STUDIES ON THE PHYSICAL STATE OF WATER IN LIVING CELLS AND MODEL SYSTEMS: IX. THEORETICAL SIGNIFICANCE OF A STRAIGHT LINE RELATIONSHIP BETWEEN INTRACELLULAR CONCENTRATION OF A PARTIALLY EXCLUDED SOLUTE AND ITS CONCENTRATION IN THE BATHING MEDIUM

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• *The experimentally observed steady-level distribution of Na^+ (25° C) and of D-glucose (O°c) in frog muscle were chosen as examples of solute distribution patterns observed in living cells, for comparison with those predicted by two theoretical models: one derived from the membrane-pump theory and the other from the association-induction (AI) hypothesis. Neither the distribution of Na^+ nor that of D-glucose follows the pattern predicted by the membrane-pump models for solutes maintained at lower level than in the external medium, in which the plot of intracellular solute concentration as ordinate against different external concentrations as abscissa bends upward with increasing external solute concentration. Instead, both Na^+ and D-glucose exhibit either straight line distribution with unchanging (below unity) slopes, or that of a hyperbola superimposed on such a straight line, both in agreement with the AI hypothesis.*

The polarized multilayer (PM) theory of cell water was introduced (Ling, 1965) as part of a broader theory of the living cells called the association-induction (AI) hypothesis (Ling, 1962, 1984). In the PM theory, the bulk of water in living cells exists in the state of polarized multilayers in consequence of direct or indirect interaction with the exposed backbone NHCO groups of a matrix of immobilized and fully extended protein chains. Such a matrix of more or less parallel linear chains carrying alternatingly positive NH sites (P) and negative CO sites (N) at proper distance apart is referred to as an NP-NP-NP system. A variant of the NP-NP-NP system is an NO-NO-NO system in which the P sites are replaced by neutral ones (O). In earlier papers, we have shown that solutions of gelatin and of ureadenatured proteins are examples of NP-NP-NP systems while solutions of polyethylene oxide (PEO), polyvinyl-pyrrolidone (PVP), polyvinylmethylether (PVME) are examples of NO-NO-NO systems (Ling et al., 1980).

A major prediction of the PM theory is the reduced rotational (and translational) motional freedom of the bulk phase water molecules (Ling, 1965, 1970, 1972). Rotational (and other) freedom of the bulk phase water molecules in living cells and model systems can be measured by quasielastic neutron scattering (QENS), nuclear magnetic resonance (NMR), and ultra high frequency dielectric dispersion (UHFD) methods. In the course of the last ten years, all these measurements have been made producing results unanimously confirming the predicted rotational motional restriction [Model studies: QENS, Rorschach (1985); UHFD, Kaatze et al. (1978); NMR, Ling and Murphy (1983); Living Cell Studies: QENS, Trantham et al. (1984); Heidorn et al. (1986); UHFD, Clegg et al. (1984); NMR (qualified confirmations, see Ling and Murphy, 1983), Seitz et al. (1980)].

Other predictions in regard to the proper-

ties of bulk phase water in the state of polarized multilayers that have also been tested and confirmed in model systems include extremely high osmotic activity (Ling, 1983), freezing point lowering not seen in solutions of native proteins (Ling and Zhang, 1983), sustained swelling or shrinkage of dialysis sacs containing NO-NO-NO polymer solutions in dilute or concentrated electrolyte and nonelectrolyte solutions respectively (Ling and Ochsenfeld, 1987), and extensive sorption of water in quantity matching that of living cells (Ling and Hu, 1987). However, it is the predicted partial exclusion from polarized water of solutes that are found at low concentration in living cells, e.g., Na salts, sugar and free amino acids, that is of prime importance. One recalls that it was the need to explain the reduced levels of these solutes in living cells that prompted the introduction of the PM theory.

THEORY

1. *The Sodium Pump Hypothesis.*

The idea that the cell membrane can regulate the contents of living cells at the expense of metabolic energy dates back to the middle of the 19th century when Theodor Schwan, the founder of the "Cell Theory", suggested that the cell membrane had "metabolic power" with which it could regulate the chemical composition of both the fluids surrounding the cells and within the cells (see Hall, 1969, p. 194). After radioactive tracer studies had disproved the atomic sieve version of the membrane theory (Cohn and Cohn, 1939; Heppel, 1939) according to which larger solutes like (hydrated) Na^+ are impermeant to the cell membrane due to limiting size of membrane pores (Boyle and Conway, 1941), the Na pump hypothesis became increasingly accepted. In the 48 years following, enormous amounts of time and effort have been spent in investigating the postulated Na pump (Glynn and Karlish (1975), Hoffman and Forbush (1983), Glynn and Ellory (1985), Clausen (1986)).

From its very start, the Na pump hypothesis has been unusual on two accounts: (i) the lack of a theoretical mechanism for the pump and (ii) the lack of an equation describing the steady level of Na^+ in living cells.

