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Predictions of Polarized Multilayer Theory of Solute Distribution Confirmed from a Study of the Equilibrium Distribution in Frog Muscle of Twenty-one Nonelectrolytes Including Five Cryoprotectants

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Abstract: We determined the equilibrium distribution of twenty-one nonmetabolized nonelectrolytes in frog muscle cells. In all cases, plots of the equilibrium intracellular concentrations of a solute in the cell water against the external concentrations of the solute yielded straight lines in agreement with the prediction of such a rectilinear plot by the polarized multilayer (PM) theory.

The slopes of these straight lines yield the equilibrium distribution coefficients or q-value of that solute. It was shown that, again in agreement with the PM theory, the q-values of fourteen nonelectrolytes vary with the molecular volumes of the nonelectrolytes, obeying the "size rule", i.e., the larger the solute, the lower its q-value. The q-values of the remaining seven nonelectrolytes also decrease with their molecular volumes but cm a separate curve.

These q-value vs. molecular volume plots (q-v plots) show strong resemblance to similar q-v plots of solutes in dialysis sacs containing proteins and polymers assuming the fully-extended conformation (extrovert models) but no, or only weak, resemblance to q-v plots of solutions containing native globular proteins (introvert models). These findings also support the PM theory, according to which some protein(s) pervasively present in cells are in the fully-extended conformation; and that these fully extended cell protein(s) polarize(s) in multilayers all or virtually all cell water.

The relationship between the q-values of the nonelectrolytes and the solutes' respective molecular volume are described by two sets of theoretical curves, calculated from an equation introduced in the preceding paper.

Both curves were computed on the basis of the same exclusion intensity (u_{vp} = 126 cal/mole). This factor measures the extra water-to-water interaction of the polarized water which acts to keep solute out of the cell water in degree according to the size of the solute. The two curves are computed on the basis of two different values of u_s, which represents the surface or solute component of the polariza-

tion energy, describing the affinity of the solute for the dynamic water structure: one curve which roughly predicts the q-value of fourteen of the nonelectrolytes studied was computed with a u_s equal to 119 cal/mole.

The second curve which roughly predicts the q-value of the remaining seven solutes was computed on the basis of a higher u_s (156 cal/mole). Of these seven solutes, five are cryoprotectants (ethylene glycol, glycerol, 1,2-propanediol, 1,2-butanediol, acetamide)—in agreement with the theory that "cryoprotectants" safeguard living cells from freezing-and-thawing injuries because these solutes have high affinity for, and therefore stabilize the dynamic polarized multilayer structure of the cell water.

The q-v plots show that at least 90% of muscle cell water is polarized, possessing solvency properties different from normal liquid water. Three sets of other independent evidence suggest that there is no free water in muscle cells.

The q-v plots also yield data proving the multiplicity in the number of layers of water molecules polarized in frog muscle cells (as well as in extrovert-model-dominated water). The same plots also demonstrate that the main difference between water in frog muscle cells and water dominated by extrovert models lies in the higher exclusion intensity (u_{vp}) of water polarization in living cells. Reasons are given that this higher u_{vp} originates from the higher degree of order *in* the *arrangement* of the water-polarizing, fully-extended proteins in the living cells.

Na$^+$ ION, SUGARS AND FREE AMINO ACIDS are as a rule maintained at lower levels in the living cell than in the surrounding medium. The importance of this (partial) solute-exclusion phenomenon in cell physiology is testified to, on one hand, by its prompt annulment upon cell death; and, cm the other hand, by the fact that all major theories of the living cell have been constructed with emphasis on this phenomenon, including the atomic sieve theory (Boyle and Conway, 1941), the membrane-pump theory (Schwann, T., see Ling, 1992, p. 9; Dean, 1941), the sorption theory (Troshin, 1958, 1966), and the association-induction hypothesis (Ling, 1962, 1984, 1992).

According to the association-induction (AI) hypothesis, or more specifically, its subsidiary, *the polarized multilayer (PM) theory of cell* water, solute exclusion and various other distinctive attributes of living cells originate partly or completely from the unusual properties of cell water, which assumes the dynamic structure of polarized multilayers (Ling, 1965; 1984, p. 167; 1992, pp. 69-l IO).

The assumption of this dynamic structure is the consequence of propagated electric polarization among cell-water molecules, *pari passu* with their spatial orientation. The polarization and orientation of water molecules emanate primarily from the fixed (positively charged) NH and (negatively charged) CO groups of certain cell protein(s), pervasively present throughout the cell, and completely or partly in the *fully-extended conformation* (Ling et al., 1980a; Ling, 1992, p. 69). In this conformation, the backbone NH and CO groups are not engaged in intra- and inter-macromolecular H-bonds, and are thus free to interact with the bulk-phase water.

Over the last two decades, investigations on interaction between water and proteins (and between water and other linear oxygen-containing polymers) have led to the recognition of two distinct types of models: *extroverts* and *introverts*. Extroverts include those proteins and polymers which for structural reasons (e.g., gelatin, various oxygen-containing linear polymers) or in response to secondary-structure-unravelling denaturants (e.g., urea and NaOH) exist in the fully-extended conformation. Included among the extrovert oxygen-containing polymers are poly(ethylene oxide) or PEO; poly(ethylene glycol) or PEG; polyvinylpyrrolidone or PVP.

Most native globular proteins belong to the category of *introverts*. *The* NH and CO groups of these native globular proteins are, as a rule, engaged in intra- or inter-macromolecular H-bonds (e.g., a-helix, P-pleated sheet) and are shielded from and thus unable to interact with the bulk-phase water (for definition of the term "native protein" see Ling, 1990, response to B.L. Gupta, on p. 760; Ling, 1992, endnote 4 on p. 37).

The first requisite step toward verifying the PM theory lies in its substantiation in vitro. That is, each physicochemical characteristic of living cells hypothesized to originate from the cell water, must be reproducible in water exposed to fully-extended proteins or other extrovert models. In contrast, it is not reproduced, or only weakly so, in water exposed to native globular proteins or other introvert models.

So far, physico-chemical properties of living cells successfully reproduced (at least qualitatively) on extrovert-dominated water include solute-distribution properties (Ling *et* al., 1980a, 1980b; Ling and Ochsenfeld, 1983, 1989), osmotic activity (Ling, 1983), volume regulation (Ling and Ochsenfeld, 1987), freezing-point depression (Ling and Zhang, 1983; Zhang and Ling, 1983), vapor sorption at physiological (i.e., near saturation) vapor pressure (Ling and Hu, 1987), obedience to the Bradley's polarized-multilayer adsorption isotherm (Ling, 1984, pp. 288-289; Ling and Negendank, 1970), NMR rotational correlation time (Ling and Murphy, 1983), Debye reorientation time (Clegg *et al.*, 1984), and rotational and translational diffusion coefficient by quasi-elastic neutron scattering (Rorschach, 1984; Trantham *et* al., 1984). Water in the presence of introvert models has been tested only for the first five physicochemical properties listed above. In all these cases, the introvert models demonstrated no or only weak effects (for a full review of all the physicochemical attributes studied, see Ling, 1992, Chapter 5, in particular Table 5.5, pp. 108-109).

Thus the first phase in the testing of the polarized multilayer theory *in vitro* on model systems has been by and large accomplished. *Results of these studies have established a close relationship between the fully-extended conformation of proteins (and linear oxygen-containing polymers). and a gamut of extraordinary physicochemical characteristics of water created on exposure of the water to the fully-extended protein (or polymer).*

The second and concluding phase in the testing of the PM theory deals with living cells. It began with the development of a quantitative theory of solute exclusion. There are good reasons for singling out solute distribution phenomenon for further studies; two such reasons have been mentioned in the opening statement of this communication. Others will be made clear in the Discussion section below.

Until recently, the theory of solute distribution in cell water has been largely qualitative, or at best semiquantitative. The quantitative theory of solute distribution in cell water just introduced (Ling, 1993) has opened the door to new and definitive knowledge about the nature of water in living cells (and model systems) not attainable before.

