THE PHYSICAL STATE OF WATER IN LIVING CELL AND MODEL SYSTEMS*

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Living cells, as a rule, contain 15 to 25 per cent proteins and 75 to 85 per cent water. We now know that it is the sequences of amino acid residues in the proteins that underlie biological specificity and that the difference in the nature of one amino acid residue (in a protein containing hundreds) may produce profound differences in the behavior of the entire tissue of which this protein is a part.[1] Yet, important as the proteins are in the living phenomena, there can be no life unless there is also water. Thus, whether in the form of contractile proteins or functioning enzyme, living protoplasm always contains water; the unique behavior it manifests, reflects not the behavior of the proteins per se but that of the protein water systems. The question arises: In what way does water serve this critical role? Does it function merely as a solvent of a suitable dielectric property?

In recent years, considerable evidence has been collected, showing that this is not so. Water molecules in the close vicinity of proteins and other biologically important macromolecules appear to exist in a physical state different from that of normal water. Forslind,[2] for example, suggested that in protein solutions, water molecules close to macromolecules may exist in a state between that of liquid and solid. Jacobsen[3] supported this basic concept with x-ray, dielectric, and nuclear magnetic resonance studies on macromolecular solutions. Szent-Györgyi[4] postulated an ice-like structure of water surrounding proteins; Klotz *et al.*[5] supported and further developed this "iceberg" concept. From x-ray diffraction studies, Beeman *et al.*[6] concluded that serum albumin is surrounded by a layer of water which does not dissolve sucrose as normal water does. Similarly, Hearst and Vinograd[7] by density measurements reached the conclusion that water closely associated with DNA excludes alkali metal ion (see also, Ritland *et al.*)[8] The nuclear magnetic resonance studies of Berendsen[9] have shown that in native collagen and partially dried collagen, water molecules are restricted in their rotation and that they form chains in the collagen fiber direction, being oriented by the peptide amide bonds.

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The views on the physical state of water in living cells, however, are widely divergent.

According to the classical membrane theory, the intracellular water is permanently and entirely (or almost entirely) in the form of normal water such as is found in a 0.1 M KCl solution. The well-known asymmetry in the distribution of ions and nonelectrolytes between the intracellular and extracellular water is attributed to the critical pore size on the cell membrane or to a continual pumping by "Na pumps" and "permeases" located in this thin structure.

Gortner[10] advocated the view that cell water is bound; his concept, however, was not accepted largely because of a lack of truly convincing evidence.[11,12]

According to Troschin's sorption theory[13] the cell water has different solubility properties for nonelectrolytes, amino acids and ions than normal water; it does not offer molecular interpretations as to the mechanism of this difference in solubility.

The association-induction hypothesis[1,14,15] which deals with a broader topic agrees in essence with Troschin's sorption theory concerning ionic and nonelectrolyte distribution problems, although the two theories were developed independently. The association-induction hypothesis offers, however, specific molecular interpretation of the differences in solubility properties of the cell water in terms of restricted rotation of polyatomic nonelectrolytes and de facto polyatomic hydrated ions[1] and of differences in the H-bond formed in the protoplasmic system.[1] The theory also stresses that the living protoplasm and hence protoplasmic water does not exist in one single physical state but as a rule, exists reversibly in more than one metastable cooperative states in the course of its normal physiological activity. Anticipating the evidence to be presented, we may state that it is our purpose in this paper to demonstrate that all or nearly all water molecules in a living cell can be considered to exist as polarized multilayers oriented on the surfaces of cell proteins. To demonstrate this, however, one cannot apply the direct approach which one uses on water sorption studies of stable inanimate systems because in living cells, the protein water systems are metastable; removal of water may bring about changes that are irreversible. Instead, we shall employ an indirect method which involves the three following steps: (1) establish multilayer adsorption of polarized water in one or more nonliving stable model systems; (2) choose properties exhibited by the water in living cells during its quiescent resting state; these properties must be significantly different from those of ordinary water and yet can be studied in the living cell without producing serious injury; (3) establish that this same property is also exhibited by the water in the nonliving model systems mentioned.[1]

In the following analyses, we have chosen equilibrium distribution of nonelectrolytes and of ions as the properties mentioned[2] and as models, we shall use strong electrolyte solutions, $Cu_2Fe(CN)_6$ gel, and in particular collagen from carp's swim bladder and sheep's wool. The experimental data on ionic distribution is complete and on this the argument rests; those of nonelectrolytes are still in progress. Nevertheless, the nonelectrolyte data are included in this presentation as it brings into focus additional and different facets of the problem.

