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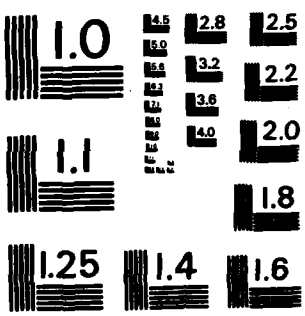

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Chapter Two

THE POLARIZED MULTILAYER THEORY OF CELL WATER AND OTHER FACETS OF THE ASSOCIATION-INDUCTION HYPOTHESIS CONCERNING THE DISTRIBUTION OF IONS AND OTHER SOLUTES IN LIVING CELLS

Gilbert N. Ling

Department of Molecular Biology
Pennsylvania Hospital
Philadelphia, Pennsylvania

DECEMBER 16, 1983

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I. INTRODUCTION

It is well known that the properties of both K^+ and Na^+ closely resemble each other. Yet to the best of our knowledge, all living cells are able to differentiate sharply between these two ions: K^+, which is found in *low* concentrations in the medium surrounding the cells, exists in *high* concentrations within living cells; Na^+, which is found in *high* concentrations in the surrounding medium, exists in *low* concentrations within living cells. An explanation of this

asymmetrical ion distribution still presents a major challenge to mechanistic biologists. A clear and correct understanding of this highly unusual and yet biologically characteristic phenomenon would shed much light not only on the solute distribution problem itself but, more generally, on the physicochemical makeup of the protein-water-ion systems which all living cells comprise. To achieve this understanding, certain theories have been formulated. These will be evaluated in this chapter.

II. THREE TYPES OF THEORIES

There are only three types of mechanisms that can produce a sustained difference in the concentrations of a chemical substance in two contiguous spaces: (1) the presence of an insurmountable energy barrier between the two spaces, (2) a continuous pumping activity, and (3) a difference in the physicochemical nature and/or conditions of the environment in the two spaces (e.g., a difference in solubilities of the substance in the two spaces). Indeed, each of these three basic theoretical mechanisms has already been investigated as the cause of the asymmetry in K^+ and Na^+ distribution, and they will be reviewed separately. The first two theories, the "sieve" theory and the Na pump theory, are offered here with evidence to disprove them. The descriptions of the early nonmechanistic equilibrium theories offer a historical perspective. The main consideration in this chapter will be the association-induction hypothesis.

A. The Sieve Theory and its Disproof

Early in this century, it was believed that asymmetrical ion distribution was the result of the impermeability of a cell membrane to all ions [1]. This view evolved into the sieve theory, which contended that the low concentration of Na^+ in cells resulted from a postulated absolute and specific impermeability of the cell membrane to this ion. On the other hand, K^+ was considered permeant and thus was selectively accumulated in the cell to balance the negative electrical charges of impermeant anions [2-4]. The sieve

theory reached the pinnacle of its development in the version by Boyle and Conway [4]; ironically, however, it became unacceptable almost immediately after its publication when contradictory experimental findings of Heppel [5] and of Steinbach [6] proved that the excluded Na^+ was not impermeant but in fact travels in and out of living cells through the cell membrane with ease.

B. The Sodium Pump Theory and its Disproof

1. *HISTORY AND Ad Hoc NATURE OF THE THEORY*

Dean [7] and Krogh [8] have been widely referred to as the founders of the Na pump theory, although a reading of their much-cited original publications leaves much doubt about whether these authors did indeed intend to launch a theory alternative to that of Boyle and Conway. The fact that neither Dean nor Krogh vigorously pursued this subject after having published their respective articles substantiates the view that the Na pump theory was in fact without a founder. Though a number of eminent electrophysiologists are enthusiastic and effective supporters of the Na pump theory, they have little or no first-hand experience in dealing with solute distribution problems. They see in this modified membrane theory as originally propounded a means of sustaining and explaining the electrical potential of living cells as a membrane potential [9] despite repeatedly confirmed experimental findings that refute the theory and that have been in print for over 10 years (see the following subsections). In my opinion, the prestige of these electrophysiologists has done much in the perpetuation of the Na pump theory.

Boyle and Conway [4] clearly perceived the asymmetry in the K^+ and Na^+ distribution as a partial expression of a more general phenomenon. Their sieve theory had predictive value. Having suggested that it is the critical-sized membrane pores which exclude the larger hydrated Na^+ but not the smaller hydrated K^+, they could, without further ad hoc introduction of new postulations, predict the behavior of other ions and molecules according to their sizes.

In contrast, the Na pump theory is ad hoc and thus has no predictive value. It is true that the pump concept has been expanding steadily, that is, more and more pumps have been added. However, as each new pump has been introduced, a new, separate theory has had to be developed. The original Na pump theory could not have foretold whether another pump would have to be postulated before the experimental observation was made.

2. WASTEFUL AND TO NO CLEAR PURPOSE

In the Na pump theory, a mechanism was postulated to move Na^+ in one direction and K^+ in the opposite direction. Because these two ions are so similar, a high degree of precision in discrimination is required. To maintain this asymmetrical ionic distribution, excessive amounts of energy must be spent continuously. It would seem unlikely that nature would have undertaken such a difficult and expensive enterprise, violating one of its own basic laws (see Sec. II.B.3.a), without a purpose. Naturally, we must assume that this activity as well as many other types of asymmetrical solute distribution is essential for the maintenance of normal cell functions. But how is it achieved?

Table 1, taken from Crane [10], shows that in transport across intestinal epithelium (which will be called *true transport* to distinguish it from the active transport of the Na pump in resting cells) only those molecules essential for the survival of the animal are actively transported, e.g., glucose and molecules closely similar to glucose. Other compounds of no benefit to the animal merely leak through the intestinal epithelium, so that energy is not wasted in actively transporting them. This basic economy is what one expects from a design of nature that has withstood the stringent tests of evolution.

Figure 1 shows the equilibrium levels of different hydroxylic compounds from muscle cells at 0°C. At this temperature the cells survive well but do not significantly metabolize even D-glucose [11,12]. It appears that a simple relation exists between the equilibrium distribution coefficient or q-value of a compound in the

TABLE 1 Absorption of Sugars and Related Compounds[a]

Compounds actively absorbed	
Glucose	4-O-Methyl-D-galactose
1,5-Anhydro-D-glucitol	D-Allose
2-C-Hydroxymethyl-D-glucose	6-Deoxy-D-glucose
D-Glucoheptulose	6-Deoxy-D-galactose
3-O-Methyl-D-glucose	6-Deoxy-6-fluoro-D-glucose
D-Galactose	7-Deoxy-D-glucoheptose
3-Deoxy-D-glucose	α-Methyl-D-glucoside
Compounds not actively absorbed	
D-Mannoheptulose	D-Gulose
D-Mannose	6-Deoxy-6-iodo-D-galactose
D-Talose	6-O-Methyl-D-glucose
1,5-Anhydro-D-mannitol	L-Galactose
2-Deoxy-D-glucose	L-Glucose
2-Deoxy-D-galactose	L-Sorbose
2-O-Methyl-D-glucose	6-Deoxy-L-galactose
D-Glucosamine	6-Deoxy-L-mannose
N-Acetyl-D-glucosamine	Mannitol
2,4-Di-O-methyl-D-galactose	Sorbitol
D-Fructose	Glycerol
3-O-Methyl-D-fructose	D-Xylose
3-O-Ethyl-D-glucose	L-Xylose
3-O-Propyl-D-glucose	D-Ribose
3-O-Butyl-D-glucose	L-Arabinose
3-O-Hydroxyethyl-D-glucose	D-Arabinose
1,4-Anhydro-D-glucitol	D-Lyxose
Gold-thioglucose	

[a]For specific references from which the above data were taken, consult the original publication: R. K. Crane, *Physiol. Rev.*, *40*: 798 (1960), reproduced by permission.