As the first of a series of solute-distribution studies on different types of living cells, we chose frog skeletal muscle, an assembly of primarily *bona fide* adult living cells.

Materials

All experiments were performed on small intact voluntary muscles of North American leopard frogs, Rana pipiens pipiens, Schreber: sartorius, semitendinosus, tibialis anticus longus, and ileofibularis. Usually four muscles, one of each kind just described from four different animals, were handled as a unit and incubated together during the experiment. At

the end of the experiment, each muscle was analyzed individually to provide four data points from which the mean and standard error of the mean were calculated.

All chemicals used were of CP grade. The source(s), catalogue number and lot number (in that order) of special chemicals (and radiochemicals) whose q-values were investigated are listedbelow:

Special Chemicals

Acetamide, Sigma Chemical Co., St. Louis, Mo. Catalogue No. A0375, Lot 119F0748; D-arabinose, Sigma A3131, Lot 116F-0609; L-arabinose, Sigma, A32.56, Lot 90F-0466; 1,2-butanediol, Aldrich Chemical Co., Milwaukee, Wisc., 17,765.2, Lot 01708HL; 3-chloro-1,2-propanediol, Sigma C0639, Lot 45F-0054; dimethyl sulfoxide (DMSO), Sigma D-5879, Lot H127F0084; erythritol, Sigma E7500, Lot 57C-0058; ethanol, Pharmaco; ethylene glycol, J.T. Baker, Phillipsburg, NJ, 9300-01, Lot 8-L715; D-glucose, Mallinkrodt, Jersey City, NJ, 4912, Lot WRAZ; glycerol, Fisher Scientific, Springfield, NJ, G33-500, Lot 871775; D-mannitol, Sigma, M4125, Lot 81F-0517; methanol, Fisher, A454-4, Lot A-412; poly(ethylene glycol) (PEG-1000), Sigma, P3515, Lot 84F-0448; 1,2-propanediol, Sigma, P-1009, Lot 11GF-0371; D-raffinose pentahydrate, Aldrich, 20667.9, Lot 00302BM; D-ribose, Sigma, R7500, Lot 25F-0481; D-sorhitol, Sigma, S-1876, Lot 34F-0016; sucrose, Mallinkrodt, 8360, Lot 68460-1M; urea, J.T. Baker, 42045, Lot 335346; xylitol, Sigma, X-3376, Lot 37C-0068; D-xylose, Sigma, X1500, Lot 76F-0193; L-xylose, Sigma, X1625, Lot 124F-0337.

Radiochemicals

Acetamide-1-^{14}C, ICN Biochemicals, Cleveland, Ohio, Lot 574462; dimethyl sulfoxide-^{3}H (DMSO-^{3}H), New England Nuclear (NEN), Boston, Mass., Lot 550-204; ^{14}C-erythritol, Amersham, Batch 35; ethanol [1-^{14}C], NEN, Lot 2255-257; D-glucose-^{14}C, ICN, Irvine, Calif., Lot 963701, NEN, Lot 2187-190; glycerol-UL-^{14}C, ICN, Lot 745765, NEN Lot 1258-206; methanol [^{14}C], ICN, Lot 2963145, NEN, Batch 1179.226; poly (ethylene glycol)-900-^{3}H (PEG-900-^{3}H), NEN, Batch 2413-077; urea-^{14}C, NEN, Lot 952-178.

Methods

Procedures to establish insignificant metabolic degradation of solutes studied

To determine accurately the q-value of a solute between cell water and the external medium, metabolic degradation of the investigated solute, which would spuriously lower the q-value measured, must be absent or insignificantly low.

Fortunately, North American leopard frogs-which yearly live through subzero temperature-are especially suited for this type of study. Thus isolated frog muscles (largely white muscle) remain entirely healthy at 0°C temperature but metabolic activities are reduced to a very low level. By conducting the experiment at 0°C, serious interference due to metabolic degradation is to all intents and purposes eliminated. The proof for this was published in 1969 by Ling *et* al. (1969): D-glucose, the major food material of (white) voluntary muscle (Lehninger, 1975, p. 768) is not metabolized at this temperature.

However, due to the critical importance of accuracy in the q-value determined, we took extra care to make certain the validity of the earlier surmise that solutes investigated in the present study do not undergo significant metabolic degradation.

Four or more muscles of the kinds described above were introduced into one glass tube (called the initial group); their pairs into another (called the final group). To each tube containing the four muscles was added cold Ringer's solution containing 30 millimoles of the compound under study. The volume of the Ringer solution was equal to the volume of the muscles in most cases but occasionally three times the volume of the muscles.

To **the** *initial* group of muscles was rapidly added either 20 volumes of distilled water and heated for 20 minutes in a boiling water bath (with a teflon marble covering the tops of the tubes) or enough 10% trichloroacetic acid (TCA) to give a final TCA concentration of 5% when diluted **by** the water in the tube including the muscle water. Further steps used in processing the hot water or TCA extracts are described below under *Incubation, Extraction and Assay Methods*. Muscles of the *final* group were incubated with gentle shaking in a 0°C water bath for six days (or occasionally in a 25°C water bath for two to four hours). At the end of the incubation period, these muscles were treated with TCA or hot water in exactly the same way as the muscles of the initial group were. From these studies, the total amount of the solute under investigation in the initial and the final group were obtained. A t-test was conducted to determine if there was significant loss of the solute during the period of incubation.

Determination of the time required for diffusion equilibrium for solutes in muscle

Since our primary objective was to determine *the equilibrium* distribution coefficient or q-value, it is essential to know how long it takes for each solute to reach diffusion equilibrium in the muscle cells. We chose two different ways to achieve this goal. The first way involved incubating many muscle units in a relatively small volume of the bathing solution containing initially an accurately known concentration of the solute under investigation. Samples of the bathing solution were taken out for analysis at different times during incubation. From the loss of solute from the bathing solution, the uptake of the solute by the cells was calculated and the time needed to reach diffusion equilibrium determined. This method is most suited to investigations with radioactively labelled solutes, where small concentration difference can be accurately determined.

The second way used to determine the time needed to reach diffusion equilibrium was to incubate the muscle in larger volumes of the bathing solution and to take muscle units out at different times and analyze for the contents of the solute under study. In either case, from the time course of solute uptake, one determined the time for the **solute** to reach diffusion equilibrium.

Incubation, extraction, and assay of nonelectrolytes and water content

The basic incubation medium contained 115 mM Na^+, 2.5 mM K^+, 1.2 mM Mg^{++}, 0.71 mM Ca^{++}, 107 mM Cl^-, 2.0 mM $H_2PO_4^-$, 1.2 mM HPO_4^-, 1.2 mM $SO_4^=$, 6.64 mM HCO_3^-. In the experimental solutions containing different concentrations of a nonelectrolyte, an osmotically equivalent concentration of NaCl was removed in order to maintain a constant osmotic activity. (However, equality of osmotic activity, a procedure chosen here for convenience, does not insure constant cell volume. See Ling, 1987a.)

Each unit of four small muscles was incubated in either 6 or 30 ml of incubation medium. In experiments using (the more expensive) radioactively-labelled nonelectrolytes, for example, the smaller volume is necessary. From the time. needed to reach diffusion equilibrium already determined, a suitable duration of incubation is chosen for each solute.

For the determination of water contents of the muscle tissue at the conclusion of most ex-

periments, one or more **muscle(s)** were weighed before and after drying at 105°C overnight. Muscles containing heat-labile compounds like urea and **ethylene** glycol were dried in a vacuum oven maintained at 68°C until constant weights were reached. In either case, the weight loss gave the water content.

Nonelectrolyte was extracted from muscle tissues with trichloroacetic acid (TCA) at a concentration which, taking into account the dilution due to the muscle cell water, gave a final TCA concentration of 5%. The TCA-treated muscles were quickly homogenized, centrifuged and aliquots of the supernatant were taken out for further analysis. (For the study of methanol and ethanol concentration in muscle cell water, the muscles were not homogenized but were extracted with TCA in an intact state.) Time course studies showed that the time needed to fully extract some compounds was longer than usual. In those cases extraction with shaking at 4°C lasted seven days.