Polarized Water in Proteins

Association of proteins with water can be demonstrated by analyzing the sorption of water vapor on purified proteins. When the amount of water sorbed is plotted against the relative vapor pressure, the data shows an S-shaped curve typical of sorption of gases in multimolecular layers on solid surfaces. Theories of such gas sorption were presented by de Boer and Zwikker[16] and by Bradley[17]; both suggested electrical polarization (induction) as the cause of the build-up of the multilayers of adsorbed gas. Brunauer, Emmett and Teller[18] severely criticized de Boer and Zwikker's theory (and of Bradley's theory on inert gas adsorption) on the ground that the inductive action on gas molecules such as argon, nitrogen, etc., is quantitatively trivial; they offered instead, what later became known as the BET theory. The BET theory is, in essence, an extension of the Langmuir adsorption isotherm to multilayer adsorption where successive layers are held by London force and such forces operate in a normal liquid. The BET isotherm can be put into a form such a plot of $p/a(p_0 - p)$ against p/p_0 should yield a straight line; where a is the amount of gas adsorbed at pressure p, and p_0 is the gas pressure at full saturation under the same condition. In their criticism of the polarization theories, Brunauer *et al.* were careful in pointing out that: "On the other hand, if the adsorbed gas has a large permanent dipole it is possible that many layers may be successively polarized by the mechanism of De Boer and Zwikker. This case has been treated by Bradley."[19] The Bradley polarization theory for gases with permanent dipole moments is quantitatively represented by the following equation:

$$\log_{10}\frac{p_0}{p} = K_1,\ K_3^a + K_4 \qquad (1)$$

where a, p, p_0 have the same meanings mentioned above; K_1, K_3 and K_4 are constants for a specified system under a specified condition. Since water has a large permanent dipole moment (1.834×10^{-18} e.s.u.) water sorption on solid surfaces should follow Equation 1.

Bull[20] who studied the water sorption of more than 10 proteins, applied the BET theory to his data and found in many cases, satisfactory fit but

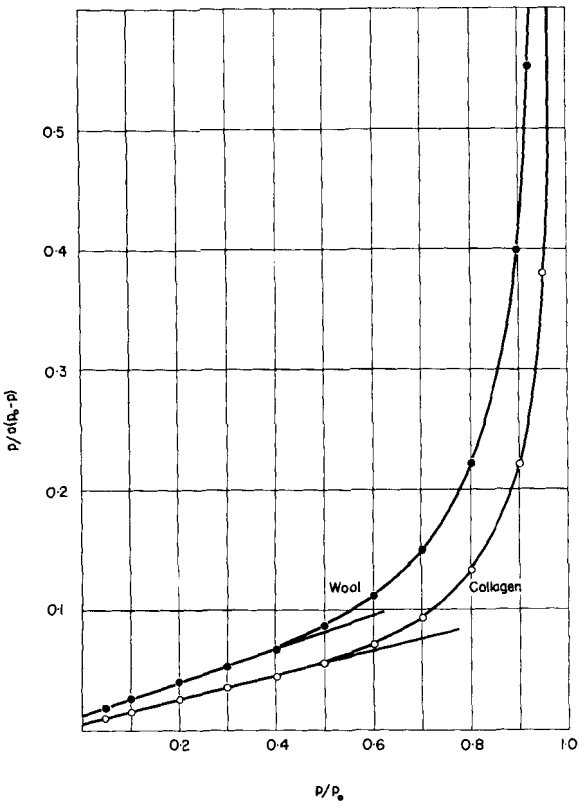

FIGURE 1. Water vapor sorption on collagen and on wool, plotted according to the BET isotherm. Data of Bull.[20]

only up to about 50 per cent vapor saturation. In FIGURE 1, Bull's data on water sorption of collagen (hide) and of wool are plotted according to the BET theory to cover the entire range of vapor pressure studied. The large discrepancy between the theory and experiments for vapor pressure higher than 50 per cent is conspicuous and typical of other data of Bull and others.[21] In 1955, Mellon and Hoover[22] noted a much greater accord between water-sorption on proteins and Bradley's isotherm and commented: "The simplest equation, the two constant equations of Bradley* fits throughout the whole range of our data from 6 to 93 per cent relative humidity. The description was so accurate. . . ." Particularly relevant for the present discussion is the adherence to Equation 1 of water sorption on polyglycine which Mellon and Hoover demonstrated; in this case the only polar sites on the polymer are the NH and CO groups on the polypeptide chain. The

*This is Equation 2 with $K_4 = 0$.

