. The six minor incomplete polyhedra

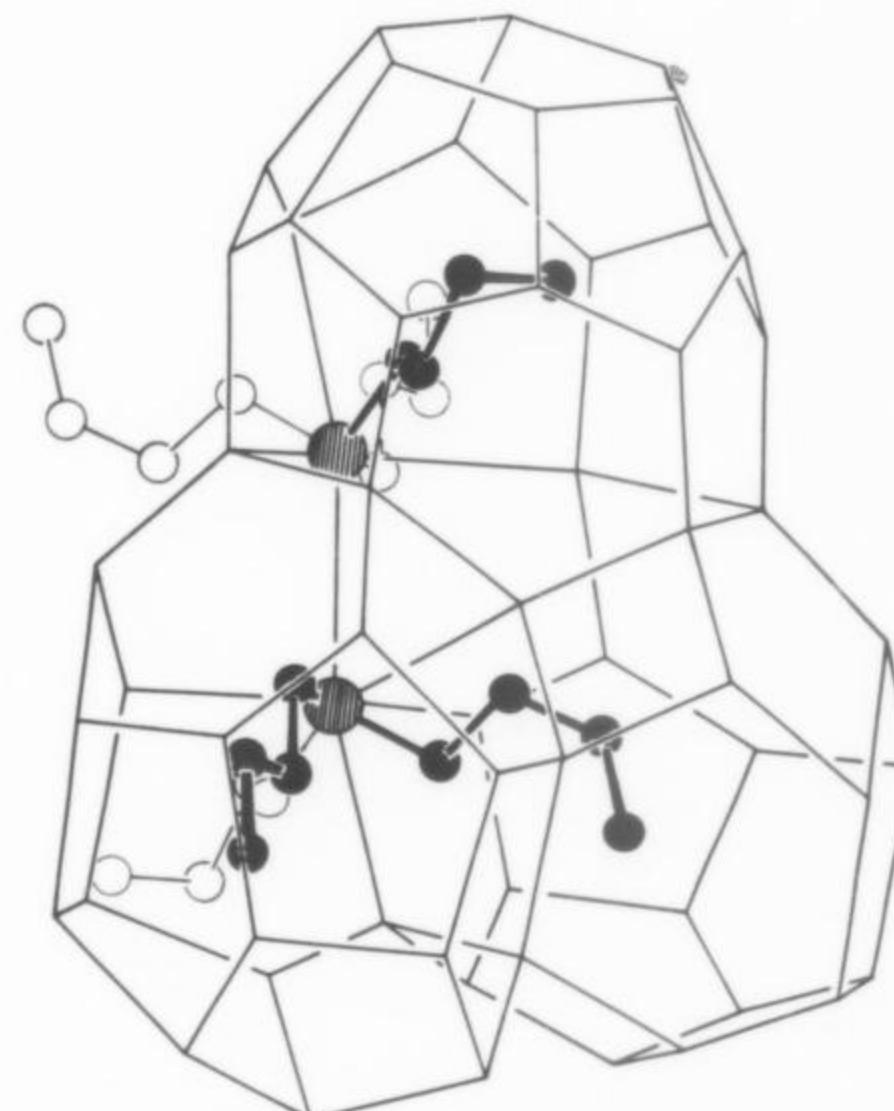


FIG. 10. Projection of clathrate cavity containing a pair of tributylsulfonium ions. Only one-half of the cavity is shown, the part into which shaded carbon chains project. The second half surrounds the open-circle carbon chains. There is a center of symmetry midway between the  $\text{S}^+ \cdots \text{S}^+$  atoms.  $\text{F}^-$  ions cannot be distinguished from O atoms of  $\text{H}_2\text{O}$ , probably because they are disordered over several water sites. (From Beurskens and Jeffrey (6).)

enclosing each of the alkyl side chains are symmetrically arranged around a center of symmetry at the midpoint of the  $\text{S}^+ \cdots \text{S}^+$  line. There are three types of these incomplete compartments with different sizes and shapes, made up of the following polygonal faces: a) nine pentagons and two hexagons; b) seven pentagons, two hexagons and one quadrilateral; and c) eight pentagons and one hexagon.

In summary, then, this structure (Fig. 10) shows that a wide variation is possible in regard to size and shape of the water cages enclosing an apolar moiety. The relatively rigid geometrical requirements that characterized the structures of the earlier hydrates can be substantially relaxed, and thus a more general form of clathration becomes possible.