Dean, often regarded as the founder of the Na pump hypothesis — though he made no such claim because the idea he discussed had long been around — pointed out that the postulated Na pump had no mechanism (Dean, 1941) and that a pumping mechanism based on the postulation of "differentially permeable membrane" (i.e., a membrane more permeable to Na^+ in one direction than in the other) is "in effect a Maxwellian demon" and as such violates the Second Law of Thermodynamics. In their 1975 review on the Na pump, Glynn and Karlish again pointed out the lack of a mechanism for the pump. However, more recently several attempts have been made to present mechanisms of just the Maxwellian-demon type which Dean cautioned against (for critical review, see Greco (1982)).

1.1. *The Cohen-Monod Equation for Sugar Distribution in Microbes.*

The lack of a simple formal equation describing the steady level of Na^+ in living cells on the basis of the Na pump is puzzling. This lack of an equation could not possibly have arisen from the difficulty in writing such an equation. After all, other scientists had no trouble in formulating a simple equation describing the steady level of nonmetabolized sugars in living cells. Thus in 1957 Cohen and Monod (1957) introduced such an equation to describe sugar distribution in microbes:

$$y \frac{[S]_{ex}}{[S]_{ex} + K} = c\,[S]_{in}, \qquad (1)$$

where y is the "pumping rate" in units of

moles/cm^2 sec. and c is the "exit constant" in units of cm/sec. K is the dissociation constant of the sugar-pump complex. $[S]_{ex}$ and $[S]_{in}$ are the extra- and intracellular concentration of the sugars during the steady state. As such, this equation is incomplete, because it implies that the cell membrane is "leaky" to the outward moving sugar molecules but not to inward moving ones, in violation of the basic law of physics. However, this oversight can be very easily remedied by adding another term to Equation 1. One then obtains

$$c\,[S]_{ex} + y\,\frac{[S]_{ex}}{[S]_{ex} + K} = c\,[S]_{in}, \quad (2)$$

or

$$[S]_{in} = [S]_{ex} + y/c\,\frac{[S]_{ex}}{([S]_{ex} + K)}. \quad (3)$$

An equation of this kind can describe quantitatively the distribution of solutes where the concentration of the sugar in the cells is higher than (or equal to) that in the external solution.

1.2. ***An Equation for the Steady Level of Na^+ (and Other Solutes) Maintained at Concentrations Lower than in the External Medium Based on the Pump Hypothesis and its Predictions.***

Unlike the sugar permeases of Cohen and Monod, the Na pump keeps the Na^+ concentration within the cell at ***lower*** concentration than that in the external medium. Thus while the postulated sugar permease pumps sugar into the cell, the postulated Na pump pumps Na^+ out of the cell. If one follows the same steps used in deriving the equation (Equation 3) for the sugar permeases, one finds no difficulty in writing such an equation for the Na^+ distribution. Indeed, all one needs to do is to switch the subscripts "in" and "ex" in Equation 3 to obtain the equation for solutes maintained at lower concentrations in the cell:

$$[S]_{ex} = [S]_{in} + y'/c\,\frac{[S]_{in}}{([S]_{in} + K')}, \quad (4)$$

where y'/c and K' have similar meanings as y/c and K except they refer to the outward pumping and the complex of Na^+ with the outward pump respectively. For the Na^+ distribution in living cells, the equation derived on the basis of the Na pump hypothesis is then:

$$[Na^+]_{ex} = [Na^+]_{in} + y'/c\,\left(\frac{[Na^+]_{in}}{[Na]_{in} + K'}\right), \quad (5)$$

where $[Na^+]_{ex}$ and $[Na^+]_{in}$ are the extra- and intracellular concentration of Na' under steady state conditions.

In Figures 1 and 2, $[S]_{in}$ is plotted against $[S]_{ex}$ for various combinations of values of y'/c and K' according to Equation 4. They

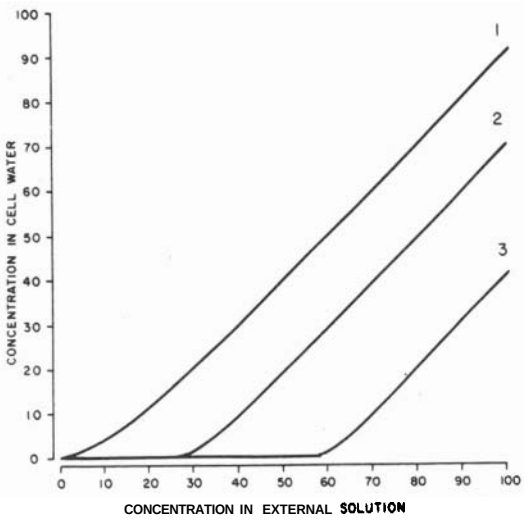

FIGURE 1. **Theoretical curves for the steady-state distribution of solutes in cell water at concentrations lower than those in the external medium calculated according to Equation 4 derived on the basis of the membrane-pump theory. K is 0.01 M for all four curves; y'/c are for curves 1, 2, and 3, respectively 10, 30, and 60 moles/cm^3.**