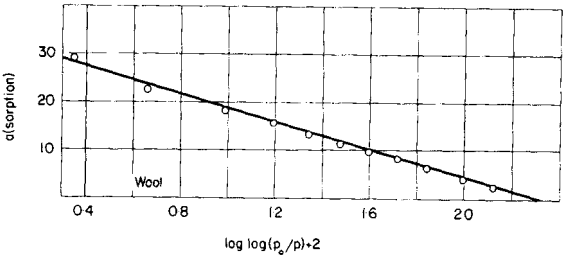

FIGURE 2. Water vapor sorption on sheep's wool, plotted according to the Bradley isotherm. Same data as in FIGURE 1.

agreement suggests that the polypeptide amide groups are inherently capable of orienting and polarizing successive layers of water molecules.

In FIGURE 2 and FIGURE 3, the same data of vapor sorption by collagen and wool that appeared in FIGURE 1 are plotted according to the Bradley equation (Equation 1).† There is good accord up to 95 per cent saturation. Since the data as a whole does not follow the BET theory, it appears probable that in collagen and in wool, water molecules exist in the form of polarized multilayers.

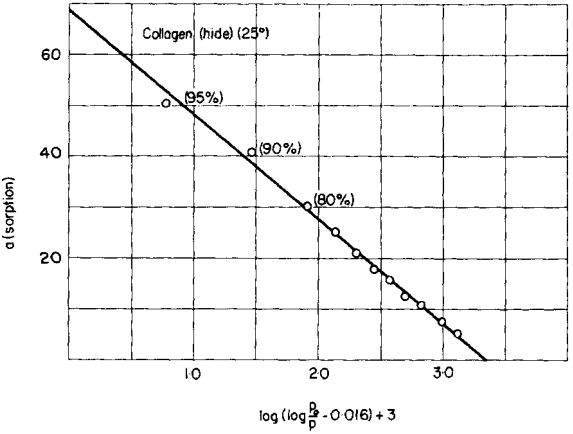

FIGURE 3. Water vapor sorption on collagen plotted according to Bradley's isotherm. Same data as in FIGURE 1.

†A refinement of the Bradley treatment is indicated for multilayer sorption within macromolecular systems such as wool and collagen; in this case the sorption is on two adjacent surfaces and the sorption must oppose forces holding the protein molecules together. Capillary condensation which is bothersome in simple solid surface sorption is reduced since sorption does not greatly alter the surface of the condensed phase.

Selective Exclusion of Nonelectrolytes

Nonelectrolyte Exclusion in Model Systems

Copper ferrocyanide gel. Sheets of animal tissues had long been known to possess what later van't Hoff named semipermeability, allowing, for example, water but not sucrose to pass through. In the middle of the 19th century, Moritz Traube[23] searched for artificial membranes that have similar attributes; among those he discovered was the copper ferrocyanide ($Cu_2Fe(CN)_6$) precipitation membranes which to this day have remained one of the best, if not the best artificial semipermeable membranes. The significant property of the $Cu_2Fe(CN)_6$ gel is that the water it contains accommodates little if any sucrose. Thus, McMahon, Hartung and Walbran[24] studied the equilibrium distribution of sucrose in an aqueous suspension of $Cu_2Fe(CN)_6$ gel; a much higher concentration of sucrose was found in the clear supernatent solution than if all the sucrose was evenly distributed in all the water within the system. Assuming the involved water is 100 per cent inaccessible to sucrose, these authors calculated that each molecule of $Cu_2Fe(CN)_6$ creates 10.6 moles of this type of water, an amount of water so large that it cannot be accommodated in the crystal lattice or on the surface of the salt. McMahon *et al.* suggested most of the water is "imbibed by the gel"; we tend to think that imbibed water, in fact, refers to water adsorbed in multilayers.

"Coacervate." The same may apply to the water in the "coacervates" e.g., a colloidal gel composed of gum arabic and gelatin. According to Troschin[13] the equilibrium distribution of sucrose in the water of this coacervate amounts to only 60 per cent of that in the bathing medium.

Strong electrolyte solutions. Although $Cu_2Fe(CN)_6$ gel and "coacervates" resemble in their physical consistency the living protoplasm and are therefore its more cogent models; selective exclusion of nonelectrolytes is not restricted to water in colloidal systems. The well-known phenomena of "salting out" of nonelectrolytes in strong electrolyte solutions is another example: the presence of salt diminishes the solubility or the distribution coefficient (against another water-immiscible reference solvent) of many nonelectrolytes including sucrose.[25,26] Although this is a familiar subject, our understanding is far from complete; one aspect, however, is clear: "salting out" owes its origin to the polarization of water molecules around the ions. It is significant that "salting out" is not only caused by strong electrolytes; nonelectrolytes (e.g., sucrose) can salt out other nonelectrolytes (e.g., ethyl acetate).[25] Thus long range electrostatic effect is not an indispensable part of this phenomenon.