Alternatively, the extraction was achieved on intact muscles with bailing water followed by deproteination with $Ba(OH)_2$ and $ZnSO_4$ according to the method of Somogyi (1930).

The TCA or water extracts were analyzed for the compound under investigation by methods described below: pentoses (Roe and Rice, 1948), hexoses (Roe *et* al., 1949), polyols with vicinal hydmxyl groups (Burton, 1957). Methanol and ethanol were assayed by the microdiffusion method (Neish, 1957; Conway, 1958). In colorimetric analyses, correction for tissue blanks was routinely applied.

Radioactively labelled compounds of the TCA extracts and the bathing solutions were mixed with Bray's scintillation fluid (Bray, 1960) and assayed on a P-scintillation counter according to the procedure described by Ling and Hu (1988).

Calculation of the equilibrium concentration of solutes in muscle cell water

The raw data obtained give us micromoles of solutes per gram of fresh muscle tissue. To obtain solute concentrations in the cells, one must correct for the solute in extracellular space and in the connective tissues elements that make up part of the normal muscle tissue.

Extracellular space: Three independent methods have been developed for the determination of extracellular space in frog muscles: the poly-L-glutamate (PLG) (Mol. Wt. 50,000) probe method (Ling and Kromash, 1967); the Br^--efflux method (Ling, 1972a); and the centrifugation extraction method (Ling and Walton, 1975). All three methods yielded the same percentage extracellular space for the frog sartorius muscle: approximately 9%. Furthermore, using the PLG method, Ling and Kromash demonstrated that the mixed muscle containing one each of four muscles used in the present experiments has a percentage extracellular space the same as in the sartorius muscle alone.

"Connective tissue elements" (including loose connective tissue, fascia, small blood vessels, small nerves etc.) of frog sartorius muscle was previously determined to be 9.1% of the muscle fresh weight (Ling, 1962, p. 210). The connective tissue elements isolated from the frog legs in the vicinity of the isolated muscles contain 85% water. The equilibrium distribution of D-mannitol, D-sorbitol, sucrose, methanol and urea in these connective tissue elements all demonstrate rectilinear distribution (Ling **et** al., 1969), showing no sign of significant adsorption, as would be revealed in the hyperbolic and sigmoid component in the uptake profile. The slopes of these curves as well as the distribution of D-glucose and chloride ion indicate that all of these compounds distribute themselves equally between water of the connective tissue element and the bathing medium. Therefore, the water in the isolated connective tissue elements, for most solutes at least, has already been represented in the 9% extracellular space measured. In other words, of the connective tissue elements,

only its solidcontent, equal to 0.091 x(14.85) = 0.0137 or about 1.4% of the **muscle** weight remains. But then this solid has already been taken into account as a part of the solid weight of the muscle. In conclusion, for all the nonelectrolytes being studied which as a rule do not adsorb onto the solid component of the muscle tissue, no correction is needed for the connective tissue elements.

Representing the concentration of the solute under study as S, its concentration in the cell water and **in** the bathing medium in millimolarity respectively as $[S]_{in}$ and $[S]_{ex}$, its concentration in the whole muscle tissue in μmoles per gram of fresh muscle tissue as $[S]_{tis}$, the percentage of dry weight of the muscle tissue as DW% and the volume fraction of extracellular space as 0.089, we calculated $[S]_{in}$ according to the equation:

$$[S]_{in} = ([S]_{tis} - \mathbf{0.089}\ [S]_{ex}) / (1 - DW\% - 0.089) \qquad (1)$$

The determination of the q-value

The equilibrium concentration of a solute in the cell water, $[S]_{in}$, **calculated** according to equation 1, is then plotted against the external solute concentration measured at the conclusion of the experiment. If the plot is rectilinear (as it is in all cases studied here), a linear regression analysis yields the slope of the straight lines. 'Ibis slope is, of course, equal to the q-value of the solute. The linear correlation coefficient (r) between the concentrations of the solute in the cell water and that in the external medium also provides a measure of how **good** the data fit a straight-line relationship.

Determination of the molar volumes of the solutes

In order to put to a test the theory that for many solutes there is an inverse relationship between the size of a solute and its q-value in the cell water, it is necessary to know the molecular volume (also called the molar volume) of all the solutes under **study. Strictly speaking,** the molecular volume of a solute is equal to the molecular weight divided by the density of the solute as a liquid at its boiling point. For the degree of accuracy we can expect to achieve, liquid density at ambient temperature suffices and was used in this study. Thus for those solutes whose density as liquid at ambient temperature was known, **the molar** volume was easily obtained by dividing the molecular weight by its density.

For solutes whose density in the liquid state was not available, the molar volumes were calculated from their elemental composition and the volume equivalents of the elements as given by Kopp (see Glasstone, 1946, p. 525): hydrogen, 5.5; carbon 11.0; oxygen (-O-)7.8. Neither Kopp nor le Bas gave the volume equivalent of the nitrogen atom due undoubtedly to its great variability. However, since the two nitrogen containing compounds, urea and acetamide, are both close derivatives of formamide and the liquid density of formamide was available, with the aid of Kopp's volume equivalents for H, C, and -O-, we calculated a volume equivalent of 4.4 for nitrogen and used this figure to compute the molar volume of urea and acetamide.

Results

Demonstration of insignificant rate of metabolic degradation by muscle at 0°C

The effectiveness, mentioned above, of 0°C temperature in suppressing metabolism of even the most favored food material of frog muscle (D-glucose) suggests that solute distribution studies conducted at this temperature do not suffer significant interference from metabolic

Table 1.

Solute	Temp °C	Duration (hrs)	Concentration (mM) Initial	Final	P
D-arabinose	0	151	16.9 ± 0.12(4)	17.0 ± 0.12(4)	0.4 < P < 0.5
L-arabinose	0	151	16.8 ± 0.15(4)	17.0 ± 0.12(4)	0.4 < P <0.5
ethanol	0	137	34.5 ± 0.95(4)	35.0 ± 1.25(4)	0.7 < P < 0.8
ethylene glycol	0	141	18.5 ± 0.37(4)	18.2 ± 0.31(4)	0.6 < P < 0.7
i-erythritol	0	140	18.8 ± 0.39(4)	18.9 ± 0.31(4)	0.3 < P < 0.4
glycerol	0	141	19.4 ± 0.78(4)	19.1 ± 0.23(4)	0.7 < P < 0.8
D-mannitol	0	140	18.6 ± 0.16(4)	19.0 ± 0.15(4)	0.1 < P < 0.2
methanol	0	137	34.4 ± 0.95(4)	35.5 ± 1.21(4)	0.7 < P < 0.8
1,2-propanediol	0	142	25.6 ± 0.14(4)	26.7 ± 0.77(4)	0.001 < P < 0.002
D-raffinose	0	47	16.6 ± 0.22(4)	16.3 ± 0.40(4)	0.6 < P < 0.7
D-ribose	0	138	16.0 ± 1.06(4)	14.4 ± 1.32(4)	0.3 < P < 0.4
D-sorbitol	25	2–4	29.4 ± 0.56(12)	27.8 ± 0.84(11)	0.1 < 0.2
sucrose	0	47	17.0 ± 0.43(3)	16.8 ± 0.31(3)	P = 0.8
xylitol	25	2–4	19.4 ± 0.86(7)	19.8 ± 0.78(7)	0.7 < P < 0.8
D-xylose	0	151	17.2 ± 0.11(4)	16.1 ± 0.14(4)	0.001 < P < 0.005
L-xylose	0	151	17.2 ± 0.11(4)	16.6 ± 0.53(4)	0.4 < P < 0.5

Demonstration of absence or extremely slow rate of metabolic degradation of fifteen nonelectrolytes by frog muscle. Concentrations are given as mean ± standard error of the mean. Number in parenthesis following is the number of individual assays made. Metabolic degradation was determined by the loss of the nonelectrolytes after (in most cases) more than six days of incubation at 0°C. For most cases this lack of significant degradation was demonstrated by the high P values for the initial and final concentrations of the solute. There are two exceptions. There are statistically significant differences between the inital and final concentrations of 1,2-propanediol and of D-xylose. However, the results must be viewed with the fact that these tissues had been incubated for 142 or 151 hours. For D-xylose, the degraded portion only amounted to 17.2 – 16.1 = 1.1 mM over a period of more than six days; the hourly rate of degradation is extremely trivial when compared to the rate of exchange across the cell surface as Ling and Ochsenfeld had clearly shown (Ling and Ochsenfeld, 1988). The total amount of 1,2-propanediol changed in 142 hours was also extremely small, and above all, it was an increase and not a decrease. Therefore, this change could not have been a result of degradation. More likely, this is an example of oddity that arose from the small sample size and the extremely small standard errors registered.