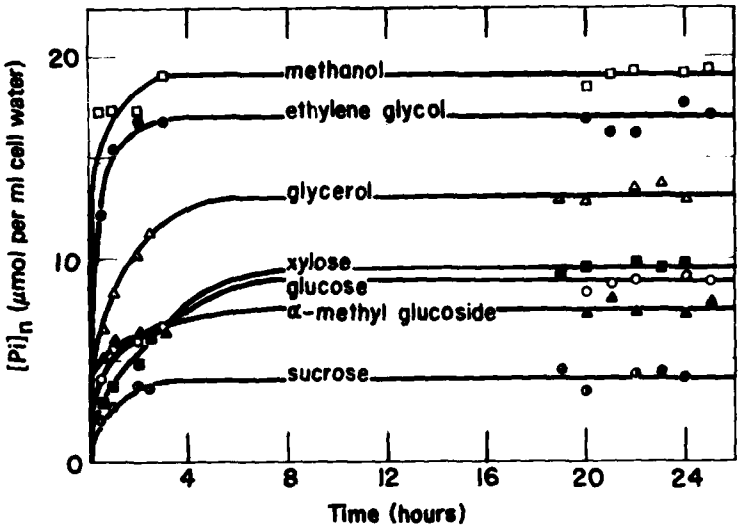

FIGURE 1 Time course of uptake of methanol, ethylene glycol, glycerol, xylose, glucose, α-methyl glucoside, and sucrose by frog muscles at 10°C. Muscles were isolated the previous day and kept overnight at 4°C. The initial concentration of all solutes was 25 mmol/liter. The final external concentrations (in mmol/liter) were as follows: methanol, 17.0; ethylene glycol, 17.1; glycerol, 18.5; xylose, 19.7; glucose, 21.4; α-methyl glucoside, 20.8; and sucrose, 22.04. The q values are 1.1 for methanol, 0.99 for ethylene glycol, 0.71 for glycerol, 0.48 for xylose, 0.44 for glucose, 0.36 for α-methyl glucoside, and 0.18 for sucrose. (From G. N. Ling, C. Miller, and M. M. Ochsenfeld [12]; reproduced by permission of *Ann. N.Y. Acad. Sci.*)

cell water and the molecular size and complexity of this compound. Small molecules like methanol reach a concentrations in the cell water equal to or exceeding that in the external medium; larger molecules are excluded. The degree of exclusion increases with increasing molecular size and complexity. In terms of the pump theory, all these larger compounds *must be pumped out* constantly in order to keep them at the steady low levels found in the cells. Here, in contrast to intestinal active transport, no relation can be found between the relative utility of the substance to the tissue and the effort with which the cell accumulates or expels it at the expense of metabolic energy. Indeed, the noxious methanol is favored while the beneficial D-glucose is vigorously kept out!

TABLE 2 Postulated Membrane Pumps[a]

Solute	Direction	System
Na,K	Coupled	Many cells
Ca^{2+}	Outward	RBC, striated muscle
Mg^{2+}	Outward	Frog sartorius
$Choline^{+}$	Inward	RBC
Amino acids	Inward	RBC, muscle, tumor
D-Xylose	Inward	Rat diaphragm
D-Xylose	Outward	Rat diaphragm
Na^{+}	Inward	Frog sartorius
Noradrenaline	Inward	Vascular smooth muscle
Prostaglandins	Inward	Mammalian liver
Curarine	Inward	Mouse diaphragm
Br^{-}, I^{-}, ReO_4^{-}, WO_4^{-}	Outward	Ascites
Cu^{2+}	Inward	Ascites
Aminopterin	Inward	Yoshida sarcoma
Cl^{-}	Inward	Squid axon, motor neurons
Mn^{2+}	Inward	*E. coli*
Cl^{-}	Outward	*E. coli*
Sugars	Inward	*E. coli*
Amino acids	Inward	*E. coli*
Tetracycline	Inward	*E. coli*

[a]Data collection was more or less arbitrary and not intended to be comprehensive. [From G. N. Ling, C. Miller, and M. M. Ochsenfeld, *Ann. N.Y. Acad. Sci., 204*: 6 (1973), by permission.]

Table 2 was the result of a rather casual survey made in 1969 of the membrane pumps actually postulated [12]. Although only 20 types are listed, the actual number of pumps is much larger, since the amino acid pumps and sugar pumps are each not a single pump but a multitude of pumps. To illustrate further the universality of the need for pumps, Table 3 shows that of all the well-known solutes in

TABLE 3 The Ionic Composition of Frog Muscle[a]

Ion	Intracellular		Extracellular		Ratio column 3 / column 5	Donnan ratio r
	Fresh muscle (mmol/kg)	Intracellular water (meq/liter)	Plasma (mmol/liter)	Extracellular water (meq/liter)		
Cations						
K	85.8 (TBF'E')	128.0	2.5 (F)	2.53	50.59	50.59
Na	24.9 (F'B)	16.9	103.8 (F)	105.0	$(6.2)^{-1}$	
Ca	4.08 (KMUB'F)	11.3	2.0 (F)	4.04	2.8	1.67
Mg	10.8 (KMUB'FC)	31.6	1.2 (B)	2.46	12.8	3.58
Amino acids	6.8 (E)	10.0	8.5 (F)	8.60	1.16	
Carnosine	11.0 (E)	10.2	0	0		
Creatine	7.2 (L)	10.7	0	0		
Anions						
Cl	10.7 (BF)	1.04	74.3 (F)	76.8	$(73.9)^{-1}$	73.9

HCO_3	10.7 (F)	9.2	25.4 (F)	26.4	$(28.7)^{-1}$	28.7
Lactate	2.8 (F)	3.5	3.3 (F)	3.42	1.02	1.02
Inorganic *phosphate*	7.2 (L)	16.2	3.1 (F)	3.21	5.05	1.85
Creatine	7.2 (L)	10.7	0	0(?)		
Amino acids	6.8 (E)	10.0	8.5 (S)	8.60	1.16	
CrP	21.8 (L)	71.0	0	0		
ATP	5.0 (E)	27.4	0	0		
Hexose-m-phosphate	1.4 (E)	4.0	0	0		
Other P	4.2 (F)	10.5	0	0		
Carnosine	11.0 (E)	6.2	0	0		

[a]The most accurate data known to the author were collected in 1946 and averaged, weighing the data from each source according to the number of determinations. The values for Na^+ and K^+ ions were taken from published values where there are at least five individual determinations and the standard error does not exceed 5% of the mean. (From G. N. Ling, *A Physical Theory of the Living State: The Association-Induction Hypothesis*, p. 217. Waltham, Mass.: Blaisdell, 1962.)

frog muscle no two items follow the same Donnan distribution ratio, r, and thus virtually all must be pumped to be maintained at the levels found.

Indeed, in making surveys of this type, it is difficult to avoid the conclusion that virtually all complex molecules distribute themselves asymmetrically between living cells and their environment and are therefore, according to the Na pump theory, in need of pumps. The only reason that not more than 30 or 40 pumps have been postulated so far is that few investigations have systematically looked for more pumps. It must also be concluded that whenever a hitherto undiscovered large, water-soluble molecule is found or synthesized, there is an excellent probability that, in accordance with the pump theory, there already exists a specific membrane pump waiting to pump it in either one direction or the other. The fact that the ad hoc pump theory leads to irrational predictions does not, in itself, constitute disproof of the pump theory. However, rigorous disproofs against this theory do exist.

3. EXPERIMENTAL CONTRADICTIONS

a. Violation of the Law of Conservation of Energy

Violation of this law was recognized in 1962, when I presented the details of experimental data showing that, of all the energy-consuming pumps in frog muscle cells, the Na pump alone would consume (under specified conditions) at least 15 to 30 times more energy than the cell can offer [13]. The essence of this finding was confirmed by Jones [14] in canine smooth muscle and by Minkoff and Damadian [15,16] in *Escherichia coli*.