It has already been mentioned that mixed hydrates are also possible, with  $\text{H}_2\text{S}$  as a second guest molecule. It is perhaps pertinent to cite one other system of this type, methane-isopropylene (46), which shows in fact that a mixed hydrate may stabilize substantially an otherwise weak one (Table 3).

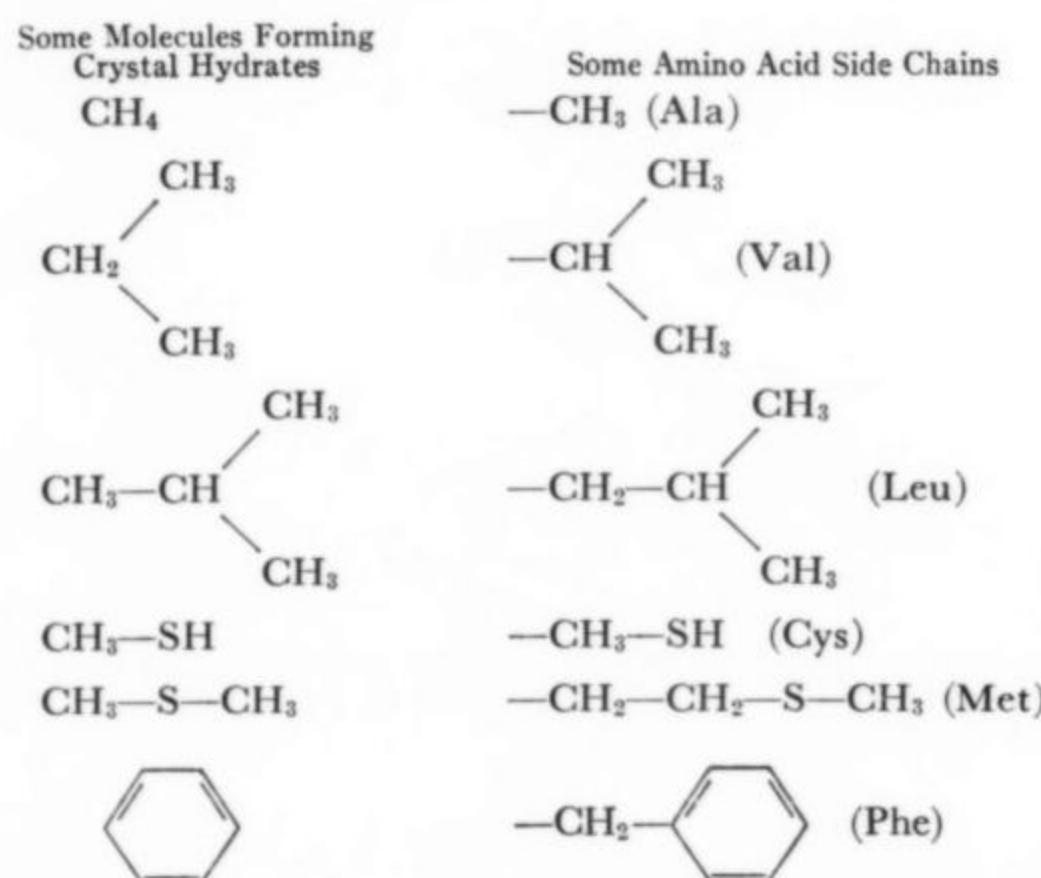
It is thus apparent that water is a remarkably versatile substance in regard to hydrate formation, being capable of forming a large variety of cagelike structures to accommodate itself to a whole gamut of apolar groups. It has been our feeling (36), therefore, that similar structures should form around apolar groups projecting from a macromolecule particularly since the local concentration of such groups in the polymer region is high and cooperative interactions should be possible.

<sup>2</sup> The  $\text{F}^-$  ions cannot be distinguished from O atoms of water molecules of the framework. It is believed (6) that they are disordered, in several different water sites, in the framework, and probably near the apolar cations.