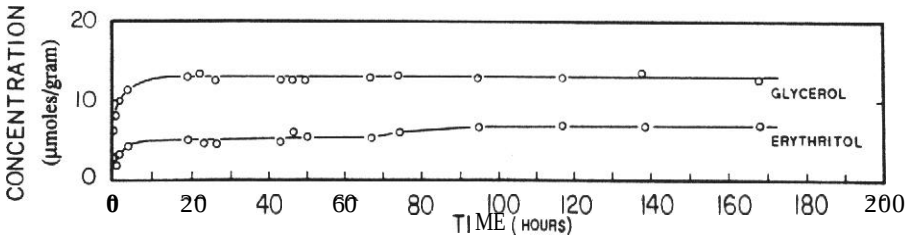

FIGURE 1. Time courses for the uptake of glycerol and of erythritol by frog skeletal muscles at 0°C.

Eight muscles, a pair each of the four kinds of small skeletal muscles (sartorius, semitendinosus, tibialis anticus longus, and ileofibularis) were isolated from each of 15 frogs. Sixty muscles, one of each pair and weighing altogether about 5 grams, were introduced into 10 ml of Ringer phosphate solution, containing respectively at the stat 24 mM of ^{14}C-labelled glycerol or ^{14}C-labelled erythritol, after a" initial 0.5 ml sample of both solutions were taken out. The flasks containing the muscles were shaken gently in a water bath maintained at 0°C. Other aliquots (0.5 ml for the first 5 samples and 0.05 ml for all subsequent samples) were taken out at intervals. The intracellular concentrations of glycerol and erythritol shown in the figure were calculated from their loss from the bathing solutions.

degradation of the solute under study. However, for **maximum** security against error on this important issue, we went on to investigate 16 nonelectrolytes (which, under favorable conditions, might be metabolized by some living organisms). The results of this investigation (see Table I) confirmed our surmise: the concentrations of these 16 nonelectrolytes (mostly sugars and sugar alcohols) before and after prolonged incubation in the presence of isolated frog muscle at 0°C are not significantly different-as indicated by high P values from t tests listed in the last column of Table I (for apparent exceptions, see legend of Table 1).

Since even compounds metabolized vigorously at ambient temperature by frog muscles are not metabolized at a significant rate at 0°C, five compounds were not investigated for metabolic degradation: acetamide, urea, dimethyl sulfoxide (DMSO), 3-chloro-1,2-propanediol, 1 ,2-butanediol. To the best of our knowledge, these compounds are not metabolized by vertebrate cells even at ambient temperature.

Determination of the time for the solutes to reach diffusion equilibrium in frog muscle

It took less than 15 hours for D-glucose to reach diffusion equilibrium in mixed muscles at 0°C (Ling et al., 1969). Figure 1 shows the time courses of uptake by similar mixed muscles of glycerol and of erythritol also at 0°C. It took 20 hours for glycerol and erythritol to reach diffusion equilibrium and, once the equilibrium was reached, the glycerol concentration in the muscle cell water remained unchanging for as long as our observations, i.e., 150 hours or 6.25 days. The glycerol type of time course is shared by many other solutes studied.

The erythritol concentration remained unchanging for only about 60 hours, and then rather abruptly rose to a new plateau maintained for at least **another 140 hours. For the** present investigation, we adopted the q-value corresponding to the first steady level observed. The significance of the transition of erythritol from a lower to a higher q-value will be discussed again below.

Table II.

Solute	Equilibration Time (hours)
water	<< 1
methanol	<20
ethanol	<20
acetamide	<10
urea	<24
ethylene glycol	<10
1,2-propanediol	24
DMSO	< 1
1,2-butanediol	24
glycerol	<20
3-chloro-1,2-propanediol	24
erythritol	<20
D-arabinose	<45
L-arabinose	<45
L-xylose	<45
D-ribose	<24
xylitol	24
D-glucose	<15
D-sorbitol	<10
D-mannitol	<24
sucrose	< 8
raffinose	10

Time for twenty-two nonelectrolytes (including water) to reach diffusion equilibrium in frog muscles. Data were obtained largely by the method demonstrated in Figure 1, although as a rule less elaborate, involving less points taken. Tbe data as a whole shows that with the exception of the three pentoses, an incubation period of 24 hours at 0°C is adequate for the other nonelectrolytes listed. (See footnote on page 191.)

Table II summarizes the minimum time required to reach diffusion equilibrium for 22 solutes investigated. The equilibration time can be roughly divided into two groups: In one group, the equilibrium was reached in less than 24 hour-some very much shorter than 24 hours; in the other, including the pentoses (especially L-xylose), it took as long as 45 hours to reach equilibrium. Therefore, for D- and L-araboinose and L-xylose, the incubation time was 6 days or about 140 hours. All others were incubated for 24 hours only.

Rectilinear relationship between solute concentration in cells and in the external medium

In Figures 2, 3 and 4, the equilibrium concentrations of different solutes in the muscle cell water are plotted against the equilibrium external concentrations. Although some scatter is observed, it seems evident that the distribution curves are straight lines. The slopes of these straight lines are equal to the q-values of the solutes.

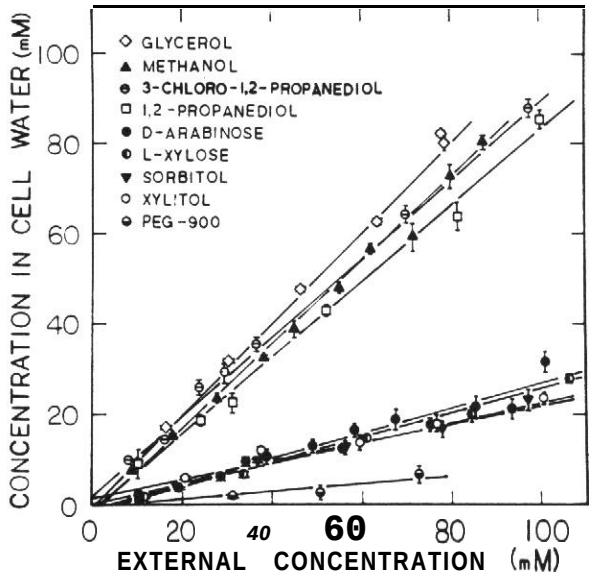

FIGURE 2. The concentrations of nine nonelectrolytes in muscle cell water at equilibrium with their concentrations in the bathing solution. The names of the nonelectrolytes as well as the symbols used to represent them are given in the graph.

Table III presents in detail the numerical values of the data plotted in Figures 2, 3 and 4. In addition, it also presents the molecular weights, the molecular volumes, as well as the q-values for all the solutes studied. The last column of Table III, presenting the q-values of the solutes, also gives (in parenthesis) the ***linear correlation*** *coefficient* (r) between the extra- and intra-cellular concentration of the nonelectrolyte. High positive linear correlation coefficients seen in most cases indicate not only that there are high positive correlations between the extra- and intra-cellular solute concentrations, but that these correlations are rec-***tilinear.***

Most of the straight lines in Figures 2, 3 and 4 have slopes less than unity. It was pointed out earlier why, on theoretical grounds, straight-line distribution curves of permeant and non-metabolized solutes with slopes below unity are incompatible with, and hence refute pumping as the mechanism for the reduced level of the solute. in the cell (Ling, 1988; 1992, endnote 1 of Chapter 8).