Ion exchange resin. A variation of the "salting out" effect of strong electrolytes is the similar phenomena observed in ion exchange resins. In this case, one species of the ions is fixed on a three-dimensional matrix; the

TABLE 1
DISTRIBUTION COEFFICIENTS OF NONELECTROLYTES BETWEEN THE WATER IN SULFONATE EXCHANGE RESINS AND ITS SURROUNDING AQUEOUS MEDIUM

	Dowex 50 (H^+) (Wheaton and Bauman)	Rexyn RG 50(H^+) (25°C)	Amberlite IR-200 (H^+) (25°C)	(0°)	ΔH° Kcal/mole	ΔS° cal/deg/mole
Urea	—	—	22.5	35.0	-2.86	-3.8
Methyl alcohol	0.61	0.94	2.2	—	—	—
Ethylene glycol	0.67	—	0.88	0.87	—	—
Glycerol	0.49	0.56	0.78	0.65	1.18	3.46
Xylose	—	0.23	0.67	0.55	1.28	3.49
Glucose	0.22	0.23	0.61	0.55	0.67	1.27
Sucrose	0.24	0.29	0.63	0.60	0.05	0.15

concentration of the fixed ions is usually high (ca. 5 M); the average number of water molecules associated with each fixed ion-counter ion pair is low, i.e., 5. Wheaton and Bauman[27] reported their findings about equilibrium distribution of various alcohols and sugars between the water in the resin and the external solution. Their data are in general confirmed by our own studies on similar sulfonate-polystyrene exchange resins (TABLE 1).

The molecular mechanism of sugar exclusion (sugar-ion exchange resin type). By studying the equilibrium distribution of these alcohols and sugars at two different temperatures (0° to 25°C.) the enthalpy and entropy of the distribution equilibrium can be estimated. Unfortunately, the best grade of ion exchange resin we could obtain was still heterogeneous, containing beads with diverse degrees of sulfonation; this produced considerable scattering of the data. Whereas the data given in TABLE 1 must still be regarded as tentative the trend is obvious: urea, which is selectively accumulated to a concentration 20 times higher than that in the external medium has an average $\Delta F^\circ = -1.89$ kcal./mole, an enthalpy value of –2.9 kcal./mole and an entropy of –3.8 cal./degree/mole (0° –25°C). The enthalpy and entropy for the various sugars are all positive; this suggests that their substitution for water is energetically unfavorable. Since the bonds which non-electrolyte molecules form with water and ions are known as H-bonds, the unfavorable enthalpy bespeaks of a restriction in the formation of H-bonds in the resin phase. A diagram of a possible orientation of a sugar molecule in ion exchange resin is shown in FIGURE 4; it illustrates the formation of fewer and/or weaker H-bonds in the resin and hence a higher degree of freedom in motion. This, however, appears not to be the only mechanism of such solute exclusion (see below).

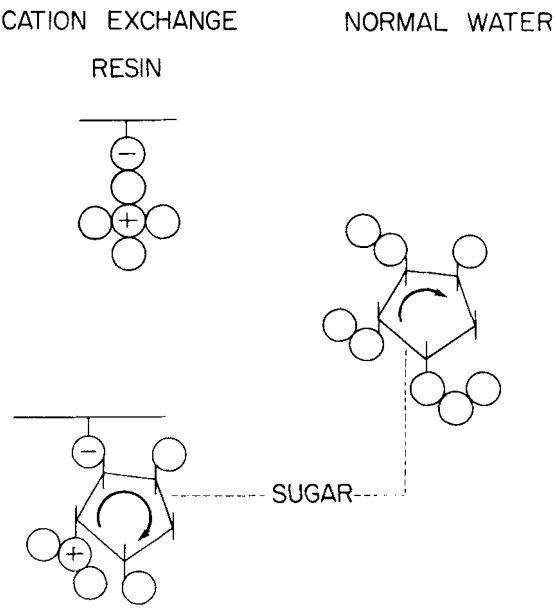

FIGURE 4. Diagram of a possible configuration of a sugar molecule in the ion exchange resin, illustrating the formation of a smaller (and/or weaker) number of H-bonds in the resin and hence a higher degree of rotational freedom. The size of arrows illustrates the relative rotational freedom of the sugar molecules.