The question was raised whether in my original calculations I had overestimated the energy need of the Na pump by assuming that the entire Na^+ efflux was due to pumping, thereby ignoring the non-energy-consuming contribution of an "exchange diffusion" fraction in Na^+ efflux. The answer to this question is as follows: It is well known that ouabain, a cardiac glycoside, slows down the Na^+ efflux of many living cells. Proponents of the pump theory have

long and vigorously cited this finding as evidence for the existence of a Na pump, since ouabain has been shown to inhibit a Na-K-activated ATPase, and this enzyme has been postulated to be an integral part of the Na pump. Experimental data of Ling and Palmer [17], shows that the application of ouabain to frog muscles lengthened the half-time exchange of Na^+ ($t_{1/2}$) from 25 to 30 min to 200 to 300 min. Thus, the rate of Na efflux was reduced 90% by the "pump-inhibiting" ouabain. In terms of the membrane-pump theory, pumping must therefore account for at least 90% of the total Na efflux. This leaves at most 10% as the maximum percentage for non-energy-consuming exchange diffusion in total Na efflux. A 10% reduction in the energy balance calculation would reduce the energy deficiency from 15-30 times too large to 13.5-27 times too large, an alternative that would hardly alter our conclusion that the Na pump would need much more energy than the cell can afford [cf. 11,13, 18,19]*.

b. Accumulation of K^+ and Exclusion of Na^+ in Membrane Pump-less Muscle Cell Preparations vs. Cytoplasm-Free Intact Cell Membrane Sacs.

Twenty-five years have now elapsed since the technique was reported for preparing a functional nerve cell membrane sheath freed of the bulk of axoplasm [20,21], a technique which has long become routine. There is little doubt that the electrical activity of these membrane sacs is beautifully preserved. If we accept the membrane-pump theory of cellular resting potential, the Na pump should be sound and healthy. Yet, to our knowledge, repeated attempts to

*The reason why this 10% correction was not made in the 1962 publication is that I do not believe that the fraction of Na^+ with a $t_{1/2}$ of 20 to 30 min is rate-limited by the intra-extracellular exchange [13,18,19]. The true rate of intra-extracellular exchange of Na^+ is much faster and is represented by a fast fraction that has long been erroneously lumped together with Na^+ trapped in the extracellular space. The slow fraction with a $t_{1/2}$ of 25 to 30 min is rate-limited by desorption from intracellular sites [11,13].

demonstrate active transport of K^+ and Na^+ have never succeeded in spite of the considerable skill of those who tried it [21,22].

This has prompted us to ask the question: Can cytoplasm without a functional cell membrane achieve what a membrane sac without cytoplasm cannot achieve? The answer is yes. Selective K^+ accumulation and Na^+ exclusion have been shown to persist in a muscle cell preparation in which part of the cell membrane was removed by amputation and the remaining intact portion of the membrane and its postulated pumps were rendered inoperative by being suspended in air and thus deprived of "sinks" for outward pumps and "sources" for inward pumps [12,23]. In evaluating the results of studies involving these "effectively membraneless open-ended cell" (EMOC) preparations, we found that (1) all muscle cells in a frog sartorius muscle run without interruption from one end of the muscle to the other; (2) no regeneration of the amputated cell membrane was observed in the environment within the duration of the experimental period; (3) the accumulation of K^+ and exclusion of Na^+ are not due to a faster diffusion coefficient of K^+ than Na^+ in the cytoplasm, since correction for this difference produces no basic change in the K^+ accumulation/Na^+ exclusion phenomenon observed; (4) the observed accumulation of K^+ and exclusion of Na^+ are not due to sequestration of labeled K^+ within and exclusion of labeled Na^+ from subcellular particles; and (5) the accumulation of K^+ and exclusion of Na^+ are not the result of leakage of labeled K^+ through the extracellular space in conjunction with the operation of the inward K^+ pumping and outward Na^+ pumping [12,23,24].

The disproof of the pump theory, by eliminating the last alternative, establishes that the asymmetry of solute distribution must be essentially an equilibrium phenomenon arising from a difference in the nature of the physicochemical environment inside and outside the living cell.

I shall now briefly review the history of equilibrium theories and then present a more detailed discussion of the association-induction hypothesis. This hypothesis, like the other equilibrium

theories, contends that the distribution of ions and other solutes is non-energy-consuming but, unlike the other theories, attempts to explain how this mechanism works.

C. Early Nonmechanistic Equilibrium Theories

1. *FISCHER'S THEORY*

At the turn of the century, Martin Fischer and his coworkers attributed the uptake of inorganic ions and organic molecules not to membrane permeability but to the colloidal adsorption properties of the whole protoplasm [25-29; cf. 30,31]; D. N. Nasonov and his coworkers [32-35] arrived at similar ideas. The work of two of Nasonov's students, Kamnev and Troschin, deserves special attention.

2. *KAMNEV'S EXPERIMENTS*

In 1938, I. Ye. Kamnev [36] reported the results of his studies of the distribution of nonfermentable galactose and sucrose in normal frog muscles. He found that the level of galactose concentrations in the cell water reached only 42.1% of that in the surrounding medium, whereas the level of sucrose concentration was 32.7%. When the muscles were killed, the cell's ability to partially exclude these sugars was lost. The intracellular and extracellular concentration then became equal. Kamnev concluded that these sugars are less soluble in the water within normal muscle cells.

3. *TROSCHIN AND THE SORPTION THEORY*

Ten years after Kamnev's paper, A. S. Troschin, another student of Nasonov, broadly extended the investigation of nonelectrolyte distribution and more formally propounded the sorption theory [37,38]. He argued that the level of a substance in the cell is determined by a combination of three factors: solubility, adsorption, and chemical binding in the living protoplasm. Troschin was able to show, based on this concept, that nonelectrolyte and amino acid concentration in living cells could be described by the following equation:

$$C_c = C_s K\left(1 + \frac{A_\infty}{C_s K + a}\right) \quad (1)$$

where C_s is the equilibrium concentration of the nonelectrolyte in the external solution, A_∞ is the limit of adsorption, and a is a constant characterizing the curvature of the rise in the adsorption isotherm [see Ref. 38, p. 82]. In plots of C_s against C_c, when C_s is sufficiently large, Equation (1) reduces to

$$C_c = C_s K + A_\infty \quad (2)$$

Then K, the equilbrium distribution coefficient of the nonelectrolyte in question between the cell water and external solution, can be determined from the slope of a plot of C_c against C_s at high C_s values. Troschin showed that in a variety of living cells K values for different sugars, the amino acid alanine, and dyes such as phenol red are below unity. (Troschin's K and the q-value mentioned above are the same.)

Troschin presented his sorption theory in Russian in 1955. Owing to the language barrier, apparently few Western scientists were aware of the work of Kamnev and Troschin until a German translation of Troschin's book became available in 1958. In his monograph, Troschin also extended his theory to deal with K^+ and Na^+ distribution in living cells, relying at that time on data from the literature.

The sorption theory cannot be considered a mechanistic theory in the sense that it does not provide a mechanism that can be derived from first principles in physics to explain the unique characteristics of diminished solubility in cell water and selective adsorption of K^+ in the protoplasm. Rather he relied on the analogous behavior of certain colloidal systems called coacervates. *Coacervates* (a descriptive term coined by Bungenberg de Jong and Kruyt [39,40] are different from other colloidal sols in that, although they contain a considerable amount of solvent, they are immiscible with the solvent and stand apart as a separate phase in contact with a pure solvent phase.

D. The Association-Induction Hypothesis

Departing from the membrane theory which I had accepted previously [41], I presented in 1951 and in greater detail in 1952 an early version of a theory later to become the association-induction hypothesis [42,43].

The new theory, shared with the independently developed sorption theory the belief that solute distribution patterns are primarily equilibrium phenomena. After presenting my reasons for rejecting the membrane-pump theory, I at once proposed a molecular mechanism for the selective accumulation of K^+ ion over Na^+ [42,43]; later, a theory concerning the exclusion of solutes like Na^+ from cell water was published [13,44].