In contrast, rectilinear distribution curves are predicted by the PM theory for solutes entirely or almost entirely in the cell water. The different solvencies of these solutes in the cell water then give rise to slopes below, equal to, or above unity. Indeed, this is the *only* ***mechanism known for rectilinear equilibrium distribution with slopes below, equal to, or above unity*** (Ling, 1992, pp. 1–2; pp. 162-166; p. 201, endnote 1)*.

* See Troshin, 1966, p. 81 for his *earlier* discovery and recognition of the rectilinear distribution of some solutes.

Table III.

Solute	Mol. WI	Mol. Vol.	$[S]_{ex}$ (mM)	$[S]_{in}$ (mM)	q (r)
1, water	18.02	18.02			1.00
2, methanol	32.04	40.25	8.70	7.59 ± 0.21	0.91
			17.8	15.0 ± 0.11	(+0.988)
			27.6	23.1 f0.34	
			38.4	32.2 ± 0.35	
			4s.o	38.5 ± 1.20	
			55.2	47.9 ± 0.74	
			62.0	56.2 ± 0.36	
			71.5	59.2 ± 3.23	
			80.0	72.8 ± 2.80	
			87.4	80.3 ± 1.64	
3, ethanol	46.07	58.39	12.4	11.1 ± 0.20	0.81
			17.9	16.9 ± 1.30	(t0.967)
			25.2	24.8 ± 0.35	
			34.8	32.0 ± 0.35	
			35.7	29.2 ± 2.06	
			53.1	45.3 ± 5.09	
			70.7	56.5 ± 1.64	
			72.7	63.9 ± 2.30	
4, acetamide	59.06	61.7	31.4	32.1 ± 0.64	1.00
			52.4	52.6 ± 0.98	(t0.977)
			73.4	76.6 f4.01	
			104.8	104.6 ± 5.20	
5, urea	60.10	49.6	9.32	9.78 ± 0.22	1.05
			18.3	19.0 ± 0.19	(t0.999)
			27.8	29.6 ± 0.47	
			35.6	36.8 ± 0.23	
			44.2	44.6 ± 0.61	
			53.1	54.4 ± 0.80	
			70.6	74.9 ± 0.17	
6, ethylene glycol	62.07	55.64	1.00	I .00 ± 0.08	1.02
			4.90	4.74(1)	(+0.991)
			6.96	6.66(1)	
			9.66	12.2(1)	
			16.9	16.7(2)	
			19.1	23.4(1)	
			28.7	33.1(1)	
			37.0	40.8(2)	
			50.0	48.4(1)	

Solute	Mol. Wt	Mol. Vol.	$[S]_{ex}$ (mM)	$[S]_{in}$ (mM)	q (r)
7, 1,2-propanediol	76.10	73.16	10.04	9.07f3.10	0.834
			23.75	18.24 ± 1.02	(+0.988)
			31.03	22.69 ± 1.10	
			52.05	43.23 ± 0.95	
			81.39	63.94 ± 3.14	
			100.4	85.41 ± 2.23	
8, dimethyl sulfoxide (DMSO)	78.14	70.96	45.5	30.4 ± 1.41	0.72
			89.1	63.2 ± 0.84	(+0.979)
			832	548 ± 17.7	
			861	571 ± 15.5	
			1783	1097 ± 36.8	
			1915	1384 ± 81.7	
			2744	1831 ± 225	
			3843	2895 ± 127	
9, 1,2-butanediol	90.12	88.44	8.62	7.19 ± 1.77	0.87
			17.4	13.1 ± 1.72	(+0.992)
			25.5	19.2 ± 1.46	
			32.8	26.4 ± 0.85	
			35.4	30.0 ± 1.50	
			69.0	55.6 ± 2.25	
			93.6	82.6 ± 2.32	
10, glycerol	92.09	73.09	16.2	17.0 ± 0.17	1.00
			30.4	31.7 ± 0.55	(+0.996)
			46.5	47.5 ± 0.72	
			63.5	62.7 ± 0.77	
			78.6	80.0 ± 1.03	
11, 3-chloro-1,2-propanediol	110.5	83.36	7.86	10.2 ± 1.29	0.893
			15.9	14.5 ± 1.91	(+0.981)
			23.8	26.2 ± 1.58	
			29.7	29.5 ± 2.65	
			36.5	35.7 ± 1.54	
			69.7	64.5 ± 1.97	
			97.9	88.2 ± 1.99	
12, erythritol	122.1	130.2	17.8	7.02(2)	0.29
			20.2	6.82 f0.28	(+0.988)
			21.0	7.44 ± 0.52	
			21.5	7.22 ± 0.66	
			21.8	7.55 ± 0.63	
			37.3	11.9 ± 1.15	
			54.4	16.1(2)	
			71.8	25.1 ± 2.7	
			94.4	27.2 ± 0.65	

Solute	Mol. Wt	Mol. Vol	$[S]_{ex}$ (mM)	$[S]_{in}$ (mM)	q (r)
13, D-arabinose	150.1	149.0	10.1	2.20 ± 0.28	0.27
			19.4	3.81 ±0.39	(+0.967)
			28.4	5.98 ± 1.18	
			34.1	9.39 ± 1.17	
			39.0	10.5 ± 1.75	
			49.1	12.9 ± 1.03	
			58.2	16.3 ± 1.32	
			67.4	18.9f2.30	
			75.3	17.1 ± 1.44	
			85.1	21.5 f2.43	
			93.x	21.0t2.25	
			101.2	31.6 ± 2.45	
14, L-arabinose	150.1	149.0	10.3	2.09 ± 0.54	0.27
			33.1	7.80 ± 1.22	(+0.942)
			50.5	14.9 ± 3.92	
			84.4	21.8 ± 1.49	
			104.1	28.2 ± 2.38	
15, L-xylose	150.1	149.0	11.3	1.81 f0.09	0.26
			33.7	6.57 f0.82	(+0.970)
			56.0	12.3 ± 1.15	
			61.0	14.5 ± 0.52	
			84.7	19.8 ± 1.43	
			106.5	27.7 ± 1.21	
16, D-ribose	150.1	149.0	1.56	0.55 ±0.11	0.26
			4.50	1.14f0.04	(+0.998)
			9.40	2.60 ± 0.22	
			16.1	3.98 ±0.25	
			26.3	6.99 ±0.80	
17, xylitol	152.2	160.0	20.6	5.52 ± 0.29	0.22
			37.8	11.8 ± 1.14	(+0.914)
			59.6	13.4 ± 1.87	
			76.9	17.1 ± 1.84	
			100.8	23.3(2)	
18, D-glucose	180.2	178.8	2.23	0.66 ± 0.03	0.227
			4.53	1.13 ±0.06	(+0.981)
			7.44	2.26(1)	
			11.2	2.70 ± 0.13	
			13.6	4.06 ± 0.32	
			23.2	5.33 ± 0.48	
			30.6	7.19 ± 0.36	
			32.7	7.85 ± 0.69	
			40.3	10.2 f0.27	
			65.6	13.4(1)	
			75.9	17.8(2)	

Solute	Mol. Wt	Mol. Vol.	$[S]_{ex}$ (mM)	$[S]_{in}$ (mM)	q (r)
19, D-sorbitol	182.2	189.8	18.5	4.16(2)	0.227
			36.6	9.92 ± 0.39	(+0.898)
			56.1	12.6 ± 1.38	
			77.8	16.8 ± 2.03	
			97.1	23.4 ± 2.53	
20, D-mannitol	182.2	189.8	5.35	0.84 ± 0.05	0.217
			11.1	1.78 ± 0.09	(+0.933)
			19.8	4.82 ± 0.65	
			39.4	8.38 ± 0.20	
			61.8	11.3zto.95	
			76.3	15.9 ± 1.28	
			99.0	19.5 ± 2.01	
21, sucrose	342.3	338.8	5.88	0.65f0.13	0.132
			9.74	1.45 f0.03	(+0.917)
			17.4	2.70 f0.70	
			21.6	2.38 kO.15	
			30.5	4.29 ± 0.03	
			35.7	5.42 ± 0.28	
22, D-raffinose	594.5	498.8	10.5	2.03 ± 0.22	0.100
			19.2	4.02 ± 0.43	(+0.777)
			29.6	4.01 ± 0.73	
			38.5	5.32 f0.56	
23, PEG-900	900	1055	10.7	1.35 ± 0.12	0.082
			31.1	1.81 ± 0.50	(+0.620)
			50.7	2.73 ± 1.19	
			72.6	6.58 ± 1.83	

Equilibrium distribution of twenty-three nonelectrolytes in frog muscles*. Included are the molecular weights, molecular volumes, and the equilibrium distribution coefficients or q-values of the nonelectrolytes. The q-values which are equal to the slopes of the rectilinear plots were obtained by the method of least squares in plots of the concentration of the nonelectrolytes in the cell water, shown as $[S]_{in}$ against the external concentration of the nonelectrolyte at equilibrium, shown as $[S]_{ex}$. The linear correlation coefficient between the two variables, r, is shown in parenthesis under the q-values, As mentioned in the text, high r's are indices of at once the closeness of correlation between $[S]_{in}$ and $[S]_{ex}$ and that of how well the data points conform to a straight line. Data of solutes 13, 14 and 15 from Ling and Ochsenfeld, 1988.