Nonelectrolyte Exclusion in Living Cells

The early cell physiologists attempted to interpret nonelectrolyte distribution in terms of the membrane permeability; permeant nonelectrolytes were assumed to distribute according to thermodynamic equilibrium; impermeant ones remain excluded completely. Advancing years brought in better techniques, which in turn, revealed that many nonassimilated sugars are found in cell water at equilibrium at a concentration considerably lower than that in the external medium.* A partial list of selected data, largely due to Hechter and Lester,[28]† is given in TABLE 2.

Thus, the exclusion of nonelectrolyte from water found in living cells is also observed in a quantitatively similar manner in various inanimate systems; in the latter cases there can be no question that there are

*More recently, workers studying selective accumulation of sugars in microorganisms introduced the concept of "permeases" (i.e., sugar "pumps") which were supposed to maintain a high intracellular sugar concentration by a continual pumping mechanism. For a critique of this mechanism, see below.

†Hechter and Lester[28] postulated ordered water lattice in living cells. They invoked Gregor's theory of ionic selectivity in ion exchange resin and attributed ionic and nonelectrolyte exclusion to swelling pressure effect. The critique of this mechanism was given elsewhere.[1]

TABLE 2
DISTRIBUTION COEFFICIENT OF NONASSIMULATED SUGARS BETWEEN CELL WATER AND EXTRACELLULAR WATER

Sugar	Frog muscle	Rat diaphragm muscle	Rat uterus (castrated adrenalectomized)	Rat adrenal gland
D-Xylose	–	0.52 (41) 0.341* (28)	0.82 (28)	0.79 (28)
Galactose	0.32 (13)	–	–	–
Sucrose	0.29 (13)	–	0.36 (28)	0.06 (28)

Data from references (13) and (41) were derived from *in vitro* studies; those from reference (27) from *in vivo* studies.

*Concentration of cellular sugar on the basis of fresh tissue weight instead of cell water.

"pumps"; yet, the water molecules differ from normal water only in that they are polarized and oriented in one way or another. Considered together, the data suggest that intracellular water may also be polarized and oriented and in this state, it excludes sugars.

SELECTIVE IONIC EXCLUSION

Ionic Exclusion in Living Cells

As in the case of nonelectrolytes, the earlier interpretation of asymmetrical distribution of ions was in terms of permeability or impermeability of the cell membrane. Thus, K^+ ion was recognized as permeant and found in high concentration within the cell; Na^+ ion, which is found at high concentration in the plasma, but low concentration in the tissue, was considered impermeant. As in the case of nonelectrolytes, however, advancing techniques (in particular, radioisotope techniques) soon proved unequivocally that Na^+ ion is in fact also permeant.[29,30] To remedy this failure of the original theory, the Na-pump was proposed. This was a very reasonable assumption at the time it was introduced, since similar "pumps" for Na^+ and for nonelectrolytes undoubtedly operate across such biological "membranes" as intestinal mucosa, frog skin and kidney tubules, etc., at the expense of metabolic energy. Thus, it was thought that Na^+ ion was an exception to the rule; by introducing the Na pump, or original

TABLE 3
MINIMAL ENERGY REQUIREMENT OF THE NA, CA AND MG PUMPS IN FROG MUSCLE CELLS IN COMPARISON WITH MAXIMAL AVAILABLE METABOLIC ENERGY

	Extracellular concentration (mM./1.)	Theoretical (Donnan equilibrium)		Intracellular concentration (experimental) (mM./1.)	"Permeability constant" (hr^{-1})
		Donnan Ratio	Intracellular concentration (mM./1.)		
K^+	2.4	53.4	128.0	128.0	0.077 ((1), p.292)
Na^+	105	53.4	5600	16.9	1.223 (31)
Ca^{++}	4.0	53.4	5704	5.7	2.45 (32)
Mg^{++}	2.5	53.4	3565	15.8	4.16 (33)

concept of a sieve-like membrane may still be considered adequate to explain the distribution of all other ions and nonelectrolytes. In reality, this turned out not to be the case.

Extensive tracer studies have long since proven that many ions, hitherto thought to be impermeant, are in fact, also permeant. These include Ca,$^{++}$ Mg,$^{++}$ orthophosphate, lactate, sulfate ion, free amino acids, and many sugars.[1] The question is: Do they all follow equilibrium distributions predicted on the basis that the intracellular water is entirely the same as in an 0.1 N KCl solution? The answer is no. Thus the anticipated intracellular concentrations (on the basis of an assumption of equilibrium distribution in normal intracellular water) are for Mg^{++} 3565 and for Ca^{++} 5704 mM. per liter of intracellular water. In reality the intracellular Mg^{++} and Ca^{++} ion concentrations are 15.8 and 5.7 mM./1. respectively.

