

A Quantitative Theory of Solute Distribution in Cell Water According to Molecular Size

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Abstract: A quantitative theory (based on the polarized multilayer (PM) theory of cell water, a subsidiary of the association-induction (AI) hypothesis) for the distribution in cell wafer of solutes of different molecular size is presented. From this theory, three predictions are derived: (1) **Linear distribution:** when the equilibrium concentration of a solute in cell or model water is plotted against its concentration in the external solution, a straight line is obtained, with a slope equal to the equilibrium distribution coefficient or q-value of that solute in the cell water. (2) **The size-rule:** The q-value is, as a rule, size-dependent: the larger the solute, the lower the q-value. (3) **Solutes with exceptionally high q-value may act as cryoprotectants.**

Earlier published data on the distribution of various nonelectrolytes in solutions of 15% poly (ethylene oxide) (PEO), 20% NaOH-denatured hemoglobin and 18% gelatin gel agree in general with the predictions of the theory. They demonstrate linear distribution and obey the size rule. The q-value vs. molecular volume plots can be fitted by theoretical curves after correction had been made for a minor fraction of free water in the system.

The new theory has made it possible for the first time to estimate quantitatively the intensities of (bulk-phase) water polarization. In the 20% solution of NaOH-denatured bovine hemoglobin, 18% gelatin gel, 15% PEO solution, and 39% native bovine hemoglobin solution, the intensities of polarization of the bulk-phase water (over and above the vastly greater water-to-water interaction in normal liquid water) are respectively estimated at 16.5, 14.9, 11.7 and 3.8 cal per mole of water. The intensity of water polarization is higher in sulfonate ion exchange resin in the Li⁺ form (55.2 cal/mole) than in the Rb⁺ form (26.6 cal/mole).

The theory has also made it possible to demonstrate that from 72% to 75% of the water in gelatin gel and in solution of NaOH-denatured hemoglobin, and of PEO are polarized and are endowed with solvency properties quite different from normal liquid water. It has also been demonstrated that each polar site in the model systems polarizes all water molecules in the first and second layer and at least some water molecules in the third layer of water molecules surrounding each polar site. The multiplicity in the number of layers of water molecules polarized is thus established in these model systems.

IN THE EARLY DECADES of the twentieth century, some biologists believed that part of the water in living cells is “bound” and “nonsolvent”. Since the level of a solute in the cell is based on the *total* cell water, an implicit prediction of this theory is that all permanent solutes that are neither rapidly metabolized nor actively pumped in or out of the cell should reach the *same* low relative level in the water of the same or similar cells as that in the surrounding medium. However, results of experimental studies contradicted this expectation. Thus Kamnev discovered in 1938 that both galactose and sucrose are found in the aqueous phase inside frog muscle cells at low but *different* levels. Based on these findings, Kamnev suggested that the low steady levels of sucrose and galactose in frog muscle reflect *a reduced solubility of the protoplasm for the sugars* (Kamnev, 1938, see also Nasonov, 1962, p. 115; Troshin, 1966, p. 111).

Troshin (1951a, b, 1966) then pointed out that similar reduced solubility for solute had already been demonstrated in nonliving “coacervate”—a colloid-rich droplet or layer immiscible with the surrounding colloid-poor phase (Holleman et al., 1934). Troshin further suggested that living protoplasm exists as coacervate and that it is the coacervate nature of the protoplasm that underlies the reduced level of solutes like sucrose and galactose (Troshin, 1966, Chapter 3). However, Troshin did not provide a molecular mechanism as to how water in coacervate differs from normal liquid water nor why coacervate water partially excludes solutes like sucrose.

The cell's ability to maintain solutes at concentrations different from those in the surrounding medium is not limited to sugars and other nonelectrolytes but includes electrolytes as well, sometimes in an even more spectacular manner.

My early research on K^+/Na^+ distribution in frog muscle soon led to the conclusion that the energy needed to operate the postulated Na pump far exceeds the energy available to the cell. This finding provided one of the main reasons for my belief that the Na pump hypothesis is not tenable (Ling, 1951: 1952; 1992a, Chapter 2).

As an alternative, I suggested that the selective accumulation of K^+ over Na^+ in living cells arises from preferential (electrostatic) adsorption of K^+ (over its competitor, Na^+) on the negatively-charged β - and γ -carboxyl groups of cell proteins. A quantitative model able to account for a 10 to 1 selectivity of K^+ over Na^+ was offered (Ling, 1951, 1952; see also Ling and Ochsenfeld, 1991). This early model eventually evolved into a general theory of the living cell, called the association-induction (AI) hypothesis (Ling, 1962, 1969, 1984, 1992a).

In 1965, I introduced the polarized multilayer (PM) theory of cell water (Ling, 1965, 1972) as an integral part of the AI hypothesis. According to the PM theory, virtually all cell water assumes the dynamic structure of polarized multilayers. The pervasive polarization and immobilization of the bulk-phase cell water underlie the partial exclusion from cell water of Na^+ as well as nonelectrolytes (Ling, 1965, 1970, 1972). Soon it was recognized that the equilibrium levels of nonelectrolytes in frog muscle cell water varied with the molecular size of the nonelectrolytes; the larger the solute the lower the level found in cell water (Ling, 1970). This relationship was referred to as the “size rule” (Ling, 1987).

I present here the first complete and *up-to-date* theory of solute distribution in cell water (and model systems) with special emphasis on the relationship between the molecular size of the solute and its equilibrium level. I will also examine, in the light of this newer version of the theory, the extent and intensity of water polarization in various inanimate models on the basis of equilibrium distribution of solutes of various sizes already in the literature (e.g., Ling and Hu, 1988). I hope what is set forth here may help the reader in assessing the true

significance of what I believe to be a set of pivotal experimental findings described in a companion paper which follows (Ling et al., 1993).

Theory

I. A brief sketch of the polarized multilayer (PM) theory of cell water

Given a polar surface carrying properly-spaced-charged sites and the physical properties of the water molecules, the adsorption of polarized multilayers of water on the polar surface is an inevitable consequence of physics (see Ling, 1992a, p. 69-71). It is little surprise that physicists have long ago derived equations for the adsorption on polar surfaces of multilayers of gaseous molecules with permanent dipole moments (Bradley, 1936; see also Brunauer et al., 1938). Next I briefly sketch my *polarized multilayer (PM) theory of cell water*.

A positively charged (P) site or negatively charged (N) site on a solid surface attracts, orients and adsorbs water molecules (*permanent* dipole moment, 1.86 debyes; polarizability, $1.44 \times 10^{-24} \text{ cm}^3$). The adsorbed water molecule, equipped now with an additional *induced* dipole moment, in turn, attracts, orients and adsorbs another water molecule. Electrical polarization produces, eventually, a row of adsorbed water dipoles, all oriented in one or the other direction depending on the polarity of the adsorbing site. (A more detailed discussion on how far the induced dipoles due to the N and P sites can go and what are responsible for the long range polarization of deeper layers of water molecules will be presented in the near future, Ling, 1994).

Since parallel electric dipoles oriented in opposite directions attract each other, every water molecule in an oriented row may attract not only its nearest neighboring water molecules in the same row but also neighboring water molecules in the adjacent rows. This lateral attraction is most favorable to adsorption if the N and P sites on the solid surface are arranged like a checkerboard with each N or P site surrounded respectively only by P or N sites and at a distance apart roughly equal to the diameter of a water molecule. Such a surface carrying a checkerboard of alternatingly N and P sites is referred to as an *NP surface*. (For an earlier view on dipolar surface and solvent polarization, see de Boer and Zwikker, 1929.)

An NP surface carries one type of charged site that produces stable multilayers of polarized water molecules. Polarized multilayers of water at least 5 molecules thick have been demonstrated on the NP surface of titanium dioxide crystals (Harkins, 1945). Deeper layers of water molecules are polarized by two closely juxtaposed NP surfaces called an *NP-NP system* and by another kind of charged-site assembly, *known as an NP-NP-NP system*: a matrix of linear polymer chains bearing properly spaced, alternately N and P sites (see below, also Ling and Hu, 1987; Ling and Ochsenfeld, 1989).

All living cells are endowed with an abundance of linear charge-bearing polymers, i.e., the proteins. According to the PM theory, some cell proteins exist in *the fully-extended conformation* with their backbone (positively-charged) NH and (negatively-charged) CO groups functioning as the properly spaced P and N sites of an NP-NP-NP system. *A very important corollary of this theory is that chains effectively alter the properties of bulk-phase water. Native globular proteins—with their backbone NH and CO groups locked in a-helical or other intra- and/or inter-macromolecular H-bonds and thus shielded from the bulk-phase water—do not. or do so weakly.*

The fully-extended-conformation of a protein may result from a specific non-helix-forming primary structure-as in the case of gelatin, and oxygen-containing polymers like poly(ethylene oxide) (PEO) or poly(ethylene glycol) (PEG) (Ling *et al.*, 1980a, 1980b; Ling, 1992a, p. 81); or in response to secondary structure-unravelling denaturants (e.g., urea, NaOH) (Ling *et al.*, 1980a; Ling and Ochsenfeld, 1989).

From these and other *in vitro* studies, I was able to sort out two kinds of experimental models: *extroverts** and *introverts*.* Extroverts include gelatin, PEO, PEG and urea-, or NaOH-denatured proteins. Introverts include most native proteins (for definition of the term, native protein, see Ling, 1990, p. 760; 1992a, p. 37). All extroverts reduce the solubility of the bulk-phase water for sucrose, Na⁺ (salts) and free amino acids, found as a rule in low concentrations in living cells; introverts are weakly effective or ineffective (Ling, 1992a, pp. 106–109).

Experimental studies of the past two decades have confirmed the theoretical expectation that a spectrum of characteristic properties of living cells-exemplified by the reduced levels of solutes maintained in cell water-are reproduced in water under the influence of extrovert models and either not at all or only very weakly in water containing introvert models (Ling, 1992a, p. 108-109).

II. Theory of solute distributions between cell water and external medium

The primary objective of this communication is to introduce a quantitative theory for the equilibrium distribution of solutes of different molecular sizes between water in living cells (or model systems) and water in the surrounding fluid. Since most of my readers are not physicists, I introduce two illustrative analogies and some elementary concepts of the branch of physics that will be needed for resolving the problem on hand, statistical mechanics. (Those already familiar with the subject can go straight to Section IIB.)