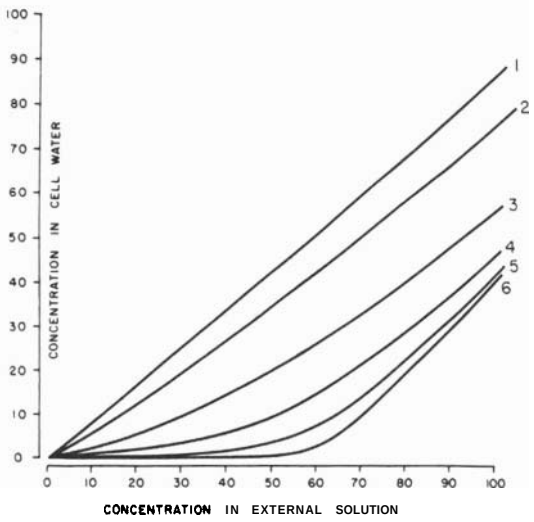

FIGURE 2. Theoretical curves for the steady-state distribution of solutes in cell water at concentrations lower than those in the external medium calculated according to Equation 4 determined on the basis of the membrane-pump theory. Curves 1, 2, 3, 4, 5, 6 were calculated with $y'/c = 100, 60, 60, 60, 60, 60$ moles/cm^3 and K = 500, 100, 20, 5, 1, 0.1 M respectively.

show that as long as y' is not zero, the curves invariably bend upward with increasing $[S]_{ex}$. It is easy to see why. As $[Na^+]_{in}$ increases, the pump sites become more and more saturated and as a result, progressively less and less efficient, until at the extremely high $[Na^+]_{ex}$, $[Na^+]_{in}$ approaches $[Na^+]_{ex}$.

Thus one can predict from this simple equation derived for the Na^+ pump as well as for other outward pumps (intended to keep the steady state, intracellular concentration of the ion or other solutes at a concentration lower than in the external medium) that a plot of the steady state intracellular concentration of the solute against the external solute concentration always bends upward. The only condition in which the plot of $[S]_{in}$ against $[S]_{ex}$ does not bend upward is when the slope is equal to unity (i.e., no pumping). Under no condition does the plot of $[S]_{in}$ against $[S]_{ex}$ bend ***downward*** (i.e., like a hyperbola) while $[S]_{in}$ remains lower than $[S]_{ex}$.

2. *The Association-Induction Hypothesis.*

Serious concern that there is not enough energy to operate the postulated pumps eventually led me in 1952 to suggest what was to evolve into the AI hypothesis: that is, the high level of K' in living cells is the consequence of selective (electrostatic) adsorption of K' on the β- and y-carboxyl groups in cell proteins (Ling, 1952, 1962, 1984). As mentioned above, in 1965, the polarized multilayer theory of cell water was added to the AI hypothesis to explain the low levels of sugars, Na^+ and other solutes in living cells. Both K^+ accumulation and Na^+ exclusion represent equilibrium phenomenon and as such require no continual energy expenditure. From the outset, the AI hypothesis differed from the membrane-pump hypothesis in its focal emphasis on ***physical mechanisms:*** i.e., why is K' selectively accumulated in the cell while Na^+ is not; why are some nonelectrolytes partially excluded from the cells (e.g., sugars) while other nonelectrolytes are not (e.g., urea, see Hill, 1930) and in being able to write simple equations describing the quantitative relationships stated in the theory.

2.1. *Equations for Equilibrium Solute Distribution in Living Cells and Model Systems.*

In the same year the polarized multilayer theory of cell water was published, I also introduced a general equation for solute distribution in living cells and in model systems. This equation and its variations can describe solute distribution in living cells and in model systems ***regardless whether the solute concentration is higher or lower than in the external medium*** (Ling, 1965a, 1969, 1984, p. 321):

$$[p_i]_{cell} = \alpha q_i [p_i]_{ex} + \sum_{L=1}^{N} \frac{[f]_L}{2} \left[1 + \frac{(\xi_L - 1)}{\sqrt{(\xi_L - 1)^2 + 4\xi_L \exp(\gamma_L / RT)}} \right]. \quad (6)$$