The same criteria (permeability and distribution not following that of Donnan equilibrium) that led to the postulation of the Na^+ pump also fully apply to these ions. If the pump theory is not an *ad hoc* but general theory which supplements the pore-size concept whenever needed, there is no choice that there must be Mg^{++} pump and Ca^{++} pump as well. TABLE 3 summarizes calculations we have presented elsewhere in greater detail.[34] Even if each of the pumps operates at 100 per cent efficiency, the overall

TABLE 3A
MINIMAL ENERGY REQUIREMENT OF THE NA, CA AND MG PUMPS IN FROG MUSCLE CELLS IN COMPARISON WITH MAXIMAL AVAILABLE METABOLIC ENERGY

	Fraction of nonenergy consuming efflux according to pump model	Electrochemical work per mole of ion pumped (volt-Farady)	Pump		Maximum available energy (cal./kg./hr.)
			Efficiency assumed	Minimal energy requirement (cal./kg./hr.)	
K^+	100%	0	–	–	–
Na^+	0.1%	0.148	100%	51	–
Ca^{++}	0.4%	0.142	100%	343	–
Mg^{++}	0.15%	0.116	100%	176	–
			Total	570	170

Data of ionic concentrations from.[36] Details of calculations given in.[34] Calculation of energy requirement for Na pump is essentially that from Levi and Ussing.[31]

minimal energy need of the three ions (Na^+, Ca^{++}, Mg^{++}) alone would be 335 per cent of the total metabolic energy delivered per unit time.

The critical analysis given above shows that if one assumes the intracellular water to be normal and the protein unreactive toward ions and nonelectrolytes neither an equilibrium nor a steady state (pump) interpretation is satisfactory because both violate fundamental physical laws.

Since 1951, the theory (the association-induction hypothesis) has been developed[1] that ionic and nonelectrolyte distribution do represent equilibrium phenomena, that the cell proteins are not inert toward ions and nonelectrolytes and that the cell water is not normal. The entire living cell is considered to constitute a bulk phase fixed-charged system; in this, ionic (and other) sites of the proteins selectively adsorb ions (and nonelectrolytes); distribution of these ions and nonelectrolytes within the cell water is, as a rule, lower than in the extracellular water due to re-

striction of rotational (and to some extent translational) motions and hence a lowered entropy of ions and nonelectrolytes found in it. The theory is summarized by the following equation (Troschin arrived at a simpler version of this equation in studying nonelectrolyte distribution):[13]

$$[p_i^+]_{in} = \kappa_i [p_i^+]_{ex} + \sum_{J=1}^{N} \frac{[f_J^-] \tilde{K}_{iJ} [p_i^+]_{ex}}{1 + \sum_{s=1}^{m} \tilde{K}_{sJ} [p_s^+]_{ex}} \quad (2)$$

where $[p_i^+]_{ex}$ and $[p_i^+]_{in}$ are the extra- and intracellular concentration of the ith cation; $[p_s^+]_{ex}$ is the extracellular concentration of the sth cation which refers to any one of the m cations in the external medium including the ith. Where $[f_J^-]$ refers to the Jth type of anionic site among a total of N types of similar sites. K_{iJ} and K_{sJ} are the adsorption constants of the ith, and sth monovalent cations on the Jth site. Since K_i is less than unity (see below) for the ith ion with a high K_{iJ} value at values of $[p_i]_{ex} \lesssim [f^-]$ one may on first approximation assume that there is only one type of site, the Jth, rewrite and simplify Equation 2 as:

$$[p_i^+]_{in} = \frac{[f_J^-] \tilde{K}_{iJ} [p_i^+]_{ex}}{1 + \tilde{K}_{iJ}[p_i^+]_{ex} + \tilde{K}_{jJ} [p_j^+]_{ex} + \sum_{s=1}^{m-2} \tilde{K}_{sJ} [p_s^+]_{ex}} \quad (3)$$

where p_j refers to another multivalent cation in the system. If in one series of experimental studies, concentrations of all cations are kept constant except the ith and jth, then,

$$[p_i^+]_{ex} = \frac{[f_J^-] K_i' [p_i^+]_{ex}}{(1 + K_i' [p_i^+]_{ex} + K_j' [p_i^+]_{ex}} \quad (4)$$

where

$$K_i' = \frac{\tilde{K}_{iJ}}{1 + \sum_{s=1}^{m-2} \tilde{K}_{sJ} [p_s^+]_{ex}} \quad (5)$$

$$K_i' = \frac{\tilde{K}_{iJ}}{1 + \sum_{s=1}^{m-2} \tilde{K}_{sJ} [p_i^+]_{ex}} \quad (6)$$