1. *SELECTIVE K^+ ACCUMULATION*

a. Theory

Selective K^+ accumulation over Na^+ is not a unique biological phenomenon: a variety of inanimate systems exhibit a similar ability [13,43]. These inanimate systems include soils, permutit, sulfonated coal, collodion, glass, and man-made ion-exchange resins. These models, in general, and the ion-exchange resins, in particular, clearly demonstrate that the seat of selective uptake is the anionic group fixed on a three-dimensional water-containing matrix of organic and inorganic polymers. Theoretical calculations have led to the conclusion that the greater electrostatic interaction energy between the fixed anion and the small hydrated K^+ could account for the selectivity seen in many living cells of K^+ over the larger hydrated Na^+ [43].

From the analysis of glutamic amd aspartic acid residues of muscle proteins it was shown that "'myosin' alone is able to provide sufficient or nearly sufficient charges to adsorb all the cations..." ([43], p. 774). These fixed anions, of course, refer to the β- and γ-carboxyl groups on the aspartic and glutamic acid side chains. More recently, however, increasing evidence indicates that under certain conditions the carbonyl groups on the protein backbone may

also adsorb alkali-metal ions [23], thereby providing possible sites in high concentrations for selective K^+ accumulation in such organisms as the halophil bacteria.

(1) The C-Value Concept

In further extensions of the earlier theory, the c-value concept was introduced briefly in 1957 and in greater detail in 1960 and 1962 [45,46; cf. 13]. The c-value is, roughly, a parameter representing the effective electron density at an anionic site. Acetic acid has a pK value of 3.76, because its oxyacid group has a high electron density, hence a high c-value. In contrast, the oxyacid group in trichloracetic acid, because of the electron-withdrawal (inductive) effect of the Cl atoms, which are more electronegative than the H atoms they replace, has a much lower pK value, hence a low c-value. Theoretical calculations led to the conclusion that selective K^+ adsorption over Na^+ occurs at low c-value sites. At higher c-value sites, preference for K^+ over Na^+ declines. At a still higher c-value, Na^+ actually becomes preferentially adsorbed over K^+. In most resting muscle cells, apparently many of the β- and γ-carboxyl groups of myosin, for example, are in an electronic state in which they have low c-values. Maintenance of this electronic state depends on the cooperative nature of the protein interactions and the presence of key agents controlling the cooperative state of these proteins. Chief among the key agents, called cardinal adsorbents, is the main metabolic product, adenosine triphosphate (ATP).

(2) The Cooperativity in Ion Adsorption

Cooperativity in ion adsorption provides another interesting perspective when thinking about K^+ accumulation only. The concept of what is "living" is obscure in a model of living cells seen in the context of the membrane-pump theory. In contrast, in the association-induction hypothesis, the protoplasm of the entire cell is seen as a system of proteins, water, and ions in close association. This system may exist at a high-energy state called the "resting"

or living state. But, much as a bent bow or a set mousetrap, the living protoplasm is metastable in nature and can be triggered into a lower energy--but still reversible--active state (e.g., contracted muscle, excited nerve), or irreversibly into a still lower energy state, death.

The discreteness of each of these states follows from the basic "cooperative" nature of the closely associated protein-water-ion system. Cooperativity in the statistical mechanical sense means that elements of the system, such as the sites on a protein chain, are not isolated but interact with neighboring elements, or sites. Based on the one-dimensional Ising method, Yang and Ling derived an equation describing the adsorption of K^+ and Na^+ [47]. The isotherm adequately described the uptake of oxygen by hemoglobin *in vitro* [48] as well as *in vivo* [49]. Similarly, the theoretical isotherm provides the basis for a general equation for solute distribution in resting cells [22]. Equation (3) is a special version of this general equation as applied to K^+:

$$\left[K^+\right]_{in} = \alpha \bar{q}_{K^+} \left[K^+\right]_{ex} + \sum_{L=1}^{N} \frac{f_L}{2} \left(1 + \frac{\xi^L - 1}{\sqrt{(\xi^L - 1)^2 + 4\, \xi^L \, ex\, \gamma_L / RT}} \right) \quad (3)$$

in which the first and succeeding terms on the right-hand side refer respectively to free K^+ in the cell water and K^+ adsorbed on the L type of sites (at a concentration equal to f_L) among a total of N types of adsorption sites for K^+. α is the water content of the cell; $\bar{q}_{K^+}$ is the average q-value of K^+ in the cell water; $-\frac{\gamma}{2}$ is the nearest neighbor interaction energy; and

$$\xi = \frac{\left[K^+\right]_{ex}}{\left[Na^+\right]_{ex}} K_{Na \to K}^{\circ\circ(L)} \quad (4)$$

where $\left[K^+\right]_{ex}$ and $\left[Na^+\right]_{ex}$ are the equilibrium concentrations of K^+ and Na^+ in the external medium, respectively; $K_{Na \to K}^{\circ\circ(L)}$ is the intrinsic equilibrium constant for Na→K exchange on the L type of sites.

(3) A New Theory for Biological Energization by ATP

This new theory was presented as part of the association-induction hypothesis [13,48]. To put the theory in proper perspective, the discussion will focus on the activity of muscle tissues.

In the theory originally introduced by Lipmann [50], ATP was thought to carry a package of energy in its "high-energy phosphate bond." When ATP interacts with the contractible protein system, the enzyme ATPase unlocks this "energy package" by splitting off the high-energy phosphate bond. This liberated energy is then transformed into mechanical energy, causing the muscle to contract. Subsequent demonstration that the enthalpy of ATP hydrolysis is not -12.0 kcal/mol, as was once thought to be the case, but only -4.75 kcal/mol [51] destroyed the foundation of this theory.

The association-induction hypothesis offers an alternative interpretation of the way in which ATP functions in living cells: ATP acts as a cardinal adsorbent; its adsorption tips the protein system into a cooperative, high-energy state. When ATP is destroyed, the system goes into a lower energy state, the contracted state. Thus, energy is injected into the system at the moment when the hydrolyzed ATP is resynthesized and readsorbed onto the cardinal site. New experimental findings in studies of the control of hemoglobin oxygen binding provide strong evidence in favor of this view. Thus ATP, like 2,3-diphosphoglycerate, by adsorption onto hemoglobin causes alteration in the affinity of a distant site (the heme site) for its adsorbent oxygen, even though ATP itself is not hydrolyzed as a result of the adsorption [52,53].

Another fact supporting this view of the role of ATP is even simpler. If, as in models based on the high-energy bond concept, the contracted state of the muscle truly represents a higher energy state, a poisoned muscle deprived of metabolic energy must end up in the relaxed state. If, on the other hand, as contended in the association-induction hypothesis, the resting state is the higher energy state, poisoned muscle deprived of the sustaining ATP should end up in the contracted state. It is well known that muscles dead

from metabolic poison as a rule are found in the contracted state, in accord with the association-induction model.

b. Experimental Evidence

(1) Evidence for Adsorption

According to the association-induction hypothesis, the β- and γ-carboxyl groups of "myosin" provide the major adsorption sites for K^+. Recently three different lines of approach including autoradiography [54,55], transmission electron microscopy [56], and electron probe microanalysis [57] have combined to offer unanimous confirmation of this hypothesis. (See also ref. 58 for older evidence.)

The demonstration of low mobility of K^+ in normal frog muscle cells [59], as well as the low intracellular conductivity in red blood cells [60] and in *Aplysia* neurons [61], also confirms the adsorption model.

(2) Evidence for Cooperativity in the K^+ and Na^+ Adsorption in Living Cells

Since Ling [49] first demonstrated cooperative K^+ uptake by frog muscles (see Fig. 2; also Eq. 3), extensive confirmation of the theory of cooperative adsorption of K^+ and Na^+ has been reported for a variety of living tissues [62-66].