The first term on the right hand side of Equation 6 refers to solute in the cell water. The second terms refer to solute that is adsorbed. a is the water content in percentage (v/w). q_i is the equilibrium distribution coefficient for the ith solute. $[p_i]_{cell}$ and $[p_i]_{ex}$ are respectively the intracellular ith solute concentration in moles per kilogram of wet weight and the extracellular ith solute concentration in molarity (i.e., moles per liter of water). $[f]_L$ is the Lth type of adsorption sites with affinity for the ith solute among a total of N types of sites. ξ_L is defined as follows:

$$\xi_L = \frac{[p_i]_{ex}}{[p_j]_{ex}} \cdot K^{oo}_{j \rightarrow i(L)}, \qquad (7)$$

where $[p_j]_{ex}$ is the external concentration of another solute competing against the ith solute for the same adsorption site. $K^{oo}_{j \rightarrow i(L)}$ is the intrinsic equilibrium constant for the jth → ith adsorption exchange. $-\gamma_L/2$ is the nearest neighbor interaction energy, i.e., the extra energy gained each time a new pair of i and j occupies an adjacent pair of sites. Both $K^{oo}_{j \rightarrow i(L)}$ and $-\gamma_L/2$ refer to the Lth sites.

If only one type of adsorption site exists in the cell and if there is no nearest neighbor interaction energy (i.e., $-\gamma/2 = 0$), then the sum of the cooperative adsorption isotherm terms on the right hand side of Equation 6 reduces to a simple Langmuir adsorption isotherm. One then obtains

$$[p_i]_{cell} = \alpha q_i [p_i]_{ex} + \frac{[f][p_i]_{ex}\tilde{K}_i}{1 + [p_i]_{ex}\tilde{K}_i + [p_j]_{ex}\tilde{K}_j}, \qquad (8)$$

where $\tilde{K}_i$ and $\tilde{K}_j$ are the adsorption constants of the ith and jth solutes respectively. Here $\tilde{K}_i/\tilde{K}_j$ is equal to $K^{oo}_{j \rightarrow i(L)}$ of Equation 7. Equation 8 has been extensively used to determine [f], $\tilde{K}_i$ as well as $\tilde{K}_j$ (see Ling, 1984, p. 322). As I have pointed out often in the past, Equation 8 is a modified version of the ***Troshin equation*** (Troshin, 1966, p. 115; Ling, 1965, p. 100; 1984, p. 85, 322).

Now if all the ith solute in the cell is found exclusively in the cell water and none adsorbed, then Equation 8 further simplifies into the following form:

$$[p_i]_{cell} = \alpha q [p_i]_{ex}, \qquad (9)$$

which can also be written as follows:

$$[p_i]_{in} = q [p_i]_{ex}, \qquad (10)$$

where $[p_i]_{in}$ is the concentration of the ith solute in moles per liter of the cell water instead of moles per kilogram of fresh cell weight as in $[p_i]_{cell}$. A plot of $[p_i]_{ex}$ against $[p_i]_{in}$ then yields a straight line with a slope equal to the equilibrium distribution coefficient, q.

In summary, in all living cells and model systems all or a part of any permeant intracellular solute exists in the cell water. As such the concentration of this part of the cell solute is ***rectilinearly*** related to the equilibrium concentration of that solute in the external medium according to Equation 10 with a slope equal to the equilibrium distribution coefficient, q. In other cases there may be one (or more) additional adsorbed fractions of the cell solute. That fraction may appear as a hyperbola or S-shaped curve superimposed on the rectilinear fraction, when $[S]_{cell}$ is plotted against $[S]_{ex}$ according to Equation 8 or 6 respectively. In those cases where the adsorbed fraction can be eliminated by physiological or artificial means, the distribution of the solute should then be reduced to the rectilinear pattern depicted in Equations 9 or 10.

3. *Comparison of Experimental Data with Theories*

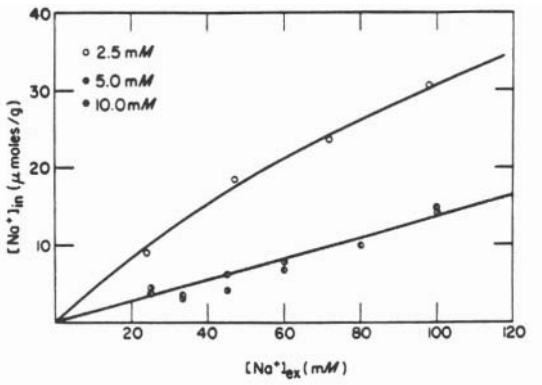

FIGURE 3. Equilibrium distribution of Na^+ ion in frog sartorius muscle in the presence of varying external K^+ ion concentrations (2.5 mM, 5.0 mM, and 10.0 mM). The data were calculated on the basis of a 10% extracellular space. The slope of the straight line going through the points at the higher K^+ concentrations is 0.14. The muscle cells contain 78% water. If all Na^+ in the cell at the higher K^+ concentrations is assumed to be in the cell water, the equilibrium distribution coefficient of Na^+ between the cell water and the external medium is 0.18. [From Ling (1969) by permission of Internat. Rev. of Cytol.]