Equation 4 can be written reciprocally:

$$\frac{1}{[p_i^+]_{in}} = \frac{1}{K_i' [f_J^-]} (1 + K_j' [p_i^+]_{ex}) \frac{1}{[p_i^+]_{ex}} + \frac{1}{[f_j^-]} \quad (7)$$

If $1/[p_i^+]_{in}$ is plotted against $1/[p_i^+]_{ex}$, a straight line should be obtained at each constant value of $[p_j^+]_{ex}$. When the ith and the jth apparent adsorption constants are similar (as in the case of K^+, Rb^+ and Cs^+ in frog sartorius muscles) this proves to be the case. The complete experimental data were presented elsewhere.[35] Now,

$$\kappa_i = \alpha q_i \quad (8)$$

TABLE 4
THE DISTRIBUTION COEFFICIENT OF Na^+ ION IN WATER OF LIVING CELL AND OF SHEEP'S WOOL

Frog sartorius muscles (0 - 25°C.)			
q_{Na}	ΔF° kcal./mole	ΔH° kcal./mole	ΔS° cal./deg./mole
0.19 (0°C.)	1.04	-0.94	-6.9
0.13 (25°C.)			

Sheep's wool (25°-37°)			
q_{Na}	ΔF° kcal./mole	ΔH° kcal./mole	ΔS° cal./deg./mole
0.16 (25°C.)	1.247	-3.28	-14.8
0.10 (37°C.)			

where α is the percentage of water in the living cells and K_i is the distribution coefficient of the ith ion between the intracellular water and the extracellular water. The value of κ_i, however, is best studied in the case of an ion which has a very low value of K_j. This is the case for Na^+ ion ($q_{Na} = 1.0$. Since if one chooses a jth ion which has a much higher adsorption constant as in the case of K^+ ($K_j' = 6.67 \times 10^2$), $[p_i^+]_{in} \sim \kappa_i [p_i^+]_{ex}$ as $[p_j^+]_{ex} \rightarrow \infty$. The value of $[p_i^+]_{in}$ at $[p_j^+]_{ex} = \infty$ can be determined by extrapolation based on several values of $[p_i^+]_{in}$ obtained at increasing concentrations of $[p_j^+]_{ex}$. From such studies the value of q_i is obtained. The value of q_{Na} so determined are given in TABLE 4.

Ionic Exclusion in Model Systems

We have made an analogous study on the distribution of Na^+ ion in two model systems: collagen of carp swim bladder and sheep's wool. Within the concentration range of ions studied (up to 1.0 M) swim bladder collagen does not adsorb an appreciable amount of Na^+ ion. q_{Na} in this case,

is found to be 0.8. However, this estimation was made on the basis of the total water of the swim bladder tissue which contains water outside the collagen tissue in the form of adhering fluid films. Thus, the true value of K_{Na} between water in collagen and the surrounding fluid must be lower.

The pattern of ion uptake in sheep's wool, on the other hand, is essentially similar to that found in muscle cells and follows both Equation 2 and Equation 7. Wool contains 33 per cent of water; in this q_{Na} has a value of 0.16 at 25°C. and 0.1 at 37°C.

Molecular Mechanism of Ion Exclusion (Ion-Wool Type)

From data just given, one can calculate the average $\triangle F^\circ$ of the equilibrium:

$$[Na]_{in} \rightleftarrows [Na]_{ex} + \triangle F^\circ$$

to have a value of 1.247 kcal./mole (between 25 and 37°C). The average $\triangle H^\circ$ is −3.15 kcal./mole and the average $\triangle S^\circ$ is −14.4 cal./degree/mole. Thus, the Na^+ ion would have accumulated in the cell water to a concentration higher than that in the external medium, had it not been for the large entropy loss which more than offset the favorable enthalpy. As was shown, by Guggenheim and Fowler[36] the hydrated ion is a *de facto* multiatomic structure; as such by far the largest partition function (and hence entropy) is the rotational partition function. The highly unfavorable entropy of Na^+ ion in the water in sheep's wool must be therefore largely due to the restriction of rotation of the hydrated Na^+ within the protoplasmic water.