(3) Evidence for the Control of Cooperative K^+ and Na^+ Adsorption by Cardinal Adsorbents

A quantitative theory based on the cooperative adsorption isotherm forming the right-hand-most term of Equation (3) could describe the experimental data of Benesch and Benesch [67] on the control of oxygen binding on hemoglobin by 2,3-diphosphoglycerate and by inosine hexaphosphate [18]. Similarly, the theory can adequately describe the control of K^+ and Na^+ distribution by low concentrations of the cardiac glycoside ouabain [62]; the control of K^+ distribution of the vascular smooth muscle of canine carotid arteries by ouabain and by Ca^{2+} were reported by Gulati [66]; and the control of K^+ distribution of the smooth muscle in teniae coli of guinea pig were reported by Jones [68].

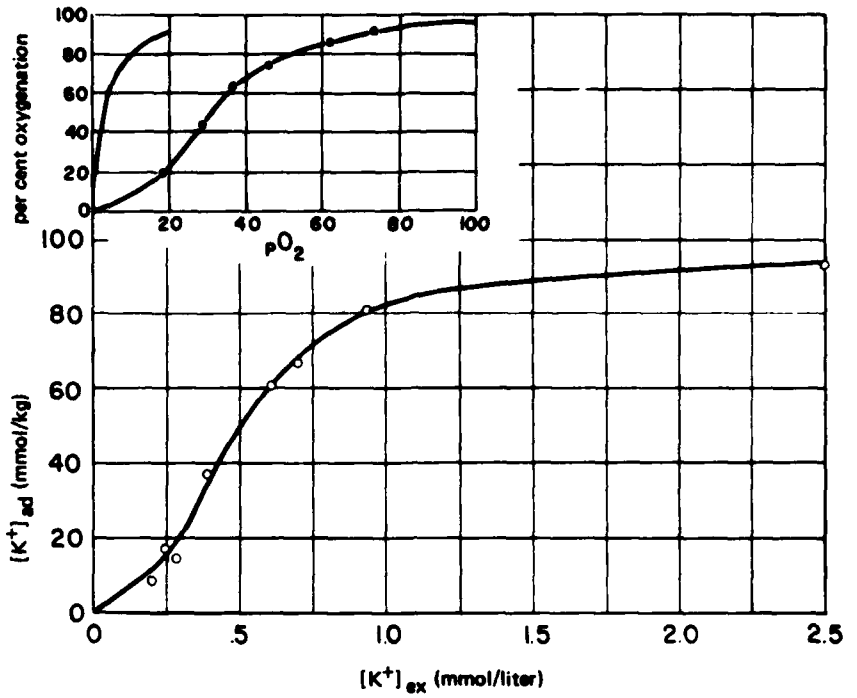

FIGURE 2 Equilibrium K^+-ion concentration in frog sartorius muscle in solutions with low K^+-ion concentrations but a high Na^+-ion concentration. Sterilely isolated sartorius muscles were shaken for 72 hr at 25°C in Ringer solutions containing a fixed concentration (100 mmol/liter) of Na^+ ion and varying low K^+ and Na^+ ions were analyzed by flame photometry on HCl extracts of the muscles. Total intracellular ionic concentration was obtained from raw analytical data after correcting for extracellular space (10%). Adsorbed ionic concentration in mmol/kg of fresh tissue was further computed from the total intracellular concentration by subtracting the interstitial ion concentration (estimated as 10.4% of the equilibrium external ion concentration; this figure represents an average of all values determined to this point). Each point represents a single determination. Inset shows oxygen uptake by human erythrocytes (line with filled circles) and by myoglobin (solid line). (From N. J. Eastman, E. M. K. Gelling, and A. M. DeLawder, quoted by R. G. McFarlane and A. H. T. Robb-Smith in *Functions of the Blood*. New York: Academic Press, 1961. The larger graph from Ling [49], by permission of *Fed. Proc.*).

(4) Evidence for a Predicted Quantitative Relation Between Selectively Accumulated K^+ and ATP in Living Cells

According to the association-induction hypothesis, each group of cooperatively linked protein regular sites (called a "gang" of regular sites) may be controlled by one cardinal site, and occupancy of the cardinal site by ATP is required to maintain a c-value of all the regular sites in this gang at which K^+ is selectively adsorbed. When ATP

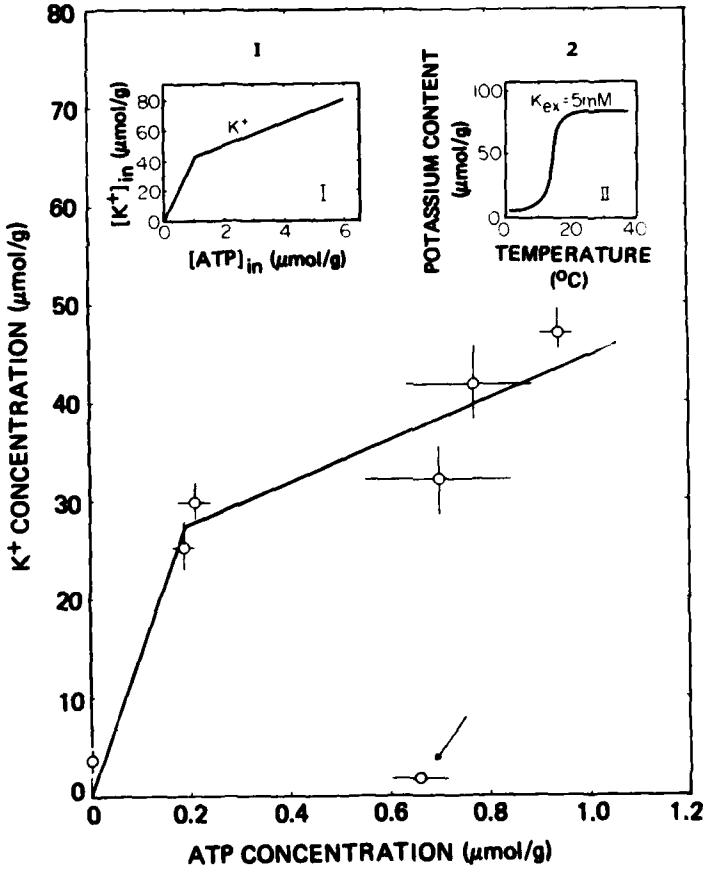

FIGURE 3 Plot of ATP vs. K^+ concentration in rat myometrium. Variations of ATP concentration were brought about by various metabolic poisons and by cooling (marked with arrow). Data from Rangachari et al. [70]. Inset 1 shows quantitative relation between K^+ and ATP in frog sartorius muscles from Ling and Ochsenfeld [59]. (Other similar data are given by Ling [13], p. 252.) Inset 2 from Reisin and Gulati [65] shows temperature transitions from K^+ to the Na^+ state in the guinea pig teniae coli. [From G. N. Ling, *Physiol. Chem. Phys.*, *6*: 285-286 (1974), by permission.]

is hydrolyzed, the regular sites give up their K^+ in exchange for Na^+, a fixed cation, or some other cations [59].

A prediction of this theoretical model is that at equilibrium there should be a quantitative relation between the concentration of ATP and that of K^+ in the cell. Such a relation was demonstrated by Ling in 1962 [13] and has been repeatedly confirmed since then [59,69]. Recently, however, Rangachari et al. [70] had contended that in uterine smooth muscle the predicted relation did not hold. A replotting of their data (Fig. 3) shows that most of the experimental points agree reasonably well with our earlier findings in frog muscles (shown in the left inset of Fig. 3. The single point that does not follow the predicted relation is indicated by an arrow). It turns out that this point was obtained by cooling the uterine muscle to 0°C. Rangachari et al. [70] were apparently not aware of the fact that Reisin and Gulati [65] from our laboratory had already shown that lowering the temperature to below 14°C causes a temperature transition in another mammalian smooth muscle, the guinea pig teniae coli [65]. If this temperature transition also occurs in uterine smooth muscle--a reasonable assumption--then the data of Rangachari et al. had in fact confirmed not one but two of the basic relations derived from the association-induction hypothesis.