3.1. Na^+ Distribution.

Figure 3 taken from Ling (1969) shows a plot of the steady level of Na^+ in frog muscle at various external concentrations of Na^+. When the external concentration of K' was 2.5 mM, the $[Na^+]_{in}$ vs. $[Na^+]_{ex}$ plot bends downward. When $[K^+]_{ex}$ is elevated to 5 and 10 mM, the $[Na^+]_{in}$ vs. $[Na^+]_{ex}$ plot becomes a straight line. Under neither concentration does the plot show upward concavity predicted by Equation 4 derived on the basis of the membrane-pump theory.

The data shown in Figure 3 agree well with the AI hypothesis, expressed in the form of Equation 8. In the presence of 2.5 mM K^+ (which is the physiological K^+ concentration) $[Na^+]_{cell}$ plotted against $[Na^+]_{ex}$ appears as a hyperbola superimposed on a straight line suggesting that part of the Na^+ in the muscle cell is in the cell water and the remaining adsorbed following a Langmuir adsorption isotherm. When the external K' is raised to 5 mM, all or virtually all of the adsorbed Na^+ was apparently chased away from the adsorption sites as witnessed by the fact that further increase of external K' to 10 mM produced no additional decrease of cell Na^+ concentration. The curve is now a straight line, described by Equation 9. The slope of the straight line, equal to 0.14 yields a q-value of 0.18 calculated on the basis of the cell's water content (a = 78%).

3.2. D-glucose Distribution

Figure 4 taken from Ling and Will (1969) shows that at O°C (at which temperature D-glucose is not metabolized) and in the absence of insulin, a plot of $[D\text{-glucose}]_{in}$ against [D-glucose]$_{ex}$ yields a straight line with a slope of 0.3. A rectilinear distribution curve with an unchanging slope below unity is again not predicted by Equations 3 or 4 derived on the

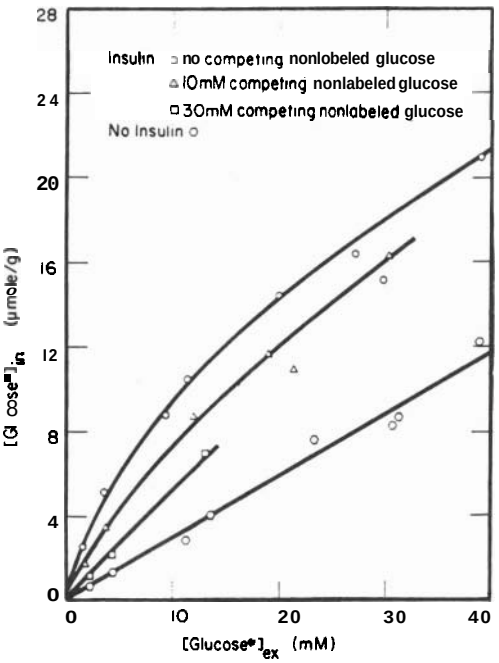

FIGURE 4. Steady level of glucose uptake at O°C in insulin-treated (upper curves) and washed (lower curves) muscles. Mixed frog muscles were preincubated in Ringer solution, containing no glucose and no insulin (lower curve) or 24 mM glucose and 0.1 U insulin/ml for 6 hr. at 25°C. They were then incubated overnight at O°C in Ringer solution containing varying concentrations of glucose and labeled glucose. [From Ling and Will (1969)].

basis of the membrane-pump theory. After prior exposure of the muscle to insulin (0.1 unit/ml.), the steady level of D-glucose in the cells rose higher but was still substantially below that in the external medium. Now the curve, like the Na^+ curve in the presence of 2.5 mM K^+, also bends downward, once more contradicting the prediction of Equation 4 derived on the basis of the membrane-pump theory.

Furthermore, as shown in Figure 4, the labeled D-glucose found in the cell water decreased with an increase of the concentration of external competing nonlabeled D-glucose. According to the membrane-pump theory, an increase of nonlabeled D-glucose could only lead to competition for outward pumping sites, causing a ***rise*** of intracellular labeled D-glucose. What were observed were exactly the opposite. Increase of nonlabeled D-glucose ***decreased*** the level of intracellular labeled D-glucose.

In the total absence of insulin, the data of Figure 4 shows a rectilinear curve in agreement with the notion based on the AI hypothesis that all D-glucose was found in the cell water.

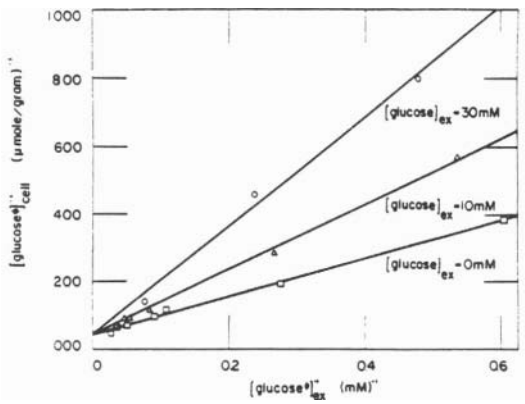

FIGURE 5. Reciprocal plot of adsorbed glucose. The amount of glucose taken up by washed muscles (lowermost curve, Figure 4) was subtracted from the glucose taken up by insulin-treated muscles (upper curves, Figure 5). [From Ling and Will (1969)].