TABLE 3. Hydrate solid solutions

Mole %		Melting Point, °C at 26 atm
CH <sub>4</sub>	C <sub>3</sub> H <sub>8</sub>	
100	0	0
99	1.4	5
97	3	7
92	8	10
70	30	13

TABLE 4. Comparison of hydrate formers with amino acid residues



Particularly in regard to proteins, there is a remarkable parallelism between its apolar side chains and corresponding small molecules known to form clathratelike hydrates (Table 4). If small molecules such as CH<sub>3</sub>—CH(CH<sub>3</sub>)<sub>2</sub> and CH<sub>3</sub>—SH form stable water cages, it seems reasonable that leucyl [—CH<sub>2</sub>—CH(CH<sub>3</sub>)<sub>2</sub>] and cysteinyl (—CH<sub>2</sub>—SH) side chains of proteins should be able to do so also. In the protein, furthermore, the local concentration of side chains is high and one would expect a cooperative effect of adjacent apolar ones to induce a stabilized arrangement of water in a microscopically crystalline array (Fig. 11). We would therefore attribute apolar-bond stabilization in these macromolecules to the formation of these ordered water regions or "hydratoids."

On this basis (Fig. 11) one can understand the normal effects of temperature in denaturing proteins. We would attribute the effect of temperature to the disorganization or melting of the hydratoids. Likewise we would attribute the denaturing effect of urea to its ability to disorganize the hydration lattice and thereby decrease the stabilization of the protein originating from the presence of apolar groups. On the basis of this picture (Fig. 11) one can also understand a variety of masking phenomena observed in proteins. For example, let us suppose that one of the side chains is an —SH group in the midst of apolar groups, and let us remember that the mercaptan itself acts as an apolar group. All of these

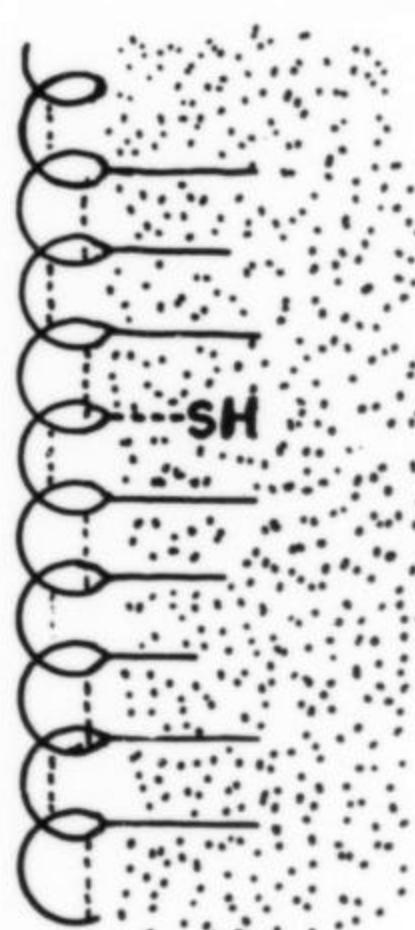


FIG. 11. Schematic diagram of "hydratoid" formation around apolar groups of protein macromolecule.

could form a hydrate or immobilized array of water molecules around that region of the protein. The —SH group then would not react readily with a mercaptant-titrating reagent, such as silver ion, since it would be difficult for the silver to penetrate the immobilized water. Likewise we can understand why the presence of urea would abolish masking; if urea breaks down the icelike lattice, silver ion can reach the —SH group.

This then is a brief exposition of the hydratoid picture, an alternative to the hydrophobic viewpoint. Both ideas are essentially speculations on how the structure of water may be involved in interactions with, and stabilization of, natural and synthetic macromolecules. Much current work is aimed toward clarification of these views.

#### METHODS OF FURTHER INVESTIGATION OF WATER-MACROMOLECULE INTERACTIONS

The physicochemical methods that are currently being applied to the elucidation of the molecular nature of these interactions fall largely into three broad categories: thermodynamic, hydrodynamic, and electromagnetic.

The thermodynamic approaches include some largely empirical and others highly theoretical. Based on free energies of transfer of apolar groups from an organic to an aqueous phase, Kauzmann (35), originally, and Tanford (54), in greater detail recently, have estimated the contribution that apolar groups would make to protein stability due to hydrophobic bonding. An alternative approach starting from a detailed model of water structure and using statistical-mechanical formulations has been made by Némethy and Scheraga (44, 45) to compute a number of thermodynamic properties attributable to hydrophobic interactions. Extensive theoretical studies of solvent interactions with macromolecules have also been made by Sinanoğlu (53). Since most of these investigators participated in this Symposium, they can best present their own views.