A. A preamble: two analogies and a little statistical mechanics

If one pours popcorn onto a frying pan, the kernels of corn will be found in the narrow space on the surface of the pan, as they are held down by the gravitational force. Now if heat is applied to the pan, some corn will pop up into the wider space over the pan. This redistribution of the corn in space is the consequence of the application of heat energy to the system and the kinetic energy gained by the corn kernels which enables them to escape momentarily the confining force of gravity.

In below-zero temperature, one can dry frozen laundry in open air. Yet, if the same frozen laundry is laid out in a small container kept at the same below-zero temperature, the laundry will not dry. This difference in drying shows that the ability of the water molecules to escape the confining molecular forces in ice depends on the volume of the space into which it can escape. When the volume of this space is enormous, as the open air, most water molecules escape by sublimation; when the volume of the space is much smaller, as in a closed container, most water molecules remain in the ice. As in the popcorn analogy, the escape of water is also the consequence of heat energy absorbed from the environment (even

* Fully extended linear polymers with properly spaced polar sites directly exposed to the bulk-phase water are called *extroverts*; similar linear polymers existing in the folded conformation with polar sites internally neutralized are called *introverts*.

though the temperature is below zero). At absolute zero temperature, no water sublimates, even when exposed to the open air.

Why should the distribution of water molecules between ice and the air depend so much on the volume of the air space and on the temperature? To answer, we resort to some elementary principles of Quantum Mechanics and Statistical Mechanics.

Quantum mechanics tells us that in response to warming the water molecules gain kinetic energy; the energy levels reached are limited to certain discrete values—equivalent say, to the popcorn being allowed to rise up to 1 inch, 2 inches, $2\frac{3}{4}$ inches from the pan surface but nowhere between and beyond these levels. With this kind of limitation, more popcorn will be found in the space over the pan if at the same elevated temperature, there are more allowed levels in the open space. While the levels of 1 inch, 2 inches, etc. mentioned above have no other meaning than a prop for my presentation, in the real world, the allowed energy levels for the distribution of water molecules in ice and in air are real, as dictated by the laws of Quantum Mechanics.

Quantization of the energy is ascribed entirely to the fact that each particle (e.g., a water molecule) is represented by a wave; only those energies are allowed whose wavelengths fit the volume in which the particle is confined. Since more waves with different wavelengths can fit into a larger space than a small one, there are more allowed energy levels in larger spaces. As a result, the larger the volume, the narrower are the spacing of energy levels: the smaller the volume, the wider the spacing between the successive energy levels.

In ice, each water molecule is confined to a very *small* volume defined by the interatomic forces that imprison each water molecule to a specific location in the ice crystal. Accordingly, the energy levels are few and spread widely apart. (And at the same temperature, water molecules are found in significant numbers only in the lowermost of them). In contrast, the energy levels in the wide open space are very close together. Here, within a narrow range of energy there is an enormous number of allowed quantized states. (And at the same temperature, water molecules are found at significant concentrations at many of these higher levels). The vastly greater number of allowed energy levels in the wide open space accounts for the success of sublimation in open space but not in a closed small container.

Summarizing, one notes that the attractive energy holding back water molecules in ice is more than offset by the much larger number of ways the vaporized water molecules can adopt in the open space than in ice. In other words, a large entropy gain is the primary cause for the sublimation of frozen water.

This entropy gained during sublimation depends on the thermal energy received and is referred to as thermal entropy. It is the thermal entropy gain that leads to sublimation. However, there is another kind of entropy, called *configurational entropy* which is independent of the thermal energy but depends on the number of different sites available to the water molecules.

In ice, each water molecule occupies a particular position or site. If one water molecule possesses 1 unit of thermal energy and another one possesses 2 units of thermal energy, this would be recognizably different from the case where the first water molecule possesses 2 units of thermal energy while the other possesses 1 unit of energy. Since a vast number of sites are available in the ice crystal, there are numerous ways or configurations that are recognizably different for the same distribution of thermal energy (e.g., 2 units for one, 1 unit for another) and the number of these recognizably different configurations give rise to the configurational entropy.

In contrast, each water molecule in air can reach anywhere in the open space. That is, they all share the same site. Consequently, for each thermal energy distribution, there is only one way or configuration. The configurational entropy of the water molecules in the vapor phase is therefore zero.

Indeed, the configurational entropy difference between water molecules in ice and in air actually discourages water molecules from leaving the ice. To sublime, the thermal entropy gain must be so large that it can overcome the combined retentive tendencies of the sublimation energy and the configurational entropy advantage.

Quantum mechanics tells us what the allowed energy levels are. Statistical mechanics tells us how particles like water molecules distribute themselves among these energy levels at a specified temperature. Thus if we represent the number of particles on a specific energy level, the r th, as n_r , and the total number of particles in the assembly as n , then

$$n_r = \frac{n \exp(-e_r / kT)}{(\text{p.f.})}, \quad (1)$$

where e_r is the energy of the r th level. k and T are respectively the Boltzmann constant and absolute temperature. (p.f.), the abbreviated symbol for "partition function", is the all-important statistical mechanical expression representing the sum taken over all the allowed energy states in that system at a specified temperature and volume:

$$(\text{p.f.}) = \sum \exp\left(\frac{-e_r}{kT}\right) \quad (2)$$

The partition functions are important in deriving quantitative relationships in equilibrium phenomenon including solute distribution problems of interest here.

B. An equation for solute distribution based on a lattice model of cell wafer

In Appendix 1, I describe the derivation of a set of equations (of these only equations 17 and 18 are displayed here) for the equilibrium distribution of solute B between phase 1 (e.g., cell water) and phase 2 (e.g., the external bathing solution). In this derivation, two major concepts were adopted: (1) liquid water, whether normal or polarized, is represented as a flexible lattice; (2) solutes are treated as chains containing different numbers of similar segments, each segment having the same dimensions as those of a single water molecule.

The equations represented here are equations 17 and 18:

$$q = \frac{{}^1C_B}{{}^2C_B}, \quad (17)$$

where q is the equilibrium distribution coefficient of the solute B in phase 1 and phase 2, while 1C_B and 2C_B are the corresponding concentrations of the solute B in phase 1 and phase 2 respectively. And

$$q = \frac{{}^1(\text{p.f.})_B}{{}^2(\text{p.f.})_B} \cdot \exp\left(-\frac{\eta}{RT}\right) \quad (18)$$

where ${}^1(\text{p.f.})_B$, and ${}^2(\text{p.f.})_B$ are the partition functions of solute B in phase 1 and phase 2 respectively. η is the energy difference of the ground state of the solute B in phase 1 and phase 2 and is referred to as the *polarization* energy.

Equation 18 as such gives no explicit relationship between the size of a solute molecule and its q -value. In the following section, I will further elaborate on this equation so that quantitative predictions of the relationship between molecular volume and q can be made.

C. *A quantitative model for the distribution in cell water of solutes of different molecular volumes*

Equation 18 shows that the equilibrium distribution coefficient, or the q -value, of a solute between cell water and the external medium is determined by the partition-function ratio, ${}^1(\text{p.f.})_B / {}^2(\text{p.f.})_B$, and by the value of the polarization energy, η . I have suggested earlier that the polarization energy η , in turn, may be broken up into two components, referred to respectively as the volume (or solvent) component of the polarization energy and the *SURFACE* (or solute) component of the polarization energy (Ling, 1984, p. 167; 1992a, pp. 77-79). Postponing further description of these components to a later section, I now proceed to resolve the full (observed) q -value of a solute into three components: q_v represents the q -value component arising from the volume (or solvent) component of polarization energy; q_s represents the contribution from the surface (or solute) component of the polarization energy; and q_p the contribution from the partition-function ratio or entropy component. Thus

$$q = q_v q_s q_p \quad (19)$$

I shall discuss the relationship between solute size-as represented by its *molecular* (or molar) volume*-and q_v , q_s , and q_p separately before looking into the relationship between the molecular volume of a solute molecule and its full q -value, i.e., the products of q_v , q_s and q_p

(1) The volume component (or solvent component) of the polarization energy

In transferring a solute molecule from the external medium into the cell water, a hole of the right size (and shape) has to be excavated in the cell water in order to accommodate the new arrival. To make such a hole, thermal energy must be spent. It is hue that thermal energy will also be recovered in filling up the hole left behind in the external solution. However, since the water-to-water interaction is stronger in the cell water (existing in the state of polarized multilayers) than in the external free water, the energy spent in excavating the hole is more than the energy recovered in tilling up the hole. This difference in the energy spent and recovered is one major component of the ground energy difference η of equation 18.

The difference between the energy spent in excavating the hole in cell water and the energy recovered in filling up the hole in the surrounding normal liquid water has been referred to up to now as the volume *component* of the energy term. Under certain conditions, the term solvent *component* may be more explicit. Thus the volume component comes entirely from the polarized cell water. The solute contribution here is indirect, being limited entirely to the size of the solute molecule, which determines the size of the excavated hole. These two names, volume component and solvent component will be used interchangeably.

Now, the larger the solute molecule, the larger is the hole to be excavated; the larger the

* The molecular or molar volume of a solute is equal to its molecular weight divided by the density of the solute (as a liquid) near the boiling point (Glasstone 1946, pp. 524-525). For the sources of the molecular volumes used in the present paper, see Methods in the companion paper following (Ling, Niu and Ochsenfeld, 1993).

hole excavated, the greater is the energy difference between energy spent and energy recovered. This then is the main cause for the predicted inverse relationship called the "size rule": *The larger the solute molecule, the lower its q-value in the polarized water.*

Quantitatively, q_v —the q-value change due to the solvent or volume component of the polarization energy—is simply:

$$q_v = \exp\left(\frac{-v\Delta E_v}{RT}\right) \quad (20)$$

v is the molecular volume of a solute under discussion. ΔE_v is the *specific solvent polarization* energy, equal to the difference between the energy spent in excavating a hole 1 cm^3 in size in the polarized (cell) water and the energy recovered in filling up a 1 cm^3 hole in the surrounding normal liquid water. ΔE_v is in units of $\text{cal}\cdot\text{mole}^{-1}(\text{cm}^3)^{-1}$.

In Figure 1, q_v is plotted against the molecular volumes of different solutes in water polarized to different intensities according to equation 20. Indicated in the figures near each curve in units of RT (where R is the gas constant and T the absolute temperature) is ΔE_v .

As shown in Figure 1 at all values of ΔE_v examined, there is an inverse relationship between q_v of the solutes and their molecular volumes, i.e., q_v follows the *size rule*.