As mentioned above, exposure to insulin increases the intracellular D-glucose concentration. In terms of the AI hypothesis, this increase is attributed to the insulin-induced availability of D-glucose adsorption sites in the cells. This explanation is supported by the fact that after insulin exposure, the distribution of D-glucose now follows Equation 8, where labeled D-glucose represents the ith solute under study and the jth solute as competing nonlabeled D-glucose. In further support of this interpretation, one finds that if one subtracts from the labeled D-glucose concentration of insulin-treated muscle, the labeled D-glucose concentration found in insulin-free muscle (at the same external labeled D-glucose concentration), one obtains a set of data, which plotted reciprocally demonstrates that the extra labeled D-glucose uptake follows the Langmuir adsorption isotherm (Figure 5). From these data we also found that the maximum number of D-glucose adsorption sites in frog muscles induced by insulin treatment is 10.3 ± 0.23 mmoles/Kg. fresh cells and that the adsorption constant of D-glucose on these sites is (8.26 ± 0.48) × 10^{-3}M.

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The following presents the comments of one of the reviewers of this paper and the author's answers. Since both questions and answers are of possible interest to the readers, the editors have decided to reproduce them as part of the effort toward making scientific publications in this journal interesting and more alive than the usual format permits.

Editor

Comments: Don't globularproteins at higher concentration demonstrate abnormally high osmotic pressures as demonstrated by Adair for hemoglobin? Thus, I don't see the need to postulare polarized multilayers of water on filamentous proteins to explain the osmotic and freezing point lowering phenomena.

Answers:

It is true that Adair has demonstrated abnormally high osmotic pressure of native hemoglobin solutions. However, an osmotic pressure described as "abnormally high" is not precise enough to settle the question involved. Indeed, anticipating possible confusion which might arise, I have in my original publication reproduced the very same data from Adair side by side with our new data from gelatin (Figure A) and from polyethylene oxide (PEO) (Figure B). These figures show clearly that even though the hemoglobin data are high in comparison with an ideal solution (which follows van't Hoff's Law of osmotic pressure) they are very much lower when compared with the data from gelatin and PEO, both of which exist at least partially in the fully extended conformation.

To answer the question whether or not it is necessary to offer the polarized multilayer theory to explain freezing point lowering phenomena, and for the convenience of readers who may not have access to my earlier publications, I have once more reproduced here some earlier graphs.

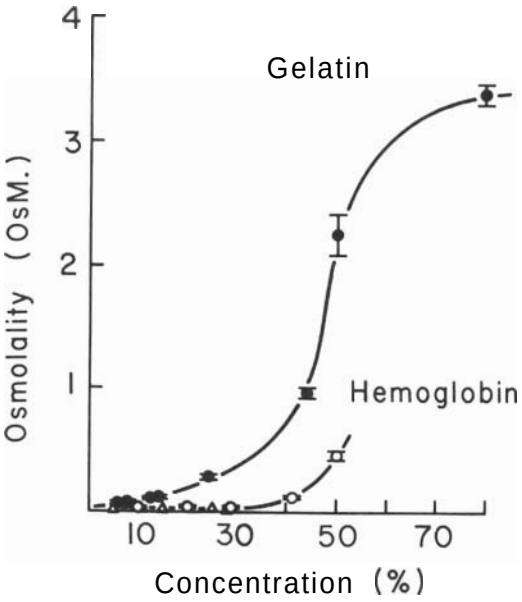

FIGURE A. Osmolality of gelatin and hemoglobin at varying concentrations. Osmolality is given in Osmolal concentration. Protein concentrations are in % (wt. wt.). Each point is the average of at least 4 independent determinations and the distances between horizontal bars are twice the standard errors. Six extra points on the hemoglobin curves shown as A's are taken from **Adair's** data (from Ling, 1983).

First, the freezing point of native hemoglobin solution (and of 5 other native proteins) is independent of the concentration of the protein and at the temperature between **−10°** to **−12° C** (263 to 261 K) (Figure C). On the other hand proteins which for structural reasons as in gelatin (the possession of large percentage of non-helix-forming amino acid residues) or in response to denaturants like urea and in oxygen-containing polymers like PEO, show pronounced **concentration**-dependent freezing point lowering, until at very high protein or polymer concentration, the solutions may not freeze at all (Figure D).