2. SOLUTE EXCLUSION

a. The Theory Proper

The theory of polarized multilayers of cell water will now be discussed. Having presented experimental data showing that maintenance of the partial exclusion of one solute, Na^+, by pumps would consume 15 to 30 times as much energy as the cell has, I concluded that the pump theory is not tenable as a general mechanism for the asymmetrical solution distribution between the inside and outside of resting cells. In the chapter on "Selective Distribution and Permeability of Nonelectrolytes" in *A Physical Theory of the Living State* [13], I suggested two types of basic mechanisms for the exclusion as well as for the accumulation of a solute in living cells:

(1) an entropic mechanism in which restricted rotational motion lowers the entropy of the solute and hence of free energy in the cell water, and (2) an enthalpic mechanism in which differences in hydrogen-bond formation lowers the enthalpy of the solute in the cell.

In 1965, the theory of exclusion mechanisms was presented in greater detail following an explicit exposition of the polarized multilayer theory of cell water [44]. It was then suggested that the bulk of cell water exists in a state of polarized multilayers and that, as such, the water uptake, a, of living cells in environments containing water at varying relative vapor pressures, p/p_0, should follow the polarized multilayer adsorption isotherm derived by Bradley [71]

$$\log \frac{p_0}{p} = K_1 \cdot K_3^{\,a} + K_4 \qquad (5)$$

where K_1, K_3, and K_4 are constants under specified conditions. When this theory was presented, no explicit experimental data existed to evaluate this theoretical prediction. Five years later, Ling and Negendank [72] reported success in such a study. They showed that about 5% of the water in frog muscle cells exists in a tightly bound monolayer; the remaining 95% does indeed follow Equation (5).

In 1972 [73], in a chapter entitled "Hydration of Macromolecules" in the monograph *Water and Aqueous Solutions*, the theory of polarized multilayered states of the bulk of cell water was further extended. It was pointed out that not all charged surfaces can polarize deep layers of water. Thus, if the surfaces are uniformly positively (P) or negatively (N) charged, water sorption cannot extend much more than a monolayer deep. However, if each positively charged surface site is surrounded by negatively charged sites, one has what is referred to as an NP system. If the distance between the sites is of the right dimension, then deeper layers of water molecules can be polarized and oriented. Still deeper, more stable water layers can be maintained when two NP surfaces are juxtaposed, as in the case of water held between polished glass surfaces. This is called an NP-NP system.

A somewhat different system, also theoretically capable of polarizing deep layers of water molecules, consists of a matrix of parallel linear chains bearing alternating positive and negative sites, each site being separated by appropriate distances from its immediate neighbors. Such a system is referred to as an NP-NP-NP system. Biologically speaking, the NP-NP-NP system is the most important system. According to the association-induction hypothesis, the one-dimensional linear chains of the cell proteins provide the positively charged NH and negatively charged carbonyl sites to polarize and orient the bulk of cell water.

It is interesting to consider the theoretical requirements of NP-NP-NP systems to polarize deep layers of water. It is well known that the same protein chain may exist in more than one conformation. The chain can fold upon itself, whereupon each backbone carbonyl group forms hydrogen bonds with the nitrogen of amine groups of a third amino acid residue farther away, thereby forming the α-helical conformations. Alternatingly, the carbonyl groups on one chain can form hydrogen bonds with N-amide groups from other neighboring polypeptide chains in the β-pleated sheet conformation. In these cases, the N-amide and carbonyl groups are internally "neutralized." Clearly the protein in either one of these or similar conformations cannot provide the sites to polarize and orient deep layers of water. To polarize and orient deep layers of water, the protein backbone must satisfy two basic criteria: (a) it must be "extended", and (b) it must be "free."

Being extended means not forming α-helix folds; being free means not forming hydrogen bonds with other polypeptide chains of the same or a different kind. Such extended and free conformations of proteins have often been referred to as "random coils." Here we contend that in the living state, certain extended and free chains are neither randomly oriented nor coiled but are arranged in essentially orderly parallel arrays in a matrix of chains.

The following question can be asked: Are there universally (or at least widely) present proteins in all living cells that function

to maintain the bulk of cell water in the state of polarized multilayers? Virtually all living cells partially exclude solutes like Na^{2+}, sugars, and so on. From the basic concepts of the association-induction hypothesis, one can expect that most, if not all, living cells are equipped with a protein system which under normal conditions can function as NP-NP-NP systems in polarizing and orienting the bulk of cell water.

From our knowledge of both phylogeny and ontogeny, we can say that all living cells have a common origin. It seems reasonable, therefore, that the same or very similar protein or proteins may serve the same purpose in all living cells. At this time there is no concrete evidence to substantiate the idea; nevertheless, I would like to suggest that such a "universally" present protein does indeed exist and that it probably includes actin as a major, if not the chief, component (and perhaps also, to some extent, myosin). One reason for this speculation is that with its high proline content, actin has a natural tendency to exist in an extended state. Another reason is that actin has already been detected in a wide variety of living cells, and it is not unlikely that it is present in all living cells.

Intriguing to note also is the fact that, on the one hand, Ambrose et al. [74] have discovered abnormally oriented protein filaments in malignant cells and that, on the other hand, there are reasons to believe that water in malignant cells is less organized [75-77]. If actin in normal cells can indeed function as the basic NP-NP-NP system in orienting cell water, as accords with the association-induction hypothesis, its disorganization can easily explain a lesser degree of water polarization in neoplastic cells. More recently Minkoff and Damadian [78] have suggested a role for actin in normal cells. They propose that actin exerts a "cytotonus" on cell water by contracting and that this pressure then causes K^+ accumulation and Na^+ exclusion, much as in Gregor's theory of ion selectivity in ion-exchange resin [79]. However, Gregor's theory cannot explain why changes of oxyacid from sulfonate to carboxyl

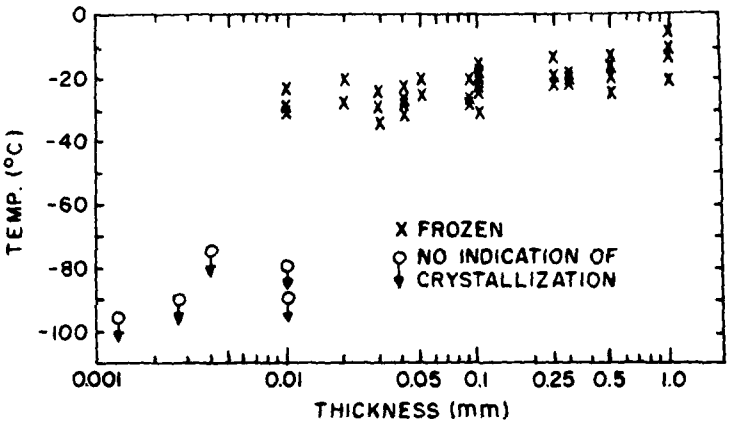

FIGURE 4 Effect of distance between flat glass plates upon the freezing point of water held between them. Distance between plates given in millimeters on the abscissa. (Redrawn after Hori [83], by permission of *Low Temp. Sci.*)

in ion-exchange resin should reverse the order of selectivity from K^+ over Na^+ to Na^+ over K^+ [80]. Nor can it explain the much greater selectivity for Tl^+ and Ag^+ over other ions of similar size (e.g., Cs^+; see Ref. 81, p. 162). On the other hand, the association-induction hypothesis offers a general interpretation of all these experimentally derived facts with no constraints (for details, see Ref. 82).

b. Properties of Water Under the Influence of Fixed Charges

It would now seem appropriate to consider the properties of water, presumably in the state of polarized multilayers.