As shown in Figure 1, for a specific solute of known molecular volume, its q_v -value in polarized water strongly depends on the magnitude of ΔE_v . At room temperature (25°C), RT is equal to 592 cal/mole. Thus the highest ΔE_v , computed and shown in Figure 1, $0.05RT$ is equal to $29.6 \text{ cal}\cdot\text{mole}^{-1}(\text{cm}^3)^{-1}$, corresponding to $29.6 \times 18.02 = 533$ cal per mole of water molecules, where 18.02 is the molecular volume of the water molecule in cm^3 's. The lowest figure of $0.0002RT = 0.0002 \times 592 = 0.12 \text{ cal}\cdot\text{mole}^{-1}(\text{cm}^3)^{-1}$ corresponding to $0.12 \times 18.02 = 2.16$ cal/mole of water molecules. It is remarkable that polarization which creates a difference in the water-to-water interaction as little as 2.16 cal/mole markedly reduces the solubility of the larger solutes.

When ΔE_v reaches one tenth the value of RT , even small molecules are partially excluded from the polarized water. And very large differences can be seen in the q_v -values of solutes different from each other by relatively small differences in the molecular volumes. This subject will be pursued in detail in a following paper on semipermeability of living cells.

To introduce the "surface (or solute) component" of the polarization energy, I call attention to the q_v -value of the water molecule itself. The theoretical curves of Figure 1 indicate that on account of the volume component of the polarization energy, a water molecule with a molecular volume of 18.02 cm^3 should have a q_v -value significantly below unity, its precise value depending on the value of ΔE_v . Yet, by definition, the q_v -value of water in polarized water must be unity, regardless of the value of ΔE_v . Therefore, some other factor(s) must enhance the affinity of the polarized water for the "immigrant" water molecule to annul completely the exclusion effect of the volume component of polarization energy, whatever the value of ΔE_v . The "surface or solute component" of the polarization energy introduced in equation 18 is the name given to this factor.

(2) The "surface component" (or solute component) of the polarization energy

When a water molecule is removed from the polarized water phase and replaced by another water molecule taken from the surrounding normal liquid water, the energy needed to excavate the hole in the polarized water exceeds the energy gained in filling the hole left behind in the external medium. However, once the water molecule from the surrounding

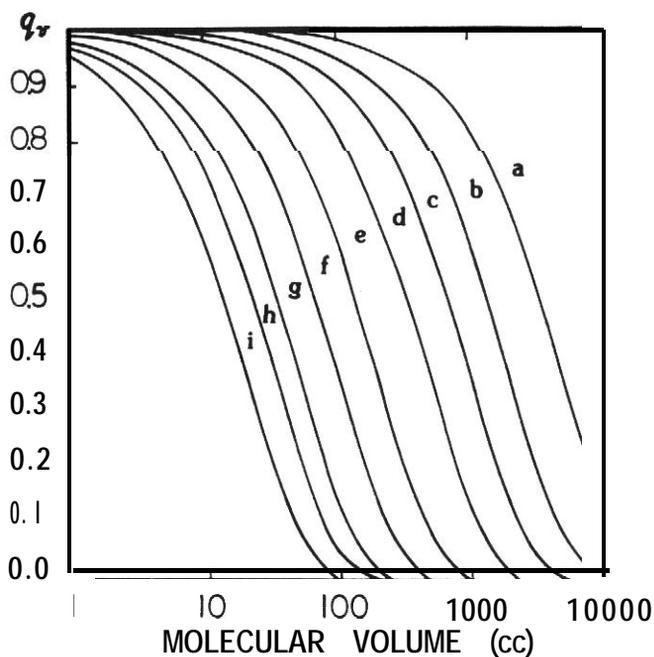


FIGURE 1. The theoretical volume (or solvent) component of the equilibrium distribution coefficient (q_v) for solutes of different *molecular* volume in water polarized at different intensity. The intensity of water polarization due to the volume component of the polarization energy is given as the *specific solvent polarization energy*, ΔE_v . The specific value of ΔE_v in units of RT per cm^3 is indicated by the letter near each curve, where a represents 0.0002; b, 0.0005; c, 0.001; d, 0.002; e, 0.005; f, 0.01; g, 0.02; h, 0.03; i, 0.05. R is the gas constant and T the absolute temperature. At room temperature (WC), RT is equal to 592 cal/mole.

medium is installed into the excavated hole, its "surface sites" interact with the surrounding water molecules, and the "immigrant" water molecule itself becomes polarized. In the end this newly introduced water molecule becomes indistinguishable from the polarized water molecule it has replaced.

Note that I have chosen to use water here not as solvent, but in the role of a solute. It is in this role that water provides special insight into the nature of the surface component of the polarization energy. Here, the "surface component" of the polarization energy exactly cancels the effect of the volume component (plus the partition-function-ratio component, see Mow). This follows from the fact that the same polarization that emanates from the surrounding water molecules and causes the polarization of the water removed, remains the same on polarizing the immigrant water molecule. And the surface geometry, charge distribution, and overall polarization of the removed water molecule are, of course, identical with those of the immigrant water molecule.

However, *when a solute molecule other than water is introduced into the excavated hole,*

the effectiveness of the surface component of polarization energy produced to counter-balance the volume component will vary according to the size, shape, polar site distribution and overall polarizability of the specific solute introduced. As a result, it is theoretically possible that the retaining effect of the surface component may be smaller than, equal to, or greater than the exclusion effect of the volume component.

Therefore, unlike the volume component which reflects exclusively the property of the solvent cell water, the "surface component" reflects the intensity of polarization of the bulk-phase water (the solvent) and especially the property of the **solute** molecule itself. For this reason, the "surface component" may also be called the "solute component". However, for the same reason that the names "volume component" and "solvent component" are used interchangeably, the names "surface component" and "solute component" will also be used interchangeably.

To derive a model for the surface component of the polarization energy and its effect on q_s , one needs to determine first the relationship between the surface and the volume of the solute. For this purpose, we return to an earlier section (or in more detail, Appendix 1), where each organic molecule under consideration is viewed as a chain of repeating segments each having dimensions not too far from that of a single solvent molecule, e.g., water. Thus in this model, each solute molecule is seen as a flexible cylinder of varying length. The surface of a cylinder with a cross-sectional radius a and length b , is $2\pi ab$, while its volume is $\pi a^2 b$. The surface is, therefore, equal to $2v/a$ where v is the volume of the solute molecule.

The molecular volume of water is 18.02. By assuming a spherical shape for the water molecule, one obtains a "molar radius" of $\{18.02(3.1416)^{-1}(3/4)\}^{1/3} = 1.626$ cm for the water molecule, and, indirectly, for all the solutes regarded as chains of segments sharing the same radius. The surface of a solute molecule is then equal to $2v/1.626 = 1.230 v \text{ cm}^2$.

The polarized multilayers of water represent a dynamic structure. As such, this structure is continually changing. Nonetheless, at any instantaneous moment, the inner surface of the cavity dug out in the polarized water is structured.

This structuring of the water lattice in general and the inner surface of the excavated hole in particular affect the interaction of solutes of different sizes. While a small molecule with proper polar groups oriented in the correct geometry may enjoy a high probability of finding "complimentary sites" for all the solute's surface sites, the probability of finding such accommodating sites on the polarized water lattices declines as the solute molecule becomes larger and its surface more expansive. When the solute is still larger, only a steady small percentage of its surface sites can find partners. With these characteristics in mind, I introduced the following equation relating q_s to the solute molar volume v , and ΔE_s , where ΔE_s is the **specific surface (or solute) polarization** energy per cm^2 in units of $\text{cal.mole}^{-1} (\text{cm}^2)^{-1}$ when the solute is moved from normal liquid water to the polarized cell water.

$$q_s = \exp \left\{ \frac{1.23v \cdot \Delta E_s [1 - (1-b) \frac{(kv)^n}{1 + (kv)^n}]}{RT} \right\}, \quad (21)$$

where b , a small fractional number describes the low and steady probability of very large solute molecules in finding adsorbing sites on the water lattice. k and n are parameters describing the steepness of the declining probability of finding adsorbing sites with increase of molecular volume.

(3) The partition function ratio or entropy component

As shown in equation 19, the (full) equilibrium coefficient, q , is resolvable into three components, q_v , q_s and q_p . We have already discussed the first two. We must now evaluate q_p , which is equal to the partition function ratio of the solutes. To evaluate the partition-function ratios of solutes of different sizes between the polarized water of the living cells and the external medium containing normal liquid water, one begins by examining the various components of the partition function of the solute molecules. The energy levels of multi-atomic molecules and hydrated ions include not only the kinetic translational energy but also two internal energies: vibrational and rotational. Thus, the full partition function can be represented by the products of three partition functions, each corresponding to the translational, vibrational and rotational energy respectively:

$$(\text{p.f.}) = (\text{p.f.})_{\text{tr}} (\text{p.f.})_{\text{vib}} (\text{p.f.})_{\text{rot}} \quad (22)$$

However, most living cells exist in an environment at temperature close to or below 300°K. At such "low" temperatures, the quanta of vibrational energy are too large to be excited. As a result, the vibrational partition function is represented by a term corresponding to the lowest vibrational state, ν_0 . Since ν_0 is a constant, it cancels out in the partition-function ratios. Therefore, q_p represents in essence the ratios of the translational and rotational partition functions of the solute in cell water over those in the external medium. Our next task is to find out how these partition-function ratios for solutes of increasing size are affected by the polarization of the cell water.

Now the translational partition function of solutes can be written as $(2 \pi mkT)^{3/2}V/h^3$, where m is the mass of the solute molecule, V is the volume in which the solute is free to move or translate, h is the Planck constant and k and T have the usual meanings. Since all the other constant terms including m cancel out, the ratio of the translational partition functions of the solute in cell water and external medium is then simply $V_{\text{in}}/V_{\text{ex}}$, where V_{in} and V_{ex} are the tiny volume the solute can move around in within the cell water and the external normal liquid water respectively.

The values of V_{in} and V_{ex} are determined not by solid partitions but by the molecular forces restricting their translational motion. Since the intracellular polarized water is more tightly held due to the polarization, V_{in} might be expected to be somewhat smaller than V_{ex} , providing a translational contribution to the reduction of q_p below unity. However, this contribution is not very large, nor prominently dependent on the size of solute. The rotational contribution is quite different on both accounts.