Living cells like Artemia cyst cells exhibit a freezing point as low as **−28°** to **−35°** C (245 to 238 K) far below the freezing point of solution of native proteins (Ling, to be published). These low freezing points of living cells strongly suggest the presence in living cell of proteins which exist in the fully extended conformation'like gelatin or **urea**-denatured proteins. Other evidence demonstrating the need for introducing the polarized multilayer theory to account for the behavior of water in living cells will be given in the

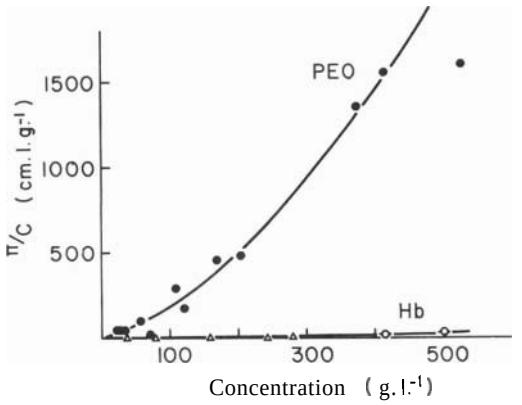

FIGURE B. π/C vs. C plots of PEO solutions. Details are similar to those described in Figure 4. Value of **virial** coefficients are those given in Table I. For comparison hemoglobin data of our own and from **Adair** are also shown (from Ling, 1984).

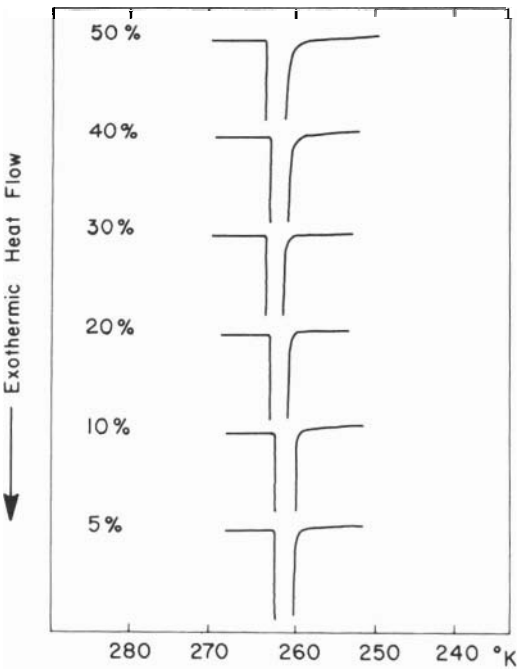

FIGURE C. Cooling **thermograms** of various concentrations of native bovine hemoglobin solutions (from Ling and Zhang, 1983).

answer to the next set of questions raised by the reviewer.

Comments: There is, to my knowledge, no good evidence for or against the concept of polarized rnultilayers of water on fully extended protein molecules in cells. Your need to postulate polarized rnultilayers of water in cells is to provide a mechanism to explain solute (Na^+) exclusion. Our recent reports and unpublished data make clear that globular proteins have a much more extensive water of hydration than previously thought and this can explain the QE(N)S, UHFD, NMR, osmotic behavior and nonfreezing water observations which show that most, and in some cases all of the water in less hydrated cells is non-bulk like in its properties (i.e. there seems no need to postulate polarized rnultilayers to explain these measures).

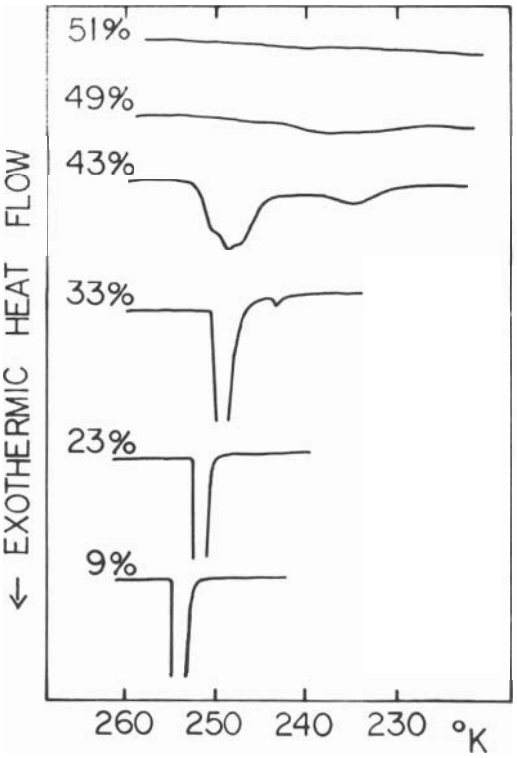

FIGURE D. Cooling thermograms of various concentrations of gelatin solutions (from Ling and Zhang, 1983).