If one recalls that we have already demonstrated that the water in sheep's wool and in collagen exist in polarized multilayers (FIGURE 3), a molecular mechanism for this restricted rotation appears readily understandable as shown in FIGURE 5. Water molecules in the "hydrated shell" and in the polarized multilayer merge and become mutually reinforcing, creating a large number of stronger H-bonds for the hydrated ion than in normal water. The hydrated ion also loses in consequence much of its freedom of rotation motion.

It now only remains to be pointed out that our data on studies on frog muscle indicates that the q_{Na} value also decreases with increasing temperamuscle indicates that the q_{Na} value also decreases with increasing temperature and hence similar signs in the values of $\triangle H^\circ$ and $\triangle S^\circ$. The overall picture therefore resembles ionic exclusion from wool water rather than the pattern of nonelectrolyte exclusion in ion exchange resin. However, the living cell is a very complicated structure; the detailed mechanisms may very well incorporate in different microscopic regions the sugar-ion exchange resin type but to a minor degree.

We shall conclude this paper by examining an interesting and highly informative experiment reported more than 30 years ago by the late Robert

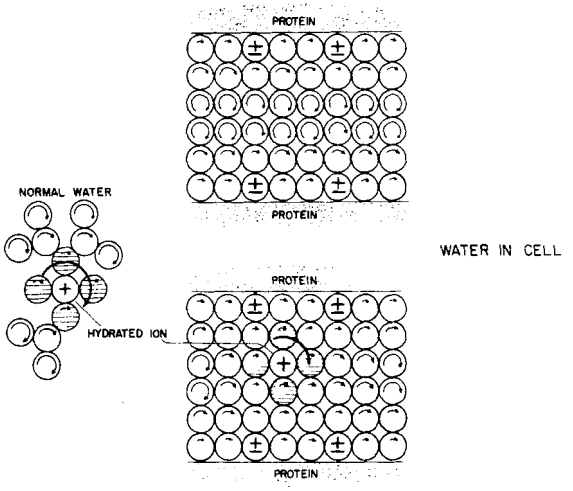

FIGURE 5. Diagram illustrating the physical state of water in the living cell in a region with hydrated ion (*lower right*) and in a region without a hydrated ion (*upper right*). Left figure is a diagram of a hydrated ion in normal water. The length of the curved arrows indicated the degree of rotational freedom of each water molecule; a progressively greater rotational freedom is shown for water molecules further away from the proteins. The merging and mutual reinforcement of the polarization (arising from the ion and from the proteins) and the anchoring effect of the fixed proteins produces a microscopic "droplet" of water molecules. In this "droplet", water molecules have greatly reduced rotational motion.

Chambers and his coworker[38] and later confirmed in more than one laboratory. A single frog muscle cell was supercooled to –6°C. Formation of one to many ice "spikes" progressed from the cut end of the muscle fiber when it was touched with an ice-tipped micropipette. The orientation of the "spikes" followed the orientation of the muscle fiber, straight when the muscle fiber was straight, twisted when the muscle fiber was twisted. The shape of ice crystal was different in other cells, being feather-like, for example, in sea urchin eggs. When the freezing was very rapid hundreds of such ice "spikes" could form in a single muscle fiber. A comparison of the cross-section of such a frozen cell[39] with a similar cross-section of a frog muscle fiber seen through an electron microscope[40] suggests that the ice formation occurs between the protein filaments which run longitudinally along the length of the muscle fiber. Thus, the frozen portion of the cell water must correspond to the water occupying intracellular space farthest away from the polarizing surfaces of the protein molecules. The fact that (1) water not in the form of ice remains in cell proteins after the completion of the intracellular freezing and that (2) no branching or horizontal propagation of ice "spikes" occurs[39] also suggests that the layer of

water immediately adjacent to the proteins is strongly oriented and that transformation of their structure to ice is energetically unfavorable. All these are in complete accord with the physical state of cellular water which is deduced from the study of ions and nonelectrolyte distribution reported in this paper.

Summary

Sorption of water vapor on collagen and on sheep's wool fits the BET theory up to 50 per cent vapor saturation; the same data fits the Bradley multilayer adsorption isotherm of polarized molecules to near saturation, suggesting that water in these systems is polarized and oriented in multilayers. It was shown that Na^+ ion is excluded from this water in wool so that it reaches an equilibrium concentration of about 0.1 that in the external medium; a quantitatively similar situation exists in living frog muscle cells. In both, the equilibrium distributions have negative enthalpy values and large negative entropy values suggesting that the water in the hydrated shell of Na^+ ion merge with and reinforce polarized water in the system and form stronger H-bonds, but this advantage favoring distribution in the wool (or cell) is more than offset by the large entropy loss in the restricted rotational freedom.

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