Monatomic molecules and some diatomic molecules have no rotational degree of freedom. As the size of solute molecules increases, it gains new recognizably different rotational motional freedom. With the gain of each degree of freedom, the rotational partition function increases by a factor of $(8\pi^2IkT/h^2)^{1/2}$, where I represents the additional *moment of inertia*. For rigid polyatomic molecules, the moments of inertia are those along the three principle axes of symmetry. However, in nonrigid polyatomic molecules-which are the subjects of most of our investigations-there are additional internal rotational degree(s) of freedom. By cancellation of similar terms on the numerator and denominator, the full rotational partition-function ratios are reduced to the ratio of the products of square roots of the moments of inertia, I 's.

$$\frac{(\text{p.f.})_{\text{rot}}^{\text{in}}}{(\text{p.f.})_{\text{rot}}^{\text{ex}}} = \prod (I_{\text{r}}^{\text{in}})^{1/2} / \prod (I_{\text{r}}^{\text{ex}})^{1/2} \quad (23)$$

The enhanced molecule-to-molecule interaction in the polarized water acts to limit both the translational and rotational degree of freedom. However, quantitatively speaking, it is primarily the rotational reduction that makes the dominant contribution to the overall loss of motional freedom and hence reduction of the partition function. Experimental evidence shows much greater reduction of rotational motional freedom in cell water than the translational freedom even for molecules as small as water.

Using quasi-electric neutron scattering methods, Rorschach and his collaborators have demonstrated that in (water-poor) brine shrimp cyst cells, the translational diffusion coefficient of water molecules is reduced by a factor of 3, while the rotational diffusion coefficient of water is reduced by a factor of 14 (Trantham *et al.*, 1984).

Not only are there reasons to believe that the rotational motion is more strongly hindered in cell water than the translational motion, there are also reasons to believe that it is the rotational motional freedom reduction that is strongly dependent on the size and complexity of the solute molecule.

Thus for monoatomic molecules and some diatomic molecules with no rotational degree of freedom, q_p has no contribution from rotational motional hindrance in the polarized water at all. For larger molecules with a single moment of inertia, the rotational motional restriction contributes a factor to q_p equal to the ratio of the square roots of this moment of inertia (I^{in}/I^{ex})^{1/2}. Let us assume that this equals 0.8. In the same cell water, another larger solute may have two moments of inertia. If it is rotationally hindered to the same degree, we then have a $0.8 \times 0.8 = 0.64$ contribution of rotational motional restriction to q_p and this goes on with increasing molecular size and the number of rotational degrees of freedom, until a large flexible molecule may have four rotational degrees of freedom and the ratios of the four moments of inertia will produce a total contribution of $(0.8)^4 = 0.41$.

Unfortunately, there is no simple way known to me to describe the relationship between molecular volume and the number of the degrees of rotational motional freedom. As a result, we have to look for short cuts to achieve the goal.

The reduction of rotational motional freedom, in consequence of interaction with surrounding polarized water molecules, bears resemblance to the type of rotational quenching seen for example in solid ortho hydrogen (and many molecular solids). Thus when the temperature is reduced to below the λ point, the rotational freedom of motion of the ortho hydrogen or other molecules suddenly disappears (see Davidson, 1962, pp. 375-378).

Decreasing temperature and hence kT has a similar effect on reducing the rotational motional freedom as that of an increase in the molecule-to-molecule interaction energy due to water polarization; in both, one may visualize the success or failure of molecular rotation to depend on the probability of the molecules involved in overcoming an activation energy barrier (e^*) and this probability is proportional to $\exp(-e^*/kT)$. This activation energy is dependent on the molecule-molecule interaction, which, in the case of solutes rotating in water is enhanced as a result of multilayer polarization of the water.

With this in mind, we assume that there are "sticky" or "anchoring points" distributed more or less uniformly over the surface of solutes and at approximately the same density. Taking into account our earlier recognition that the surface of a solute molecule can be represented as $1.230 v$, where v is the molecular volume of the solute, one can write a simple equation relating the molecular volume of the solute and the q_p of the solute in polarized water:

$$q_p = \exp \left\{ \frac{-(1.23v\Delta e^*)}{RT} \right\}, \quad (24)$$

where as shown earlier, $1.23 v$ is equal to the surface area in cm^2 of a solute molecule with a molecular volume of v . Δe^* is the *increment of the activation energy* for overcoming the greater rotational restriction per unit surface area in units of $\text{cal.mole}^{-1}(\text{cm}^2)^{-1}$, when a solute is transferred from normal liquid water phase to the polarized water phase.

Equation 24 differs from equation 20 only in that ΔE_v is replaced by $1.23 \Delta e^*$. Bearing this in mind, one may regard Figure 1 as plots of equation 24 as well. Like the volume component, the entropy component also tends to reduce the level of a solute according to the size of the solute in the polarized water.

(4) The full q-value

As described by equations 20, 21 and 24, the full q-value of a solute of molecular volume v is the product of its three components and described as follows:

$$q = \exp \left\{ \frac{1.23v\Delta E_s \left[1 - (1-b) \frac{(kv)^n}{1 + (kv)^n} \right] - (\Delta E_v + 1.23\Delta e^*)v}{RT} \right\} \quad (25)$$

A new notation is introduced:

$$\Delta E_{vp} = \Delta E_v + 1.230 \Delta e^* \quad (26)$$

ΔE_{vp} , in *units* of $\text{cal.mole}^{-1}(\text{cm}^3)^{-1}$, will be referred to as the exclusion *intensity* of bulk-phase water polarization. It is a **more** complete expression of the overall effectiveness of the polarized water's ability to exclude solutes.

Three curves plotted according to equation 25 are presented in Figure 2. The parameters used are given as \mathcal{U}_{vp} equal to ΔE_{vp} multiplied by the molecular volume of water, $18.02 (\text{cm}^3)$; and as \mathcal{U}_s equal to ΔE_s multiplied by both the molecular volume of water, $18.02 (\text{cm}^3)$ and the surface/volume ratio, $1.23 (\text{cm})^{-1}$. \mathcal{U}_{vp} and \mathcal{U}_s are respectively the "exclusion intensity" and "surface polarization energy" **for one mole of water. Both are in units of cal.mole^{-1}**

Discussion and Predictions

I shall begin with a discussion of three predictions of the theoretical model presented above. This will be followed by effort aimed at finding out if the published data on solute distribution in six inanimate models can be explained in terms of the present quantitative theory. New experimental data on living cells will also be presented in the paper immediately following, and compared with the predictions of the present theory (Ling et al., 1993).

I. Comparison with Predictions of the Theory

A. Linear distribution

Under the same physical conditions, the partition functions and the polarization energy, η , of equation 18 are constant. Therefore, in a plot of 1C_B against 2C_B , we expect to find a straight line with a constant slope (equal to the q-value of the solute) (equation 17).

Solute distribution between cell water and the external medium may be considered a spe-

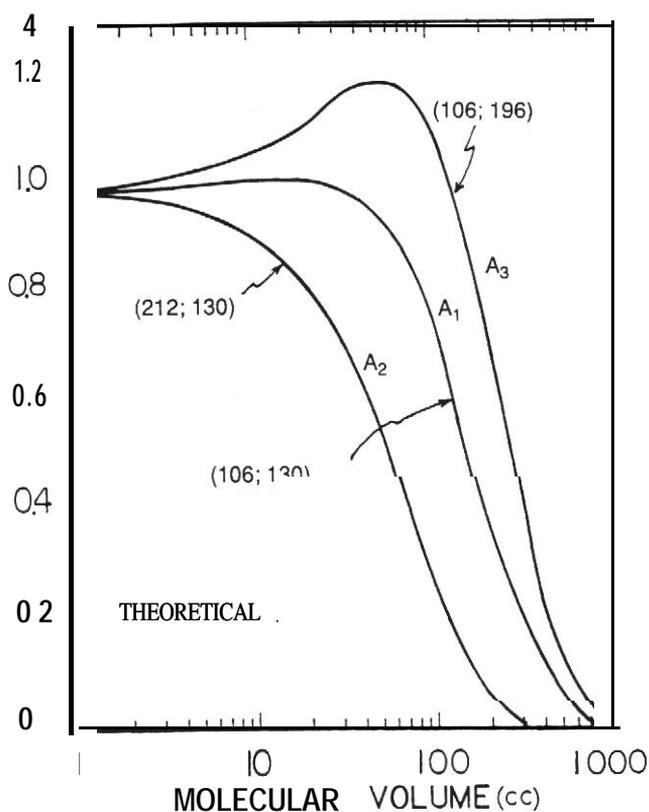


FIGURE 2. Theoretical (full) equilibrium distribution coefficient (q) of solutes of different molecular volume in water polarized to different degrees as indicated by u_{vp} and u_s . For each of the three theoretical curves, A_1 , A_2 , A_3 , calculated according to equation 25, the first number in parenthesis in this and following figures, is u_{vp} and the second is u_s . Both are in units of calories per mole of water. Other numerical values used in the construction of all three theoretical curves are: $n = 1$, $k = 0.01$, and $b = 0.2$

cial case of the Berthelot-Nernst partition law first introduced in 1872: "If to a system of two liquid layers made up of two immiscible or slightly miscible components, is added a quantity of a third substance soluble in both layers, then the substance is found to distribute, or divide, itself between the two layers in a definite manner." If C_I and C_{II} are the concentration of the substance in layers I and II respectively, then at constant temperature,

$$C_I / C_{II} = \text{constant} , \quad (27)$$

which is analogous to equation 17. The constant here is equal to the q -value, or the equilibrium distribution coefficient of the solute in question.

I suggest here to use, when appropriate, the word "solveny" to represent the (non-self explanatory) q -value or the (tongue-twisting) "equilibrium distribution coefficient". "Sol-

veny” is likely to be understandable to nonspecialists, and superior to “solubility”, which was used by Kamnev and Troshin, cited in the opening section of this communication, but is inaccurate because solubility refers to solvency at one specific concentration, (i.e., that at saturation), which may or may not be equal to the q -value describing solvency at different lower concentrations.

B. The size rule

As pointed out repeatedly above, both q_v and q_p obey the “size rule”. That is, both q_v and q_p decrease with increase of molecular volume. It is only when a solute exhibits exceptionally high ΔE_s value that its full q -value exceeds what its molecular volume alone would have predicted.

A high ΔE_s usually originates from the solute’s specific surface structure that fits the dynamic structure of the polarized cell water; and from its overall high polarizability which brings **about** effective mutual polarization of the solute and its surrounding water molecules.