Answers:

I agree with only half of what is said in the first sentence, i.e., no good evidence (exists) *against* the concept of polarized multilayers of water on fully extended protein molecules in cells. I strongly disagree with the other half of the sentence, i.e., no good evidence (exists) *for* the concept of polarized multilayers etc. Indeed one set of such good evidence for the polarized multilayer concept has already been presented above in regard to freezing-point depression and requires no further reiteration.

I also only partly agree with a portion of the second sentence, i.e., globular proteins have a much more extensive water of hydration than previously thought (as we have made similar observation, using methods not used before). Nonetheless, I don't think that neither your finding nor ours on this point has disproved all previous estimates — which are extensive both in terms of kinds of proteins studied and in the diversity of methods used (see Table A reproduced here from an earlier review, Ling, 1972). Rather I believe that they differ from your recent data (and from ours) because the methods used to measure the water of hydration are different and because there is no sharp line of demarcation between what one may regard as normal water. As a result, with some methods more hydration water is measured and with other methods, less hydration water is measured. To illustrate my point, I return to Figure **A** (and other similar data from the studies of 5 other native globular proteins, see Ling and Zhang, 1983), where a 50% hemoglobin solution freezes at the same temperature and at the same rate as a similar solution containing no hemoglobin at all. Taken at face value, this finding would indicate that there is no hydration water at all. Yet the data collected in Table A shows clearly that using other methods, one does indeed detect and measure considerable amounts of water of hydration on the same proteins.

Since no quasielastic neutron scattering (QENS) or ultra-high frequency dielectric (UHFD) measurements have been made on solutions of native globular proteins at concentrations comparable to those made on living cells, it seems premature to say that behaviors of globular native proteins can explain the known QENS, UHFD behaviors of living cells.

However, entirely different situations exist in regard to a comparison of the QENS and UHFD behaviors of fully extended polymer models and the living cells. The QENS behaviors of solutions of PEO matches those of Artemia cyst cells and so do the UHFD behaviors of PEO and other polymer solutions (Rohrschach, 1983; Kaatze et al., 1978).

Since proteins themselves cause NMR relaxation of water protons, it is not possible to meaningfully compare NMR relaxation time data from protein solutions in vitro with those of living cells. However, oxygen-containing polymers like PEO and polyvinylpyrrolidone (PVP) do not directly cause water proton relaxation. Their presence in aqueous solutions do, nonetheless, cause the shortening of NMR relaxation time and the lengthening of NMR correlation time, in general

TABLE A. Hydration of Proetins in Solution modified after Ling, **1972, *by permission of Wiley-Interscience).***

Protein	Technique Identified at End of Table	Hydration (g H_2O/g protein)
Serum albumin	A	0.2
	B	0.30 – 0.42
	C	0.19 – 0.26
	D	0.31
	E	1.07[a]
	F	0.75[a]
	G	0.43
	F	0.40
	H	0.48
	A	0.18 – 0.64
	A	0.15
	A	0.23
Avg.		0.32
Ovalbumin	I	0.18
	A	**0.1**
	B	**0 – 0.15**
	G	**0.31**
	E	**0.45'**
	A	**0.18**
Avg.		**0.17**
Hemoglobin	B	0.20 – 0.28
	D	0.10
	G	0.45
	E	0.36[a]
	F	0.69[a]
	A	0.14
	A	0.2
Avg.		0.25
8-Lactoglobulin	B	**0 – 0.20**
	E	**0.72"**
	F	**0.61'**
	A	**0.4**
	A	**0.24**
Avg.		**0.25**
Lysozyme	B	**0.25 – 0.32**
	D	**0.24**
	G	**0.36**
	E	**0.89'**
	F	**0.33 – 0.35**
	A	**0.23**
Avg.		**0.29**
Ribonuclease	E	**0.35**
	F	**0.59**
	C	**0.34**
Avg.		**0.43**
Myoglobin	A	**0.2 – 0.3**
L-Chymotrypsin	G	**0.37**
Chymotrypsinogen	E	**0.52**
Catalase	E	**0.70**
Urease	E	**0.53**

'Maximum hydration value, not used in calculating average for protein.

Techniques

A Dielectric dispersion.
B X-ray scattering.
C Sedimentation velocity.
D Sedimentation equilibrium.
E Diffusion coefficient.
F Intrinsic viscosity.
G NMR.
H Frictional coefficient.
I $^{18}0$ Diffusion.

accord with what we have learned from QENS and UHFD studies on the motional freedom of bulk phase water in living cells.

Finally, I refer to the answers I provided to the reviewer's first question. These answers have offered strong evidence against the claim that the presence of native globular protein can explain the osmotic and freezing-point depression (which is not the same as non-freezing).

In conclusion, it would seem that contrary to the claim of the reviewer, there is indeed compelling evidence that the presence of only globular protein in the cells is not able to explain the unusual properties of water in the living cells and that in contrast, the polarized multilayer theory is in full accord with the facts.