(1) Freezing, for example, is pertinent. Figure 4 shows that a thin film of water held between polished glass plates will not freeze at temperatures as low as -190°C [83]. This is a case of water in a typical NP-NP system.

It is also known that 60% gelatin will not freeze at -190°C. This is a case of water in a proteinaceous NP-NP-NP system [84].

Like these model systems, normal living cells repidly frozen will not form ice crystals [85] and muscle cells will form only long spikes rather than snowflake-like hexagonal structures when the supercooled cytoplasm is brought into contact with ice [86,87]. In

my opinion, the long spike is an artifact produced by the injury suffered as a result of the protein-filament splitting action of the front end of the advancing ice spike [18]. The best evidence for this conclusion is the fact that if the ice spikes form and then thaw, new spikes will re-form at exactly the same locations on further cooling [86]. Thus water in normal living cells, like that in concentrated gelatin and thin water films held between glass plates, is fundamentally not freezable. Only when injury produces a shift of the protoplasm to a nonliving state can ice formation follow; even then it is limited to freezing in "one" dimension following the orientation of the actomyosin filaments.

(2) While we must await future investigation to know more about the structure of water in the state of polarized multilayers in NP-NP and in NP-NP-NP systems, certain conclusions can already be drawn from existing information. According to the basic theory itself, the structure of water in polarized multilayers must be significantly determined and constrained by the structure of polarizing sites of the NP-NP or NP-NP-NP systems. That is to say, water in the state of polarized multilayers is not expected to be in the open tetrahedral structure of ice I.

One can also deduce some information about cell water structure from its behavior when it freezes. Thus the freezing patterns of living cells quite clearly show that there is no ice I preformed in normal cells. If there were, it would have seeded itself in ice formation in supercooled cells. Actually supercooled cell water forms ice spikes only after being brought into contact with external ice I crystals, thus proving the lack of similar ice I structures in the cells normally [86,87].

It is not possible to "catch," so to speak, the normal liquid water state by suddenly cooling ([88], p. 2), but it is possible by sudden cooling to catch water in the state of polarized multilayers [85]. This difference would suggest that water in the state of polarized multilayers is not closer to the ice I structure than liquid water but farther away from it. In other words, water in

polarized multilayers has distinctive cooperative features of its own, different from the structure of the major components of normal liquid water.

(3) In presenting a reasonable interpretation of ion behavior in living cells based on the polarized multilayer theory of cell water, it is essential to show that water in living cells can exist in deep enough polarized layers to fit the need in explaining solute exclusion patterns. While more definitive results will be forthcoming from work now in progress in our laboratory, the data of Jellinek and Fok [89] on the freezing properties of the NP-NP-NP system of polyvinylpyrrolidone (PVP) is highly informative. These authors have shown that PVP can cause the polarization of water molecules and render the water nonfreezable at below freezing temperatures. The amount of water so polarized reaches a level of 20 g of water per gram of the polypeptide. This amount is five times greater than that found in most living cells, in which there are uaually only 4 g of water per gram of protein. It is true that many of the proteins in living cells are globular and thus contribute little to water polarization. Nevertheless, the model PVP behavior shows that if the actin (and partly myosin) in living cells is as effective as PVP, there would be sufficient long-range ordering to render all cell water nonfreezable. This follows from the fact that actin, which alone constitutes 15% of the total muscle cell protein, is capable of polarizing 75% of all the water in the muscle cells. In fact, other proteins, particularly myosin, may also polarize water in deep layers, thereby providing the basis for the polarization and orientation of all the cell water.

(4) At one time it was believed that the significantly reduced nuclear magnetic resonance (NMR) relaxation times of water protons in living cells were due entirely to the structure of water molecules in the cells [90,91; cf. 18]. I now believe that a minor quantity of water associated with paramagnetic ions and other substances may also make a substantial contribution to the reduction of the spin lattice relaxation time (T_2) of water protons. Indeed, under certain

conditions, the contribution from the minor component may mask the acceleration of relaxation times caused by the orientation and polarization of the bulk water.

A simple theoretical consideration of the association-induction hypothesis will show that severe reduction of the T_1 and T_2 of water protons is not called for in the case of water existing in the state of polarized multilayers. Thus, to account for an equilibrium distribution coefficient (q value) of 0.1 for Na^+ (as sodium chloride), the partition function of the hydrated Na^+ in the polarized multilayered water needs to be different by only one order of magnitude from its partition function in free water. The partition function of a complex hydrated ion is difficult to calculate, but a rough estimate would indicate a figure in the thousands, if not much higher ([13], p. 24). That is to say, to have a degree of "structuring" sufficient to account for the exclusion of Na^+, one needs only a relatively small degree of motional restriction, which would then be compatible with a T_1 reduction of moderate magnitude. Preliminary experimental data for work in progress support this expectation.

(5) There are several characteristic features of solute exclusions; for example, the stability of polarized water depends upon the degree of cohesion among the neighboring water molecules therein. Thus, polarized multilayers of cell water demand a greater enthalpy in the water-water interaction. The theory also anticipates a concomitant lower entropy of the water molecules, particularly rotational entropy.

Now, if a solute molecule is transferred from the normal aqueous solution into water in the state of polarized multilayers, three possible sources of free-energy changes accompany this transfer: (a) a cavity has to be created in the polarized water entailing an expenditure of enthalpy larger than that gained in filling the hole left in the normal aqueous solution; (b) at the periphery of the hole, the enthalpy gained in forming bonds with the surrounding polarized water molecules may be less than that lost in the severing of the bonds before the transfer; and (c) the entropy of the solute,

especially the rotational entropy of the transplanted solute, may suffer restriction in the more structured water, causing a reduction of entropy.

Each of these three mechanisms may cause an unfavorable free-energy change for the transfer of a solute from a normal aqueous environment into the polarized water. Each of these mechanisms tends to be enhanced for the larger and more complicated molecules and ions: the larger molecule demands a greater cavity in the polarized water; it has more sites of interaction at its peripheral surface, and hence a greater chance of mismatching; and it also has more rotational entropy to begin with, and thus a greater probability of lower rotational entropy. For these reasons, one would anticipate that the q value would decrease with increasing size and complexity of the individual solute, as in the normal frog voluntary muscles shown in Figure 1. The same sequential order of q value is seen in the model sulfonate ion-exchange resin (Figure 5).*

In other work recently published [92], we also have shown that in the case of sulfonate ion-exchange resin this variation in q value with solute molecular size has nothing to do with pore size. When the counterion of the resin is Li^+ or Na^+, the water in the resin excludes D-arabinose effectively, as shown by a ρ value of 0.4. (The ρ value may be equal to the q value but is a more descriptive term, since it may include solutes adsorbed; thus the ρ value may equal or exceed q, but q cannot exceed ρ.) Changing the counterion to Cs^+ causes a large loss of resin volume and water content, hence a reduction of average pore size. Thus, if pore size is the mechanism for the exclusion of larger solutes like D-arabinose but not the smaller solute molecules, the ρ value should be significantly lower for the Cs^+ resin than for the Li^+ resin. The experimental data show exactly the opposite. The ρ value for D-arabinose in the Cs^+ resin

*The Universality Rule: In the association-induction hypothesis, exclusion of all solutes in the cell water is due to the same cause. Thus if the water structure in a living cell changes, the q value for all excluded solutes will change *pari passu*.