* Due to the forced closing of our laboratory (Ling, 1989; 1992, p. xv) and the loss of radioactivity measuring capability, we were not able to complete the time course study on one nonelectrolyte, PEG-900. The distribution ratio of PEG-900 shown in Figure 2 and Table III obtained after a 24 hour incubation are therefore not included in Figures 5 and 6, since we have no clear-cut evidence that the 24 hour incubation time was long enough to reach diffusion equilibrium. It should be added, however, inulin (Mol. Wt., 4000 to 5000) which is larger than PEG-900 (Mol. Wt. ca. 900) reached diffusion equilibrium in frog muscle in only a little over 10 hours at 0°C (Ling and Kromash, 1967, Figure 3).

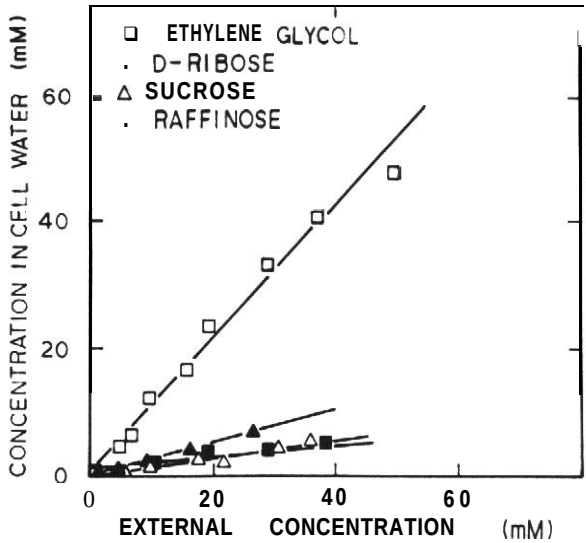

FIGURE 3. The concentrations of four nonelectrolytes in muscle cell water at equilibrium plotted against their concentrations in the bathing **solutions. The names of the nonelectrolytes as well as the symbols used to** represent them are given in the graph.

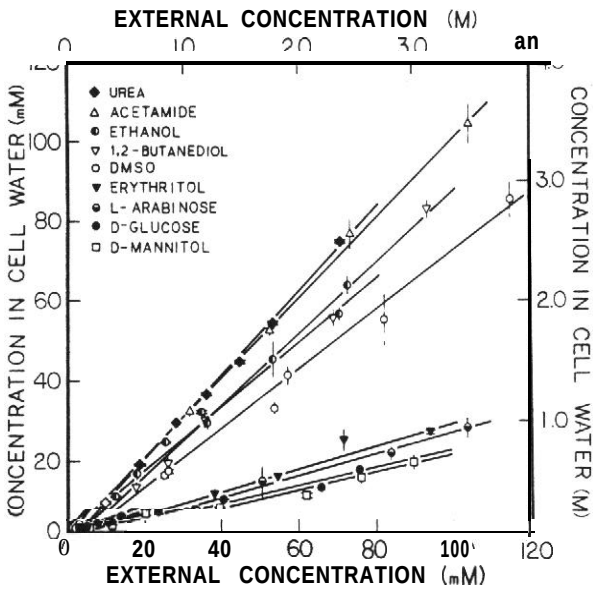

FIGURE 4. The concentrations of nine nonelectrolytes in muscle cell water at equilibrium plotted against their concentrations in the **bathing solutions.** The names of the nonelectrolytes as well as the symbols used to represent them are given in the graph.

The straight-line plots of solutes in frog muscle cell water, shown in Figures 2, 3 and 4, strongly resemble similar straight-line plots of solute distribution in solutions of NaOH-denatured hemoglobin, and the oxygen-carrying polymer, poly(ethylene oxide) (PEO) (Ling and Hu, 1988). This similarity supports the (PM) theory that water in both the living cell and extrovert model systems has solvency properties altered by fully-extended protein or polymer chains.

The relationship between the q-values of solutes in muscle cell water and their respective molecular weights

The q-values of the different nonelectrolytes in frog muscle obtained from the slopes of the rectilinear curves shown in Figures 2, 3 and 4 (and Table III) are plotted against the logarithm of the molecular weights of the nonelectrolytes in Figure 5. The solid lines going through or near the experimental points in the main graph and insets were obtained from visual inspection.

Of the 21 solutes studied-including water which by definition has a q-value of unity—14 fall on or near a smooth inverted S-shaped curve. However, seven other q-values represented as solid circles are way off the smooth curve. Of these seven nonconforming solutes, four (urea, ethylene glycol, glycerol, acetamide) have q-values close to unity. Three others (1,2-butanediol, 1,2-propanediol and 3-chloro-1,2-propanediol) have lesser but still much higher q-values than those dictated by their respective molecular weights and the smooth curve approximately describing the relationship between the molecular weights and q-values of the 14 other solutes.

In Insets A and B of Figure 5, we have reproduced the q-values vs. molecular-weight plots (q-w plots) of similar solutes in 39% (w/v) native bovine hemoglobin solution, 20% NaOH-denatured bovine hemoglobin solutions, 18% gelatin gel and 15% solution of polyethylene oxide (PEO) from an earlier publication (Ling and Hu, 1988). As mentioned earlier, a solution of native hemoglobin represents an ***introvert*** model with most of its backbone NHCO locked in inter- and intra-macromolecular H-bonds. In contrast, gelatin, PEO as well as NaOH-denatured bovine hemoglobin are all *extrovert* models with fully extended linear chains bearing properly-spaced. charged sites directly exposed to the bulk-phase water.

Comparing the main figure of Figure 5 with the four sets of data in Insets A and B, one finds that the q-w plot of solute distribution in frog muscle bears only slight resemblance to that of the q-w plot of introvert model of native bovine hemoglobin. In contrast, the q-w plot of solute distribution in frog muscle bears striking resemblance to similar plots of all three extrovert models. Keeping in mind that the dry weight of the cell is, in essence, a collection of proteins, one can hardly avoid the conclusion that ***some protein(s)*** *in frog* ***muscle cells as a representative of all living cells,*** *exist(s)* ***in thefully-extended*** *conformation.*

Relationship between q-values and molar volumes of the solutes in muscle cells and model systems; assessment of the intensity of water polarization

Plots of q-values against the molecular weights of the solutes in Figure 5 are presented above, for among other reasons, to back up earlier preliminary reports in which similar q-w plots were briefly presented (e.g., Ling, 1992, p. 91). However, to compare rigorously with theoretical predictions (Ling, 1993), requires plots of q-values against the molar volumes of the solutes. Figure 6 presents such q-value vs molecular volume plots (q-v plots).