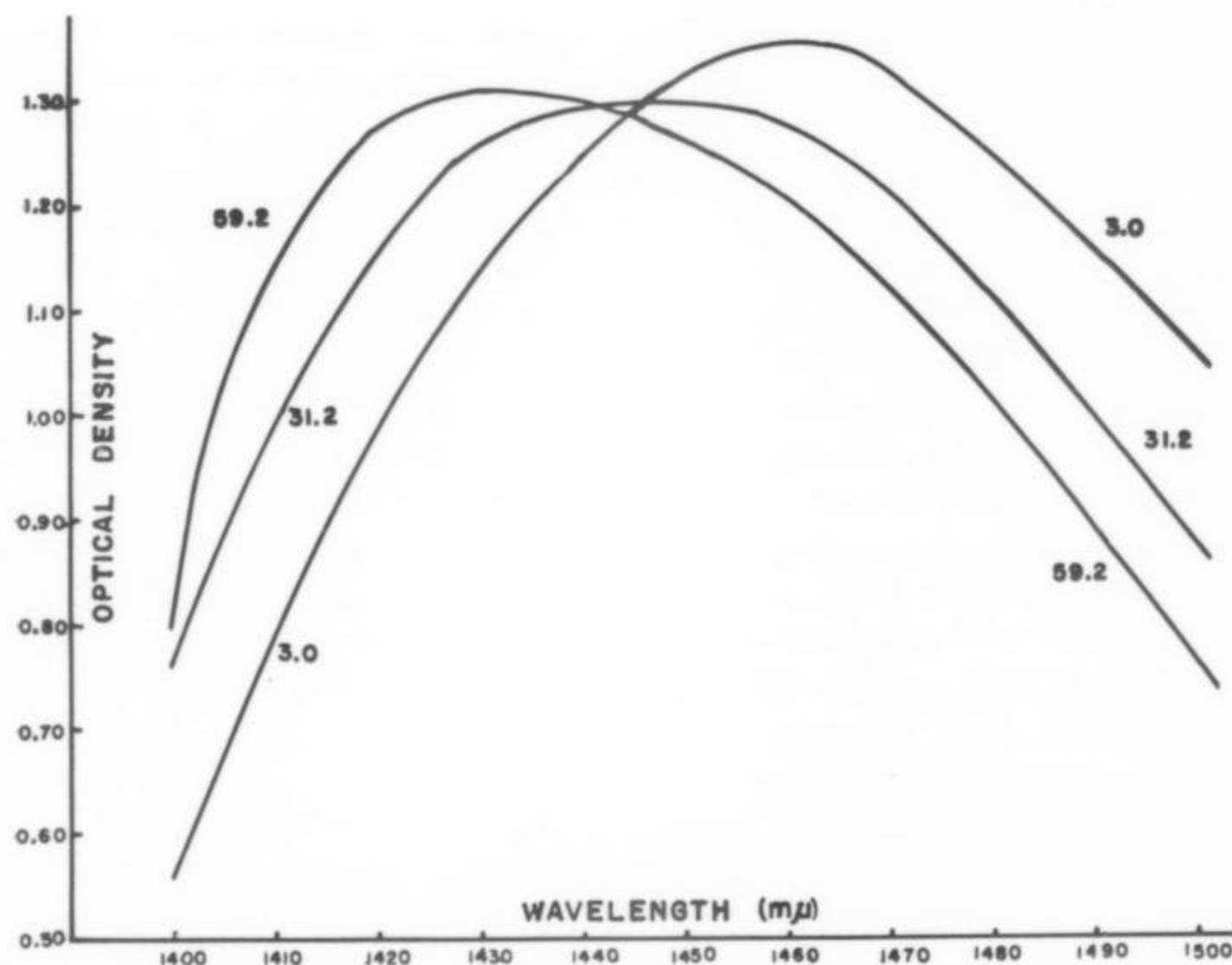


FIG. 12. Absorption spectra in the near infrared for water at 3.0, 21.2, and 59.2 °C.

Ultracentrifugation studies (13, 21, 26, 29, 41, 49, 50) have been widely used in different ways to gain some insight into the preferential binding of water by a macromolecule. In most cases the properties of a system at equilibrium, rather than hydrodynamic parameters, are actually used to estimate the amount of bound water. Buoyancy experiments have been carried out under a variety of conditions and preferential hydration calculated for proteins, such as ribonuclease, bovine serum albumin and collagen, DNA, and tobacco mosaic virus. Even in the theoretically soundest experiments, however, one obtains only an estimate of preferential hydration, not an indication of the nature of the interaction.