The theoretical calculations, like those demonstrated in Figure 2, point **out further that** prominent departure from the size rule also tends to be related to the molecular volume in the sense that a very small-fitting and polarizable solute would show a q -value not too far from what its molecular volume predicts. Similarly, very large-fitting and polarizable molecules have low q -values anyhow. It is in the middle range of molecular volumes that the solute’s surface is large enough to have an overall positive contribution to the overall q -value, while it is not **so large that the** gain in the surface polarization energy component is not all overcome by the q -value-reducing effect of the high $v \Delta E_{vp}$.

C. Cryoprotective activities of exceptional solutes

According to the PM theory, in the cell water, more so than in normal liquid water, each oxygen atom-with its lone pair of electrons-is surrounded by positively charged H atoms belonging to the same water molecule or neighboring ones. In this kind of an environment, one expects that, as a rule, the overall polarity and geometry of the surface structure of most solute molecules would not fit perfectly into the polarized water structure surrounding the dissolved solutes. As an example, the hydration water molecules on the surface of a hydrated ion, like Na^+ or Mg^{++} , are all oriented centrifugally around each ion (see Figure 5a in Ling, 1972, Figure 5.1A in Ling, 1992a) and therefore are not expected to dovetail into the order and structure of the alternatingly negative and positive charges of the polarized water structure of the cell. However, there are exceptions to this rule.

Consider the case of a special solute molecule. On the basis of its size alone, one would have predicted a q -value distinctly below unity. However, this special solute also possesses a high overall polarizability. In its surface are groupings of such polarity, geometry and affinity that the solute molecule can interact with the charged groups on the exposed surface of the excavated hole in the polarized cell water as well as, or better than, the polarized water it has displaced. In such a case, the solute molecule will work like a **splice**, stabilizing and preserving the 3-dimensional polarized water structure. If this favorable **surface energy component** is so strong that it can annul or exceed the combined unfavorable volume energy component and entropy component (on the accommodation of the solute in the polarized water), a q -value of unity or even higher may result. From this expectation, a new prediction emerges.

According to the association-induction hypothesis, being alive signifies the existence of the protoplasmic protein-water-ion system in a high (negative) energy and low entropy state called the living state (Ling, 1992a, Chapter 3). As an integral part of this living state, the bulk of cell water exists in the state of polarized multilayers. That being alive signifies the maintenance of a state, rather than continued functional activities (the Emergentist view), is supported by the successful development of a normal human baby from an embryo once kept for a long period of time at liquid-nitrogen temperature. At such a low temperature, all biochemical and physiological activities could not have continued. Life, if correctly defined by these continued activities, **would** have irreversibly terminated (Ling, 1992a, Chapter 1).

However, if one had only taken a living embryo and plunged it into liquid nitrogen, the embryo would not have survived. It was the use of *cryoprotectants* like glycerol and ethylene glycol in the freezing-thawing medium that had made **the survival** of the frozen and thawed cells possible (Luyet and Hartung, 1941; Rostand, 1946; Polge, Smith and Parkes, 1949; Lovelock, 1953; Rall, 1987).

According to a new theory of cryoprotectants* based on the AI hypothesis, one main reason for the cryoprotective activities of certain solutes lies in their ability to stabilize and preserve the polarized-multilayered dynamic structure of cell water (and hence the living state) **during** freezing and thawing (see Ling, 1992a). Combining this theory of cryoprotection and the present theory of solute distribution, one predicts that a solute which demonstrates a (close-to-unity or still larger) q-value above the q-value predicted on the basis of its molecular volume alone may be an effective cryoprotectant.

II. Comparison of theory with experimental data on solute distribution in various inanimate model systems; assessment of the intensity of water polarization and the number of layers of water molecules each polar site polarizes and orients

A. Extrovert and introvert models

(1) *Intensity of polarization:* In 1988, Ling and Hu published their study of the equilibrium distribution of 14 sugars, sugar alcohols and other nonelectrolytes in one introvert model (native bovine hemoglobin) and two extrovert model systems: poly(ethylene oxide) and NaOH-denatured bovine hemoglobin. In each case, the plots of the concentrations of solutes in the model water against the concentrations of the solute in the external medium yield straight lines for all solutes studied. This demonstration confirms the prediction of rectilinear distribution pattern derived from the theory.

* According to this theory (Ling, 1992b), cryoprotectants fall into three categories: (1) The glycerol type: as described above, cryoprotectants of this type enhance the stability of the polarized water structure by their polarizing and splicing actions. Cryoprotectants of this type as a rule have high q-values. (2) The dimethylsulfoxide (DMSO) type: cryoprotectants of this type enhance stability of the water structure by polarizing the water molecules in the same sense that N and P sites polarize the water structure. Only they are not fixed but are roaming N (or P) sites. Cryoprotectants of this type tend to have q-values not too far from those dictated by their molecular volumes. (3) The poly (ethylene glycol) 8000 or PEG-8000 type: cryoprotectants of this class have very low q-values due to their enormous molecular size. Unlike Na^+ and Cl^- which rapidly enter the cells under stressful conditions, PEG-8000 enters the cells very slowly. As a result, its presence in the surrounding medium, replacing part of the Na^+ and Cl^- of the incubation medium, removes cell water set free from its normal state of polarization. Removal of liberated water prevents intracellular formation of ice crystals from water that may be liberated during the freezing process. Prompt removal of liberated cell water also prevents accumulation within the cell of Ca^{++} , Na^+ and Cl^- which at high concentrations may be harmful to the cell.

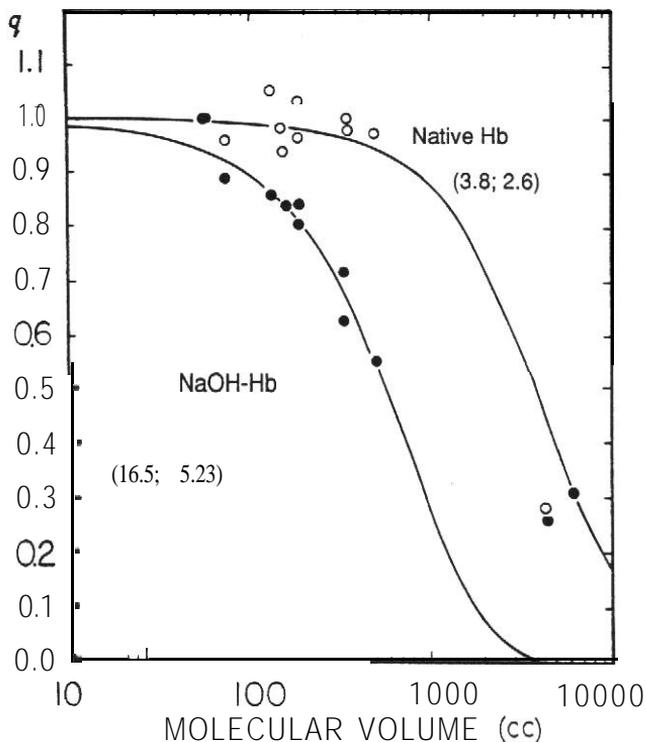


FIGURE 3. Experimental (full) equilibrium distribution coefficient (q) of solutes of different molecular volumes (as indicated on the abscissa) in solutions of native hemoglobin (39%, w/v) (empty circles) and NaOH-denatured hemoglobin (20%, w/v) (solid circles). Points are experimental and derived from linear plots of equilibrium solute concentration in protein solution in dialysis bags and in the external bathing solution. Lines are theoretical, according to equation 25. Numerical values used in calculating the theoretical curves are: NaOH-denatured hemoglobin ($u_{vp} = 16.5$ cal/mole; $u_s = 5.23$ cal/mole; $n = 1$, $k = 0.001$, $b = 0.2$); native hemoglobin ($u_{vp} = 3.8$ cal/mole; $u_s = 2.6$ cal/mole; $n = 1$, $k = 0.0001$, $h = 0.2$). The q -value and molecular volume (in that order) for solutes in native hemoglobin solution are: ethylene glycol 0.998, 55.6; glycerol 0.958, 73.1; erythritol 1.053, 130.2; D-xylose 0.980, 149.0; xylitol 0.936, 152.2; sorbitol 1.035, 182.2; D-mannitol 0.961, 182.2; trehalose 0.997, 338.8; sucrose 0.976, 338.8; raffinose 0.971, 504.5. The q -value (and molecular volume when not given above) of solutes in 20% solution of NaOH-denatured hemoglobin are: ethylene glycol 0.998; glycerol 0.887; erythritol 0.856; xylitol 0.837; sorbitol 0.840; trehalose 0.713; sucrose 0.627; raffinose 0.552; inulin 0.308; PEG 0.283; 4681. (Data from Ling and Hu, 1988).