Figure 6 differs from Figure 5 in yet another way: whereas the lines going through or *near*

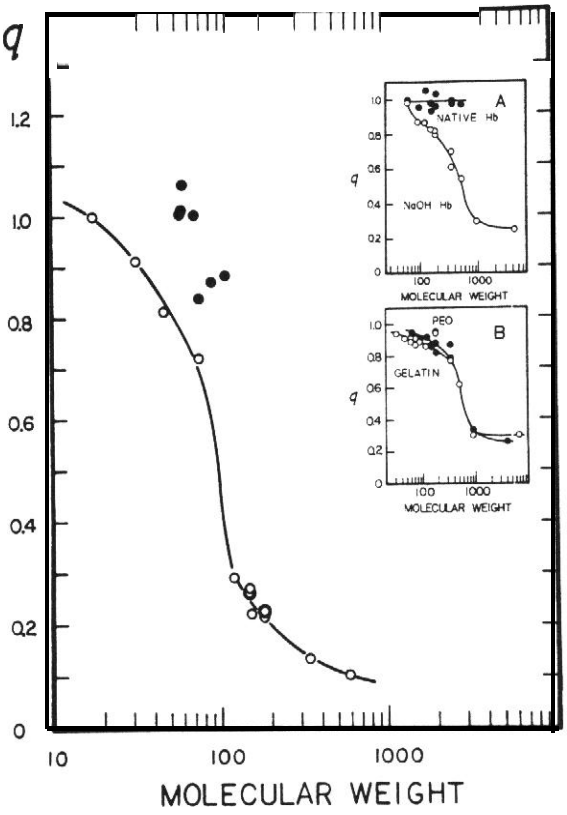

FIGURE 5. The equilibrium distribution coefficients or q-values of twenty-one nonelectrolytes in frog muscle cells (shown on the ordinate) plotted against their respective molecular weights (shown on the abscissa). For comparison, plots of q-values against molecular weights (q-w plots) of similar nonelectrolytes in solutions of NaOH-denatured bovine hemoglobin (NaOH Hb) (20%), gelatin gel (18%) and the oxygen-containing polymer, PEO (15%) and native bovine hemoglogin (39%)* against the molecular weights of the nonelectrolytes. q-values of nonelectrolytes in both frog muscles and the inanimate models are equal to and were obtained from rectilinear plots like those shown in Figures 2, 3 and 4 for frog muscles, and entirely similar plow published earlier by Ling and Hu (1988) and from whom the data shown in the inset were taken. For the native hemoglobin data, the q-value of another nonelectrolyte, that of poly(ethylene glycol) or PEG-4000 was added to a later version of the curve discussed in the preceding paper (Ling, 1993) but not included here.

* For a minor but significant change of the q-values plot of solutes in 39% native bovine hemoglobin solution due to the introduction of the new data point representing the large solute (PEG-4000), see pp. 164–165, Ling, 1993).

the data points in Figure 5 are the best-fitting curves drawn by visual inspection, there is no theoretical foundation for those curves. In contrast, the two lines going through or near most of the data points in Figure 6 are theoretical curves drawn according to equation 25 of the preceding paper (Ling, 1993), but labelled equation 2 here.

$$q = \exp\left\{ \frac{1.23\ v\ \Delta E_s \left[1 - (1 - b)\frac{(kv)^n}{1 + (kv)^n} \right] - \Delta E_{vp} \cdot v}{RT} \right\}. \qquad (2)$$

where

$$\Delta E_{vp} = AE, + 1.230\ \Delta e^* \qquad (3)$$

The symbol, v, represents the molecular volume; the symbol ΔE_s, the *specific surface (or solute) polarization* energy per cm^2 when the solute is moved from normal liquid water to the polarized cell water. ΔE_v is the *specific volume (or solvent) polarization energy per* cm^3 equal to the difference in energy spent in excavating a hole 1 cm^3 in size in the polarized water and the energy recovered in filling up a hole of the same size in the surrounding normal liquid water. Δe^* is the increment of *the activation energy* for overcoming the greater rotational restriction per unit surface area (cm^3) of a solute when it is transferred from normal liquid water to the polarized water phase. ΔE_{vp}, the *exclusion intensity* of water polarization is defined in equation 3, comprising the sum of the volume component and the entropy component of the polarization energy. Rand T have the usual meaning. b, n and k are constants. b is a fractional number describing the low and steady probability of very large solute molecules in finding adsorbing sites on the water lattice. k and n are parameters describing the steepness of the declining probability of finding adsorbing sites in the water lattice with increasing molecular volume of the solute.

The lower curve which goes through or near 14 of the experimental points was calculated with the exclusion intensity, $\mathcal{U}_{vp}$ equal to 126 cal/mole, and a surface polarization energy $\mathcal{U}_s$ equal to 119 cal/mole. The upper theoretical curve was calculated with the same exclusion intensity, 126 cal/mole, but a higher surface component $\mathcal{U}_s$ of 156 cal/mole. $\mathcal{U}_{vp}$ equals ΔE_{vp} multiplied by the molecular volume of water. 18.02 cm^3; $\mathcal{U}_s$ equals 18.02 × 1.23 × ΔE_s.

Both the upper and lower curves show decreasing q-values for solutes of increasing molecular volumes. That the experimental data points conform to one or the other curve confirms the second of the predictions of the theory: the "size rule".

In the upper right corner of Figure 6, the names of the seven aberrant solutes represented as solid circles are given. Four of these are well known cryoprotectants: ethylene glycol (Luyet and Hartung, 1941; Polge *et al.*, 1949) glycerol (Rostand, 1946; Polge *et al.*, 1949); acetamide (Lovelock, 1954) and 1,2-propanediol (Polge *et al.*, 1949; Vos and Kaalen, 1965). Our own preliminary study suggests that 1,2-butanediol is also a cryoprotectant. At this moment, there is no data on whether or not the remaining two (urea and 3-chloro-1,2-propanediol) have cryoprotective activities. While the well-known protein-denaturing effect of a high concentration of urea argues strongly against its having a cryoprotective effect at very high concentrations, the fact that urea's close "relatives", formamide [$HCO(NH_2)$] and acetamide [$CH_3CO(NH_2)$] are both cryoprotectants (Lovelock, 1954) argues for its having a cryoprotective effect. A correct answer to whether or not urea and 3-chloro-1,2-propanediol are cryoprotectants awaits future investigations.

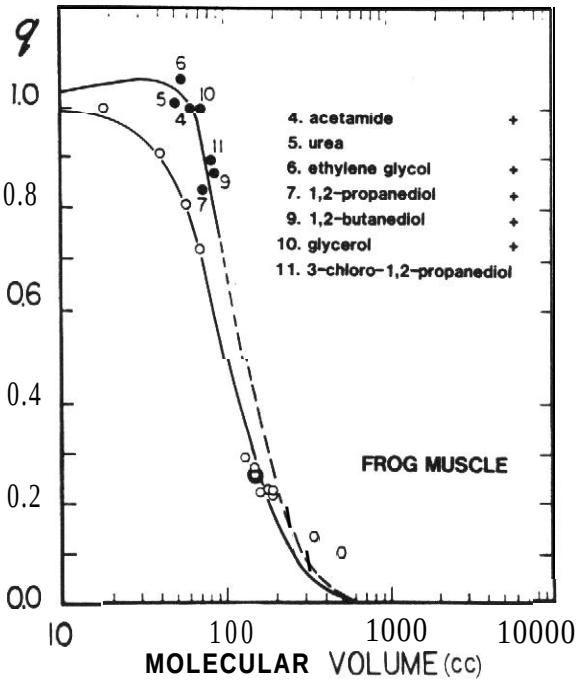

FIGURE 6. The equilibrium distribution coefficients or q-values of twenty-one nonelectrolytes (the same as those in Figure 5) in frog muscle cells plotted against their respective molecular volumes (q-v plots). The points are experimental; the lines are theoretical according to equation 2. u_{vp} for both the upper and lower curve is 126 cal/mole. u_s for the lower curve is 119 callmole; that for the upper curve is 156 cal/mole. Chemicals with established cryoprotective activity are indicated by the plus sign (+).

Whatever the eventual outcome of these future investigations, there is no question that the majority of these seven solutes with aberrantly high q-values do exercise cryoprotective activities in agreement with the third prediction of the theory: There is a higher probability of cryoprotective activity of compounds with exceptionally high q-values.