In principle, electromagnetic methods should be the most promising in examining water in macromolecular systems. Radiation from the X-ray to the megacycle regions has been used. Ideally if X-ray diffraction could be applied to macromolecules in solution, one could derive the detailed structure of the biopolymer and thereafter deduce, directly or indirectly, the state of water molecules in the neighborhood of various groups in the macromolecule. Such a possibility however is not even on the horizon as yet. Nevertheless some general information on preferential hydration can be obtained from low-angle X-ray diffraction, and for DNA relatively large amounts (70 % by weight) of bound water, impenetrable to ions, have been found (43). More detailed information, although not definitive answers, has been obtained from diffraction studies with wet crystalline or fibrous proteins. Structurally organized water, contributing to the stabilization of the protein molecule, has been deduced from X-ray studies of ribonuclease (1). Similarly X-ray patterns of collagen show that water molecules are arranged in a semiregular pattern very close to the chains and play a role in the stabilization of macromolecular configuration (16). One is of course also reminded of the observation that the

helical configuration of DNA fibers is dependent on the presence of water at high humidities (23, 40).

Recent advances in the utilization of the resolving power of electron microscopy and improvements in the preparation of specimens have increased immensely the detail which can be seen in biological systems. Low-temperature technics (19, 20) have made possible, at least in some cases, direct visualization of macromolecular structures even in the hydrated state. Particularly interesting are recent photographs of Fernandez-Moran (20) of ferritin subjected to high pressures (100 atm) or argon,