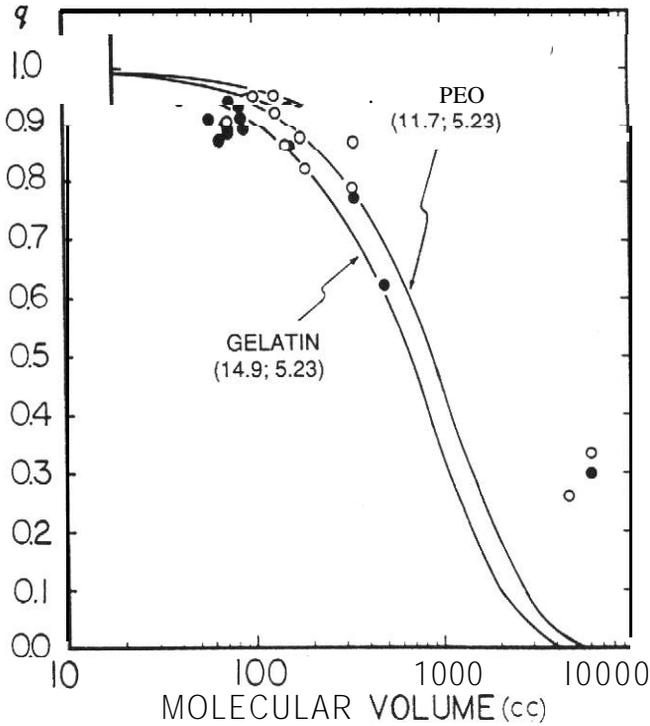


FIGURE 4. Experimental (full) equilibrium distribution coefficients (q) of solute of different molecular volumes in 15% poly (ethylene oxide) (PEO) (empty circles) and of 18% gelatin gel (solid circles). Lines are theoretical, according to equation 25 with u_{vp} equal to 14.9 cal.mole⁻¹ for gelatin and 11.7 cal.mole⁻¹ for PEG. u_s is 5.23 cal.mole⁻¹ for both gelatin and PEG. Points are experimental. Those for solutes in PEO solutions are from Ling and Hu (1988) and were obtained from the slope of rectilinear plot of solute concentration in PEO solutions in dialysis bags against the equilibrium concentration in the external bathing solution. Data points for gelatin gel are taken from Gary-Bobo and Lindenberg (1969). They represent single data points at single solute concentration. The q -value and molecular volume of solutes (in that order) in 18% gelatin gel are: methanol 0.94, 40.3; ethanol 0.91, 58.4; n-propanol 0.93, 74.7; sec-propanol 0.91, 76.5; ethylene glycol 0.87, 55.6; n-butanol 0.91, 94.0; tert-butanol 0.91, 94.0; 1,2-propanediol 0.89, 73.2; 2,3-butanediol 0.89, 88.4; glycerol 0.90, 73.1; pinacol 0.86, 158.6; D-glucose 0.94, 178.8; fructose 0.95, 178.8; sucrose 0.77, 338.8; raffinose 0.62, 498.8; inulin 0.30, 6104; hemoglobin (not shown) (0.30, 67,000 = mol. wt.). The q -value (and molecular volumes) for solutes in PEO solutions are: ethylene glycol 0.949; glycerol 0.909; erythritol 0.920; D-arabinose 0.861; D-xylose 0.864; D-glucose 0.879; D-mannitol 0.820; trehalose 0.870; sucrose 0.768; inulin 0.332, 6104; PEG-4000 0.257, 4681. (Data from Ling and Hu, 1988)

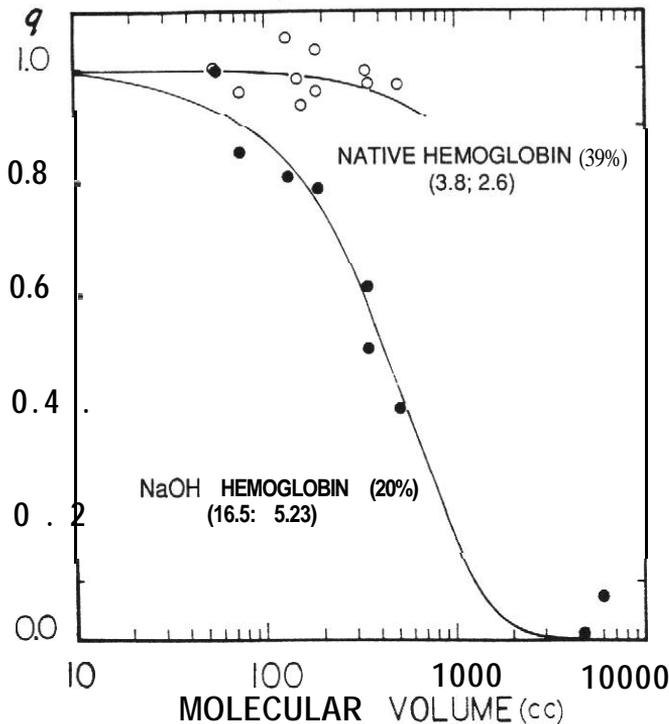


FIGURE 5. The “corrected” experimental equilibrium distribution coefficients or q -values of solutes in 39% native hemoglobin solution (empty circles) and in 20% NaOH-denatured hemoglobin solution (solid circles) plotted against the solute’s molecular volume. Data on native hemoglobin is the same as shown in Figure 3 except that only part of the data is shown here. Data on NaOH-denatured hemoglobin has been recalculated following the assumption that 25% of the water in the protein solution is free with unity q -values for all solutes and this 25% of water and the solutes dissolved in it is deleted and the q -values of the solutes in the non-free water recalculated. The lines are theoretical, calculated according to equation 25, with the following numerical data: $U_{vp} = 16.5$ cal/mole and U_s equal to 5.23 cal/mole. Values of other parameters used remain the same as those indicated in Figure 3. No correction was applied to the data from native hemoglobin solution given in Figure 3. (Data from Ling and Hu, 1988)

The q -value of the solute in cell water is equal to, and thus obtained from, the slope of the rectilinear plots. A similar set of data on a third extrovert model, gelatin, was taken from the publication of Gary-Bobo and Lindenberg (1969). In this case, the q -values were determined from single points obtained at a single external solute concentration rather than from the slope of rectilinear plots of multiple points corresponding to different external concentrations. Nonetheless, Ling and Hu gave reasons that led them to believe that Gary-Bobo and Lindenberg’s data points could not be too far from being true q -values.

Table I.

	Bovine hemoglobin		Gelatin (18%)	PEO (15%)	Sulfonate Ion Exchange Resin	
	native (39%)	NaOH-denatured (20%)			Li ⁺ -loaded	Rb ⁺ -loaded
\mathcal{U}_{vp} (cal/mole)	3.8	16.5 (26.6)	14.9 (16.5)	11.7 (15.9)	55.2	26.6
\mathcal{U}_s (cal/mole)	2.6	5.23 (13.0)	5.23 (3.94)	5.23 (5.23)	26.1	13.0
"			1	1		
k	0.001	0.0001	0.001	0.001	0.01	0.01
b	0.2	0.2	0.2	0.2	0.2	0.2
free water			(28%)	(25%)		

Numerical values for the different parameters in equation 25, used in calculating the theoretical curves shown in Figures 3 to 8. Data in parentheses refer to "corrected" Figures 5, 6, and 7 (see text).

In the original publication of Ling and Hu, these three sets of data were all plotted against the molecular *weight* of the solute (rather than molecular volume). These plots demonstrate general but less precise obedience to the "size rule", since molecular volume rather than molecular weight is the determining factor.

In Figures 3 and 4, the q -value data mentioned above are replotted, this time against the molecular volumes of the solutes. The lines going through or near most of the experimental points are theoretical according to equation 25 (and equation 26). From these best fitting curves, the intensity of bulk-phase water polarization represented as \mathcal{U}_{vp} are for 15% solution of poly (ethylene oxide), 11.7 cal/mole of water, for 18% solution (gel) of gelatin, 14.9 cal/mole and for 20% NaOH-denatured bovine hemoglobin, 16.5 cal/mole (Table I).

In addition to the data from NaOH-denatured hemoglobin, Figure 3 also includes a set of experimental q -values of solutes in a 39% solution of a "invert model", **native bovine hemoglobin**. In this set, all the data points except the one corresponding to the largest solute, PEG-4000 (poly (ethylene glycol) with a molecular weight of 4000), were taken also from the Ling and Hu paper mentioned above. (The data on PEG-4000 has not been presented before and will be published in a short note soon.)

Notice that the inclusion in Figure 3 of this new point has changed the overall picture significantly on the degree of water polarization in the 39% native hemoglobin solution. Thus without the PEG-4000 point, one could only conclude that native bovine hemoglobin at a concentration almost twice that of the NaOH-denatured bovine hemoglobin has no detectable influence on the solvency of the bulk-phase water; all the data points hover around a q -value of unity. With the inclusion of the PEG-4000 point ($q = 0.283$), however, the best fitting curve indicates that the earlier conclusion was not completely correct. At the high concentration of 39%, native bovine hemoglobin increases the exclusion intensity \mathcal{U}_{vp} by a very small increment of 3.8 cal/mole. Putting it differently, a "small" exclusion intensity as low as 3.8 cal/mole is sufficient to significantly reduce the q -value of very large solutes.

Comparing this value (3.8 cal/mole) with that of the NaOH-denatured bovine hemoglobin data mentioned above (26.6 cal/mole), one notices a sevenfold increase of the intensity of polarization when the protein unfolds to assume the fully-extended conformation. Clearly, the observed increase of intensity of polarization would be even more pronounced if the concentration of the NaOH-denatured matched that of the native hemoglobin (39%), **rather than** only a little over half of that (20%).

Here we pause to ask a question: "Since in liquid water, the water-to-water interaction is equal to the heat of vaporization, equal to about 10 Kcal/mole, how can we measure a difference in the water-to-water interaction energy amounting to only 0.038% of this grossly larger figure?" The answer is: the larger normal water-to-water interaction energy is cancelled out exactly. Indeed, what we witness here is equivalent to what we do routinely in the laboratory: weighing samples a few milligrams in weight on a pan (of an analytical balance) 10^5 times heavier.

(2) Percentage of polarized water

In each of the extrovert models, the agreement between the theoretical curves and the data points are good for solutes that do not exceed a certain molecular volume. However, in each case, the q-values of the larger solutes tend to be much higher than what its molecular volume and the theoretical curves predict. There are at least two possible explanations for this departure (for a more detailed discussion on these issues, see Ling *et al.*, 1993):

(a) A "free water" explanation is based on **the** assumption that there is heterogeneity in the degree of polarization of water and part of the water has attributes of normal liquid water. All solutes big and small have unity q-value in this part of the water.

(b) An "hydration water" explanation is based on the assumption that larger solutes can effectively orient water in their immediate neighborhood to suit their own structures rather than that dictated by the cell protein(s) or its extrovert models. In other words, for these very large solutes, each molecule carries with it a halo of water in which the **solute** enjoys a very high q-value much like Na^+ ion carries its own shell of hydration water. In contrast to free water, only this specific solute (and **others** similar to it) enjoys a high q-value in this hydration water.

In the case of solute distribution in gelatin gel and solutions of PEO and NaOH-denatured hemoglobin, we have independent evidence that "free water" is the primary if not exclusive cause for the aberrantly high q-value for the large solutes:

(i) *Stirring*, which should reduce the degree of heterogeneity of water polarization, does indeed decrease the q-value of Na^+ sulfate and citrate in solutions of poly (vinyl pyrrolidone) (PVP) (see below, Ling *et al.*, 1980a, 1980b), a finding that is in accord with a similar finding of Woessner and Snowden (1973): the NMR relaxation times, T_1 and T_2 of waterprotons in a solution containing macromolecular polysaccharide (Kelzan^R) decreased with stirring.

(ii) The atypical high q-value of the large solutes are relatively insensitive to the specific molecular structure and weight of the solutes. Thus from the data of Gary-Bobo and Lindenberg (1969) on gelatin, the q-values of inulin (Mol. Wt. ca. 4000) and hemoglobin (Mol. Wt. 67,000) are the same (0.30).