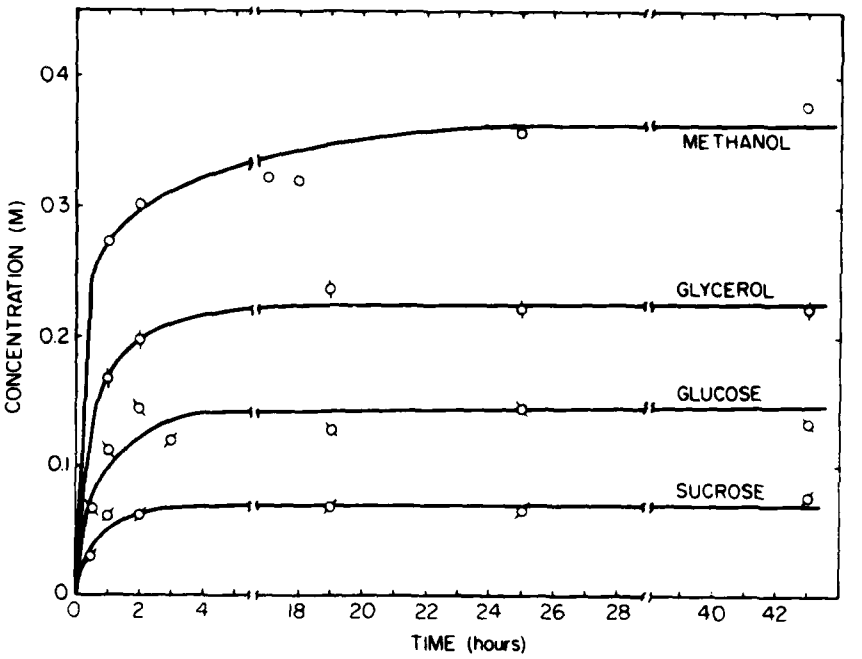

FIGURE 5 Time course of uptake of methanol, glycerol, glucose, and sucrose by sulfonate ion-exchange resin. Rexyn RG50 (H^+ form) at 0°C. Approximately 5 g of wet resin in 5 ml of solution containing 50 mmol/liter acetate buffer at pH 3.75. Final concentration was 0.383 *M* for methanol, 0.382 *M* for glycerol, 0.485 *M* for glucose, and 0.458 *M* for sucrose. The equilibrium distribution coefficients (q values) were 0.97 for methanol, 0.464 for glycerol, 0.309 for glucose, and 0.153 for sucrose. [From G. N. Ling, C. Miller, and M. M. Ochsenfeld, *Ann. N.Y. Acad. Sci., 204*: 6 (1973), by permission.]

shows no exclusion. The data, while disagreeing with the pore concept, agree well with the water structure interpretation offered in the association-induction hypothesis.

c. Theories of Protein Hydration

Let us now turn to other theories of protein hydration. In 1972, while preparing the review on the hydration of macromolecules [73], I came upon an apparent paradox: the rapidly accumulating experimental evidence concerning hydration was supporting two diametrically opposite theories! In Pauling's theory [93], the side-chain polar groups of proteins are the only sites of hydration; in Lloyd and

Phillips' theory [94], both side-chain polar groups and the backbone (peptide bond) groups are sites of hydration. A resolution for this paradox came from a discovery of the difference in the origins of the two groups: biochemists in the former case, and industrial chemists in the latter. Thus, soluble proteins, being pure and readily available, were preferred by biochemists and hydrate only on polar side chains. Fibrous proteins, which are of prime interest to industrial chemists, hydrate both on polar side chains and on the backbone. These conclusions played a role in the more detailed formulation of the theory of polarized cell water, in which alternatingly positive N-amide and negative carbonyl groups of extended backbones provide the basis for long-range ordering of the NP-NP-NP type described earlier.

The theory of polarized multilayer cell water is, of course, fundamentally a specific theory of long-range ordering of water molecules by a matrix of more or less parallel extended backbones. Other theories of protein hydration have been proposed and will now be compared with the polarized multilayer theory.

(1) B. Jacobson's [94a] lattice-fitting theory of aqueous macromolecular solutes states that "if the macromolecule has many oxygen and nitrogen atoms on the surface in such a position that they fit into the ideal water lattice, a very pronounced ordering effect is obtained and results in an almost ideal four-coordinated structure." The basic function of the macromolecular surface is lattice fitting, not long-range multilayer electrical polarization. Jacobson's theory presents no explicit mechanism for the long-range effect of the water lattice-fitting macromolecular surface.

(2) Another theory of protein hydration is A. Szent-Györgyi's theory of biological energy transfer, invoking an "iceberg" concept. In his monograph *Bioenergetics*, Szent-Györgyi [75] asked the questions: How does energy drive life?... How does it move the living machine? Demonstrating the remarkable effect of freezing on the fluorescence behavior of water solutions of various dyes, Szent-Györgyi then argued that if water near protein surfaces is ice-like,

it will have physical properties that permit forbidden transitions into the triplet state of fluorescent substances.

Szent-Györgyi did not elaborate on the nature of the "iceberg" but referred to Jacobson's 1955 paper just cited [94a]. In a later article in 1971 [95], Szent-Györgyi also cited Klotz's theory (discussed next), which, though called "iceberg," has certain distinctive features that differ from Jacobson's theory.

(3) I. M. Klotz's apolar iceberg theory of protein hydration will now be considered. His theory of macromolecular hydration was based on the "iceberg" concept of Frank and Evans [96], who presented evidence that water is ordered around apolar solutes. Citing the example of clathrate formed around different apolar molecules, Klotz suggested that a similar structure of water can then form around side chains of proteins and is, therefore, a part of the "native" conformation of a protein [97].

The profound difference between this theory and the polarized multilayer theory is self-evident.

(4) It seems appropriate to comment on these alternative theories. On the basis of logic alone, the disproof of the two alternative theories has quite clearly established the equilibrium theory as the basis of selective K^+ accumulation or Na^+ exclusion. That is to say, for solutes, the physicochemical environment of the cell is different from that of the surrounding medium. Considering that both charged ions and uncharged molecules are excluded from the cell water, one sees a compelling reason for a difference in the solubility properties of the medium of the cell, i.e., water.

The most explicit theory of protein hydration is that of Klotz [97,98], which refers to native globular proteins. Existing evidence, however, suggests that in most globular proteins the water of hydration is primarily oriented by polar rather than apolar side chains [93,98, 99; cf. 73]. Nonpolar groups tend to be folded toward the inside of the molecules and are thus inaccessible and not able to act as centers around which the clathrate-like water cages may form, as suggested in Klotz's theory [97]. Certainly the different methods of measurement

have produced considerable consistent evidence that monolayers of water no deeper than 0.2 to 0.3 g water per gram of protein exist around globular proteins. However, none of these findings rules out the applicability of Klotz's theory to other proteins not yet extensively studied or even to that part of the hydration in proteins already examined in detail.

Jacobson's [94a] lattice-fitting model is based on the concept that, as a result of macromolecular surface hydrogen bonding, "a very pronounced ordering effect is obtained and results in an almost four-coordinated structure." Of course, it is well known that in ice I each water molecule is hydrogen bonded to four others in nearly perfect tetrahedral coordination. Thus, in fact, Jacobson's theory is even more accurately described as an iceberg theory in the literal sense. The fact that externally introduced ice is necessary for initial freezing of supercooled cell water argues against application of this theory to water in living cells. While the polarized multilayer theory appears to suffer no major contradictions at this time and seems to be in harmony with much of the existing data on long-range ordering of water, its establishment must rest upon more concrete, unequivocal proof of long-range ordering of water molecules in simple model systems. In our laboratory, ongoing research to reach this goal is beginning to show promise. (For a preliminary note see ref. 100.)

ACKNOWLEDGMENTS

This work was supported in part by NIH grants I-ROI-CA16301-01 and 2-ROI-GM11422-11A1 and ONR Contract Number N00014-71-C-0178. The John A. Hartford Foundation provided many of the basic facilities. We thank Marilyn DeFeo for her valuable help.

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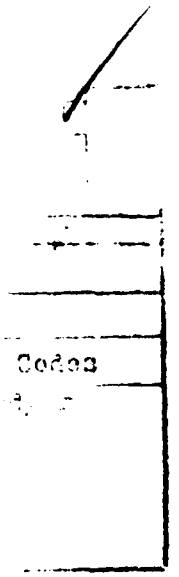

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