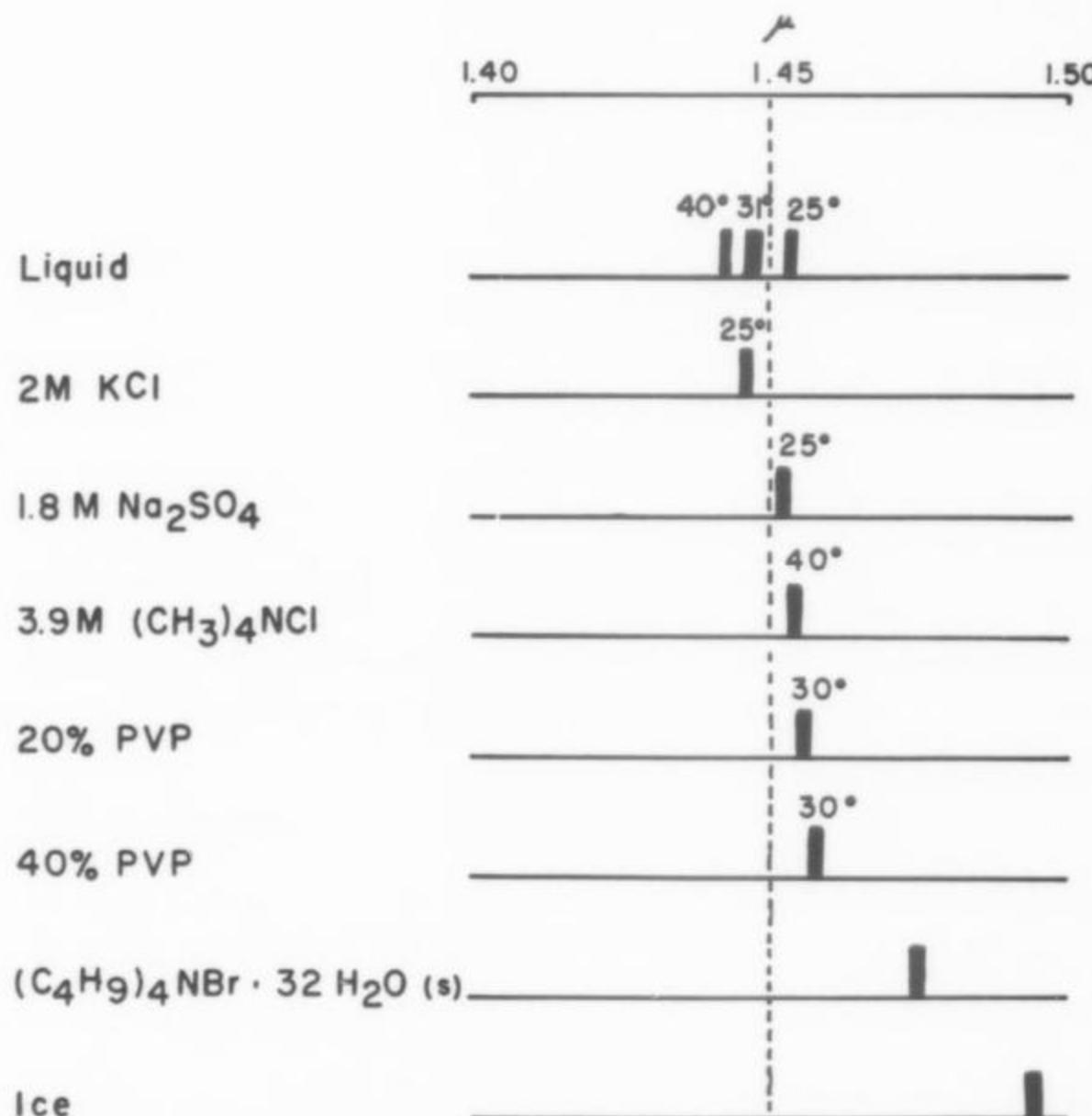


FIG. 13. Absorption peaks in the near infrared for water, aqueous solutions, an apolar crystal hydrate, and ice. PVP represents polyvinylpyrrolidone.

which indicate the presence of hydrate microcrystals in the protein shells of the ferritin molecules.

There is a long list of investigations of bound water in biological macromolecules by nuclear magnetic resonance. In principle this is a very attractive method since the proton resonance should definitely reflect any change in the state of the water. The expected broadenings in signal have been observed repeatedly (12, 27, 30-32, 51, 52). Nevertheless it is still not certain that the increased line widths reflect only the immobilization of water molecules (2, 8, 33). Much more detailed studies have been made for collagen (5) in which both the magnitude and angular dependence of the splitting of proton resonance have been measured. These indicate that a large part of the water associated with the collagen reorients, restrictively, chains of water molecules forming parallel to the fiber axis with a repeat distance of 28.6 Å. Such studies, however, cannot be carried over to aqueous solution. In any event much remains to be done before nuclear magnetic resonance results can be confidently evaluated.

Finally I should like to describe briefly a few unpublished exploratory investigations (14) which we have made using infrared absorption in the region of 1.4-1.5 μ. The absorption by the O-H group of water in this range is fairly broad. Nevertheless there is a substantial shift in position of the peak as one changes the temperature of the liquid (Fig. 12) or if one compares ice with liquid H<sub>2</sub>O (Fig. 13). As expected, the more mobile, less hydrogen-bonded system has a peak at a lower wavelength (or higher frequency). Thus it is evident from the near infrared (as has been long known from other studies) that as the temperature of water is increased, its degree of organization is decreased.

It is also evident from Fig. 13 that the addition of KCl to water breaks down the structure of the solvent, confirming the conclusions of Dr. Frank from other studies. It is of interest to note in passing, in view of its behavior on polymers described at the outset of this discussion, that Na<sub>2</sub>SO<sub>4</sub> does not seem to perturb the structure of water in the same manner as KCl. Even more striking is the effect of (CH<sub>3</sub>)<sub>4</sub>N<sup>+</sup>Cl<sup>-</sup>, which clearly,

from the results in Fig. 13, has an ordering effect on the structure of water. Similar ordering effects are observed in solutions of polyvinylpyrrolidone (PVP), a polymer with a structure similar to that of polyvinylmethyl-oxazolidinone, (1). This apolar polymer is evidently able to immobilize water molecules in its vicinity. Other infrared studies (15, 17) indicate that water is involved in stabilizing the helical configuration of DNA.

These experiments are very crude exploratory studies. The overlapping of various fundamental vibrations and combination bands in the spectrum of pure H<sub>2</sub>O complicates interpretations very much. It is apparent that we ought to follow the lead of Hornig (28) in Raman spectra and use dilute solutions of HOD in D<sub>2</sub>O. Some preliminary observations of the OH stretching vibration in such solutions show much sharper spectra and look very promising.

This is a sampling of the physicochemical methods that are being used to gain some insights into the structure of water around macromolecules. Being a sampling, this discussion has not mentioned many other pertinent studies. Furthermore no description has been given of chemical approaches, such as in our own studies (38) of the shifts in acid-base properties of substituents conjugated to proteins.

It is apparent from the results obtained from a very diverse group of experimental techniques that interactions of biological and synthetic polymers with water molecules must play an important role in establishing macromolecular configuration. However, despite widespread interest and much experimentation based on several different hypothetical models, the molecular nature of the influence of water on macromolecules and of the reciprocal effect of the polymer on water molecules remains uncertain. In fact, even the structure of liquid water itself is still an unsettled problem, and a solution to this question may be a prerequisite to the understanding of macromolecular hydration.

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