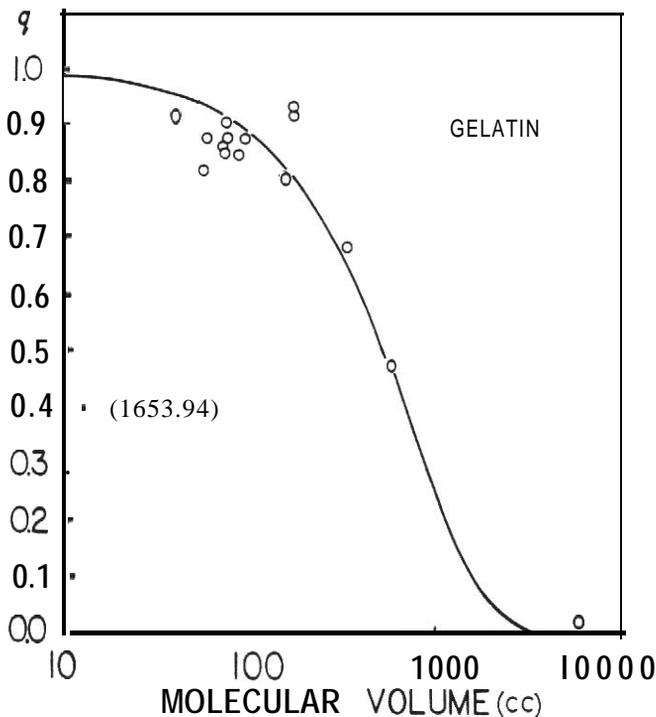


FIGURE 6. The "corrected" experimental equilibrium distribution coefficients or q -values of solutes in 18% gelatin gel plotted against the molecular volume of the solutes. "Correction" similar to that described in the legend of Figure 5 except that the free water fraction was 28%. The line is theoretical, calculated according to equation 25 with $u_p = 16.5$ cal/mole and $u_s = 3.94$ cal/mole. Other numerical data used are the same as those used in Figure 4 for gelatin. (Data from Gary-Bobo and Lindenberg, 1969).

On the basis of this evidence, I reached the tentative conclusion that the aberrantly high q -values of the very large solutes are entirely due to the presence of heterogeneity of the degree of polarization and that this heterogeneous population of water can be approximated by two subpopulations: a larger fraction of uniformly polarized water comprising the majority of the water present and a smaller fraction of normal liquid water.

Since all solutes have a q -value of unity in the minor fraction of normal liquid water, one can subtract from the total that amount in the free water and, with what remains, readily calculate the q -value of all the solute in the major fraction of polarized water. These new q -values are then plotted against their respective molecular volumes and shown in Figures 5, 6 and 7. The percentage of "free water" used in the calculation is 25% for 15% PEO and for 20% NaOH-denatured hemoglobin, and 28% for 18% gelatin. Each of these percentages were obtained by assuming that the true q -values of the aberrant large solutes are those dictated by the respective molecular volumes and the theoretical curves that fit the remaining data points.

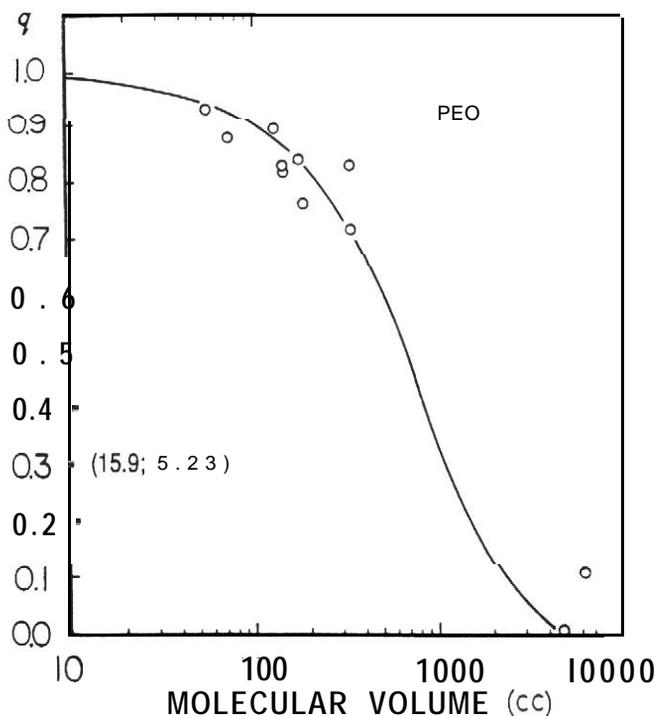


FIGURE 7. The “corrected” experimental equilibrium distribution coefficients or q -values of solutes in 15% PEO solution plotted against the molecular volume of the solutes. “Correction” similar to those described in the legend of Figure 4. The free water fraction here assumed is also 25%. The line is theoretical, calculated according to equation 25 and with $u_{vp} = 15.9$ cal/mole and $u_s = 5.23$ cal/mole. Other numerical data used are the same as those used in Figure 4 for PEO. (Data from Ling and Hu, 1988).

From the q -value vs. molecular-volume plots one obtains a new set of u_{vp} and u_s values, which are given with the new figures (4, 5, 6) and in parentheses in Table I.

The correction for free water also defines the minimum percentage of water polarized and oriented by the various polymers and proteins. They are 75% for 15% PEO solution, 72% for 18% gelatin gel, and 75% in 20% NaOH-denatured bovine hemoglobin.

To fully appreciate how unusually high these figures are, one can compare them with the established figures for hydration of normal native globular proteins (e.g., 0.2 to 0.3 grams of water per gram of dry proteins, averaging 0.25 g/g. For review, see Ling, 1972, p. 673). Based on this figure, an 18% native globular protein would have a total hydration water of only 4.5%; and a 20% protein, only 5%.

In following papers the same method used here to estimate the percentage of polarized water in the extrovert models will be used to estimate the percentage of polarized water in living cells.

(3) *The minimum number of water molecules polarized by each site*

15% *PEO*: The monomer weight of poly (ethylene oxide) (PEO) is approximately 44. Since each monomer here contains only one site, (i.e., one oxygen atom), the total number of sites in one liter of the 15% polymer solution is 150 divided by 44, equal to 3.41 M. The total molarity of water in the solution is $(1-0.15) \times 1000/18.016 = 47.2$ M. Of this, $75\% \times 47.2 = 35.4$ M is polarized. Therefore each oxygen atom polarizes at least $35.4/3.41 = 10.4$ water molecules.

Now each oxygen atom in PEO has two lone pairs of electrons. Each lone pair can potentially form an H-bond with one water molecule. Each one of these two first-layer, polarized water molecules can in turn form H-bonds with three water molecules, leaving at least two more molecules of water to participate in a third layer.

20% *NaOH-denatured hemoglobin*: The average residue weight of a sampling of proteins is 112 (Ling, 1962, p. 48). The total molarity of amino acid residues in a 20% hemoglobin solution is therefore approximately $200/112 = 1.79$ M. The total molarity of water in the 20% protein solution is $(1-0.2) \times 1000/18.016 = 44.4$ M. Of these, $44.4 \times 0.75 = 33.3$ M is polarized. Each amino acid residue carries one peptide comprising one NH group and one CO group. The number of water molecules polarized by each peptide is therefore $33.3 / 1.79 = 18.6$. Each NH group can form an H-bond with one water molecule; each CO group can potentially form H-bonds with two water molecules via its two lone pairs of electrons. Therefore, the first layer of polarized water around each peptide contains three water molecules; the second layer, nine: leaving more than eight water molecules to participate in a third layer of polarized water molecules.

18% *gelatin*: Assuming the same average residue weight of 112, an 18% gelatin contains $180 / 112 = 1.61$ M of peptides. The total polarized water amounts to $(1-0.18) \times 1000 \times 0.72/18.016 = 32.8$ M. Each peptide therefore polarizes $32.8/1.61 = 20.4$ water molecules. Following the same reasoning above, each peptide polarizes three water molecules in its first layer, nine in the second and eight molecules in a third.

I have chosen here to use the three sets of data analyzed to demonstrate clearly that ***the polar sites of extrovert proteins, as well as PEO, polarize and orient multiple* layers of water molecules. Water so polarized acquires solvency properties entirely different from normal liquid water. These findings offer unequivocal evidence for the multilayer* polarization of water molecules by proteins and other linear polymers offering properly spaced, exposed polar groups.***

The present analysis of the three models studied demonstrates that some water in the third layer surrounding each polar site is polarized and oriented. However, the exact figures arrived at ***are minimum estimates*** for the following reasons.

(i) ***Random orientation of protein and polymer chains reduces water polarization***: As mentioned above, the degree of water polarization was increased when the randomly distributed extrovert model was made more orderly by stirring. Thus when solutions of polyvinylpyrrolidone (PVP) was stirred with a to-and-fro moving silicone-rubber-coated lead shot in narrow but long dialysis **sacs, the apparent equilibrium distribution coefficient** or ***p-value*** for

* According to the Webster New Collegiate Dictionary, the word "multiple" is defined as "consisting of, including or involving more than one".

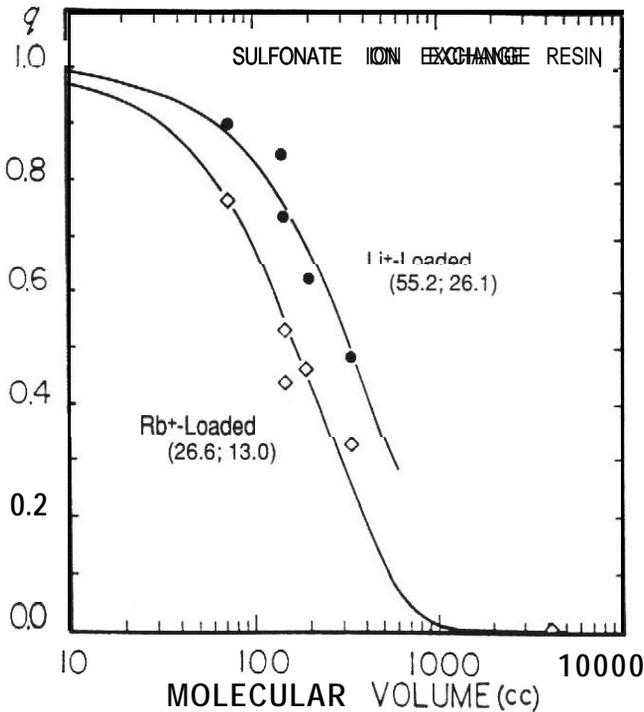


FIGURE 8. The experimental equilibrium distribution coefficients or q -values of solutes in the water in sulfonate ion exchange resin loaded with Li^+ and with Rb^+ . The data points are experimental and taken from Ling (1987). The lines are theoretical, calculated according to equation 25. For the Li^+ -loaded resin, the numerical values used are: $u_{vp} = 55.2$ cal/mole; $u_s = 26.1$ cal/mole. For the Rb^+ -loaded resin: $u_{vp} = 26.6$ cal/mole; $u_s = 13.0$ cal/mole. Other numerical values used for both are: $n = 1$, $b = 0.2$, $k = 0.01$ for Rb^+ -loaded resin, and 0.001 for the Li^+ -loaded resin. The q -value for solutes in the Li^+ -loaded resin, that in the Rb^+ -loaded resin and molecular volume (in that order) are respectively: glycerol 0.766, 0.893, 73.1; D-ribose 0.529, 0.849, 149.0; D-arabinose 0.432, 0.735, 149.0; D-sorbitol 0.462, 0.614, 149.0; sucrose 0.325, 0.482, 338.8; inulin 0.0213, 0.0740, 6104.

Na citrate decreased by as much as 40% (Ling *et al.*, 1980a, 1980b). Such a decrease of ρ -value would significantly increase the computed number of water molecules polarized by each polar site. However, the degree of ordering achieved by a moving lead shot obviously cannot be compared with what could be achieved by Nature in living cells. This issue will be brought up again in the paper immediately following (Ling *et al.*, 1992).

(ii) Some peptide NH and CO groups may remain masked. Of the three models examined above, gelatin and NaOH-denatured hemoglobin are closer in nature to their counterparts

postulated to exist in living cells. The calculations above are based on the assumption that all the peptides of the proteins are engaged in inter- or intra-macromolecular H-bonds and are thus free to react with the bulk-phase water. Whether or not all the peptides are truly free in the NaOH-denatured hemoglobin is not known. However, the amount of water polarized per peptide is very close to that of gelatin. In gelatin, it is well known that part of the gelatin chain is not in the fully-extended conformation but is engaged in what is known as "collagen folds" (Veis, 1964, p. 274). If these masked NH-CO groups are taken into account, the calculation would have produced an even larger number of polarized water per peptide than shown above.

B. Ion Exchange Resin'

Finally, in Figure 8, I have replotted the solute distribution data in Li^+ - and Rb^+ -loaded sulfonate exchange resin (Ling, 1987). Here the intensity of water polarization in both kinds of resin is significantly higher than that produced by NaOH-denatured hemoglobin, gelatin and PEO; and the intensity of water polarization is stronger in the more hydrated Li^+ -loaded resin (55.2 cal/mole) than in the less hydrated Rb^+ -loaded resin where the intensity factor is only 26.6 cal/mole. Note that inulin, the largest solute studied, exhibits a q-value as its molecular volume dictates and there is no need of correction as in the case of gelatin gel and solutions of extrovert models. The high density of N (and P) sites (i.e., 2-3 M) and the relatively high intensity of polarization have apparently contributed to this more uniform water polarization and "normal" q-values for small as well as large solutes.

The numerical data of the E_{vp} and E_s for the data presented in Figure 8 are also given in Table I.

Appendix 1

Near their freezing points, liquids resemble solids in some important aspects. Thus each water molecule in liquid water is also confined to a small volume not much bigger than that occupied by a water molecule in ice. For this reason, we can assume that cell water and water in the surrounding medium exist as lattices like those of a solid but of a more flexible nature. Let us consider a solute which has molecular dimensions similar to those of water molecules and can comfortably fit into the lattice sites of the liquid water. Let the number of water molecules in the liquid be n_A , the number of the solute molecules be n_B . Then W_{cf} , the total number of ways (or configurations) of arranging the water and solute molecules in the lattice is

$$W_{cf} = \frac{(n_A + n_B)!}{n_A! n_B!} \quad (3)$$

* The present theory is not the only theory that has been offered to explain size-dependent solute distribution in various inanimate models systems, including ion exchange resins, heavily cross-linked dextran and gelatin. These alternative theories have been discussed by Ling and Sobel (1975), Ling (1987) and Ling and Hu (1988) leading to our opinion that each of these alternative theories is in conflict with some known experimental facts and that only the present theory (as well as its earlier version) agrees with all the facts known.

The logarithm of W_{cf} , multiplied by the Boltzmann constant, k , is equal to ΔS_{cf} , the entropy of mixing (i.e., the gain of configurational entropy from mixing the solvent and solute). We can simplify equation 3 by using Stirling's approximation ($\ln n! = n \ln n + n$), and obtain the following equation for the entropy of mixing:

$$\Delta S_{cf} = k \ln W_{cf} = -k(n_A \ln x_A + n_B \ln x_B), \quad (4)$$

where n_A and n_B are the numbers, and x_A and x_B , the mole fractions of water and solute respectively.

Equation 4 is based on the assumption that the solute and water molecules have approximately the same size. Since it is our primary goal to find out how molecules of *different* size distribute themselves between cell water and external medium, as such this equation is not applicable. What we need is a similar equation that does not suffer this molecular-size restriction. Luckily, such an equation is already available, derived not for the purpose we have in mind but for predicting properties of solutions of linear polymers.

In deriving such an equation, a linear polymer molecule was considered as a chain of σ repeating segments; each individual segment equals in size that of one solvent molecule. With this assumption, Flory derived the following equation for the configurational entropy of mixing solvent with linear polymers (see Flory, 1953, pp. 497-503):

$$\Delta S_{cf} = k \ln W_{cf} = -k(n_A \ln v_A + n_B \ln v_B), \quad (5)$$

where n_A and n_B are the numbers of water and solute molecules respectively and v_A and v_B are their respective volume fractions, and

$$v_A = \frac{n_A}{n_A + \sigma n_B}; \quad v_B = \frac{\sigma n_B}{n_A + \sigma n_B} \quad (6)$$

We will use this equation for the entropy of mixing of solute *molecules of any size*, assuming that they can all be approximately represented as a flexible chain of σ segments, each segment having roughly the size of a water molecule.

Limiting ourselves to dilute solutions, we write down the total Helmholtz free energy of the system of living cells and its surrounding medium as follows:

$$F = -kT \{ {}^1n_A \ln {}^1(p.f.)_A + {}^1n_B \ln {}^1(p.f.)_B + \ln {}^1W_{cf} + {}^2n_A \ln {}^2(p.f.)_A + {}^2n_B \ln {}^2(p.f.)_B + \ln {}^2W_{cf} \}, \quad (7)$$

where 1n_A and 1n_B represent the number of water and solute molecules in the living cell, while 2n_A and 2n_B represent the number of water and solute molecules in the surrounding medium. ${}^1(p.f.)_A$ and ${}^1(p.f.)_B$ represent the partition function of the water and solute molecules in the cell water, while ${}^2(p.f.)_A$ and ${}^2(p.f.)_B$ represent the partition functions of water and solute in the external medium. $k \ln {}^1W_{cf}$ and $k \ln {}^2W_{cf}$ are the configurational entropy of the solute in cell water and the surrounding medium respectively.

When dm water molecules are transferred from cell water to the external medium, we have

$$dm = d {}^1n_B = -d {}^2n_B, \quad (8)$$

or

$$\frac{\delta}{\delta m} = \frac{\delta}{\delta {}^1n_B} - \frac{\delta}{\delta {}^2n_B}$$

From equation 7, we derive

$$\frac{\delta}{\delta^1 n_B} (\ln {}^1 W_{cf}) = (\sigma - 1) {}^1 v_A - \ln {}^1 v_B; \quad -\frac{\delta}{\delta^2 n_B} ({}^{1''} {}^2 W_{cf}) = \ln {}^2 v_B - (\sigma - 1) {}^2 v_A. \quad (9)$$

and

$$\frac{\delta F}{\delta^1 n_B} = -kT [\ln {}^1 (p.f.)_B - \ln {}^2 (p.f.)_B + (a - 1) {}^1 v_A - \ln {}^1 v_B - (o--1) {}^2 v_A + \ln {}^2 v_B]. \quad (10)$$

The condition for equilibrium in the distribution of the solute between cell water and its surrounding medium is $\delta F / \delta^1 n_B = 0$, or

$$-kT [\ln {}^1 (p.f.)_B - \ln {}^2 (p.f.)_B + (o-1) {}^1 v_A - \ln {}^1 v_B - (\sigma-1) {}^2 v_A + {}^{1''} {}^2 v_B] = 0 \quad (11)$$

Since σ is a constant for each solute, and for the (low) v_B range being studied, we may assume

$$(0-1) {}^1 v_A = (0-1) {}^2 v_A \quad (12)$$

We then have

$$\ln \frac{{}^1 (p.f.)_B}{{}^2 (p.f.)_B} + \ln \frac{{}^2 v_B}{{}^1 v_B} = 0. \quad (13)$$

where the two partition functions, ${}^1 (p.f.)_B$ and ${}^2 (p.f.)_B$, are referred to the same zero of energy. I now introduce partition functions, ${}^1 (p.f.)_B$ and ${}^2 (p.f.)_B$, each referred to its own zero energy. Choosing the zero energy of solute B in the external solution as zero of the whole system, the zero energy of B in the cell water ${}^1 (p.f.)_B$ now lies below the new zero energy by a'' amount equal to χ in ergs per molecule. Therefore

$$\frac{{}^1 v_B}{{}^2 v_B} = \frac{{}^1 (p.f.)_B}{{}^2 (p.f.)_B} \cdot \exp\left(-\frac{\chi}{kT}\right), \quad (14)$$

where k is the Boltzman constant. Now

$${}^1 v_B I {}^2 v_B = {}^1 n_B I {}^2 n_B = {}^1 C_B I {}^2 C_B, \quad (15)$$

where ${}^1 C_B$ and ${}^2 C_B$ are the concentration in moles per liter of the solute B in cell water and in the water of the external medium respectively.

Therefore,

$$\frac{{}^1 C_B}{{}^2 C_B} = \frac{{}^1 (p.f.)_B}{{}^2 (p.f.)_B} \cdot \exp\left(-\frac{\eta}{RT}\right), \quad (16)$$

where $\eta = \chi L$, $R = kL$ and L is the Avogadro number. Since the equilibrium distribution coefficient of B between cell water and the external solution is represented by the symbol q , or

$${}^1 C_B / {}^2 C_B = q, \quad (17)$$

we then have a'' equation for the solute distribution in living cells:

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