To describe the positions of these seven aberrant nonelectrolytes on the q-v plot, a higher u_s is required. This requirement supports the new theory of cryoprotection based on the PM theory (Ling, 1992a, 1993): cryoprotectant activity arises from a strong and stabilizing influence of the solute on the dynamic polarized water structure, i.e., higher than usual u_s.

Discussion

Fully-extended protein chains and other extrovert models can markedly reduce the solvency of the bulk-phase water for large solutes (Liig et *al.*, 1980a; Ling and Hu, 1988; Ling and Ochsenfeld, 1989). In the opening paragraph of this paper, we have already cited two

broadly significant reasons for choosing solvency for further in depth testing of the PM theory: a marked sensitivity to the state of health of the cell; and a central role it has played in the formulation of all major cell theories. To these we now add the more specific advantage provided by the study of this attribute: ***solvency studies offer clear-cut quantitative insights into both the amount of polarized water and the intensity of its polarization in living cells and model*** systems-insights not achievable by other currently available methods known to us. It is not surprising that the present study of cell-water solvency has yielded affirmative answers to most if not all the major postulates of the PM theory of cell water.

Correcting the cause of a major "wrong turn" made in cell physiology in the early nineteen-thirties

From the much more limited swelling of frog muscle in hypotonic solutions than that predicted by the membrane theory, E. Overton (1902, p. 273) suggested that at least a part of the water in frog muscle exists as "Quellungswasser" (or simply bound water). Gortner, Newton and others assumed that such bound water has lost its normal solvency for sucrose and used this reduced solvency for sucrose to gauge the amount of bound water in plant saps, gelatin gel and other colloidal systems (Newton and Gortner, 1922).

The discovery that urea distributes equally between frog muscle cell water and the external medium led Hill to conclude that in frog muscle there is no bound water and that all muscle cell water is free (Hill, 1930). Hill's view gained strength from confirmatory demonstration that ethylene glycol also distributes itself equally between water in human erythrocytes and the external medium (MacLeod and Ponder, 1936), and between water in frog abdominal muscle and external water (Hunter and Parpart, 1938).

Published as the opening article of the eighth volume of the Cold Spring Harbour Symposium on Quantitative Biology was a review by K.C. Blanchard on "Water, Free and Bound'-ten years after Hill's article. Blanchard summarized his analysis of the evidence on hand, including Hill's finding mentioned above, in these words: "After considering the various methods utilized for the determination of bound water, one must conclude that as yet no method has been devised which is capable of yielding precise information on the existence of this entity. In consequence there is no certainty that even in the presence of lyophilic substances bound water has any real existence" (Blanchard, 1940, p. 8).

Ernst, who lived long enough to give an eyewitness account another 23 years later, testified how it was Hill's finding just described that led to the "overnight" abandonment of the colloidal approach to cell physiology and the world-wide acceptance of the membrane theory in which free cell water is one of the basic tenets (Ernst, 1963, p. 112).

We will now give a totally different interpretation to Hill's finding on urea distribution. The bottom line of this interpretation is: Hill was mistaken in believing that equal partition of urea and of ethylene glycol between muscle cell water and the external medium proved that muscle cell water is free.

Indeed, soon after the introduction of the polarized multilayer theory of cell water it became apparent that, on theoretical grounds, one could expect that the larger the solute, the lower is its q-value and that small solutes may not be excluded at all (Ling, 1970, p. 147; Ling and Sobel, 1975). In 1987, this inverse relation between molecular size and the q-value was first explicitly referred to as the "size rule" (Ling, 1987b).

When it was found later that in various extrovert model systems, the. solvency for (small) urea is indeed like that in free water (i.e., $q = 1$), while the solvency of larger solutes like

(hydrated) Na^+, sucrose and free amino acids are much lower, as they are in cell water-the size rule seemed able to reconcile Hill's finding in muscle cells with those of Newton and Gortner on expressed plant juice and gelatin gel (Ling *et al.*, 1980a; Ling and Ochsenfeld, 1988). However, the experimental data presented in the present paper show that the size role does not pmvide the full answer.

Returning to Figure 6, one realizes that neither urea nor ethylene glycol stays on, or near the lower curve which the majority of the points are on or near. From their respective molecular volumes, both urea and ethylene glycol should have q-values much higher than that of sucrose. Nonetheless, their q-values should definitely be *below unity*. Yet the finding of Hill and others, further confirmed by our present study, show clearly that the q-values of both urea and ethylene glycol in frog muscle cell water are not below unity but are at or above unity.

While the q-values of urea and ethylene glycol do not conform to the theoretical curve describing the majority of nonelectrolytes studied, they do definitely conform to the upper theoretical curve, which differs from the lower one *only* in that the surface component of the polarization energy ($\mathcal{U}_s$) is 30% higher.

Thus the reason that urea and ethylene glycol have near unity q-values must be attributed in part to their smaller molecular volume (when compared to, say, sucrose), and in part to their specific molecular and electronic structure that enable them to fit better into the dynamic polarized multilayer structure of the cell water. Their larger sizes when compared to the water molecule they displace (and their polarizability) provide them with an affinity for the polarized cell water high enough to offset the exclusion force due to the combined volume and entropy component of the polarization energy ($\mathcal{U}_{vp}$). How molecules like these may in turn exercise a "splicing action" thereby stabilizing the polarized-multilayered dynamic structure of the cell water and protecting it from freezing injury has been discussed in the preceding paper (Ling, 1993) and elsewhere (Liig, 1992a).

Is there free water in muscle cells?

In contrast to the membrane theory or the membrane-pump theory-according to which cell water is all or virtually all free like that in dilute aqueous solutions-a basic tenet of the PM theory is that all or virtually all cell water assumes the dynamic structure of polarized multilayers. In other words, there is no or little free water in the cell.

Based on the distribution of nonelectrolytes in gelatin gel (Gary-Bobo and Lindenberg, 1969) and in solutions of extrovert models held within dialysis sacs (Ling and Hu, 1988), it has been shown in the preceding paper (Ling, 1993) that the minimum amounts of polarized water in 20% NaOH-denatured hemoglobin, 15% solution of PEO and 18% gelatin gel are respectively 75%, 75% and 72%.

These figures of 72% to 75% of water made nonsolvent to the larger solutes are very high figures indeed. For comparison, one may point out that they are some *fifteen* times higher than what one would have obtained if one assumes that *all* proteins bind from 0.2–0.3 gram of water of hydration per gram of dry protein, as determined by a gamut of other methods *on native globular proteins* (see Ling, 1972b, Table 4).

The much larger amount of water altered by the full-extended proteins also demonstrates *the unusual power of the q-v plots in revealing quantitatively the amount of water polarized.* The same set of data also clearly defines the maximum amount of free water in these model systems. And, more importantly, as will be made clear below, in living cells as well.

The largest solute, whose q-value in muscle cell water is shown in Figures 5 and 6, is raffinose (Mol. Vol., 594.5 cc)*. It has a q-value of 0.10 in the cell water of frog muscle. Since free liquid water must have a q-value of unity for all water-soluble solutes, clearly no more than 10% of muscle cell water could be free water. In other words, at least 90% of the cell water has lost nearly all its normal solvency for raffinose.

However, the following independent evidence argues that there is no free water in frog muscle cells:

In frog muscle, 9% of its volume is occupied by the extracellular space containing normal liquid water. Centrifugation of a hermetically-sealed, frog sartorius muscle for 4 minutes at from 400 to 1500 g, (or at 1000 g for from 2 to 16 minutes) removed the same amount of water as that found in the extracellular space by other methods (for details of these methods, see section on "extracellular space" under Methods above). While such centrifugation removes "surgically" all the (free) water in the extracellular space, no *intracellular* **wafer is** removed from either intact muscle or from muscle that had been cut into 2- and 4-mm wide open-ended segments (Ling and Walton, 1976). Since electron microscopy and other methods revealed no membrane regeneration at the cut ends of the muscle cells which run all the way from one end of the muscle to the other end (Ling, 1992, p. 53), Ling and Walton concluded that there is no significant amount of free water in muscle cells. The following findings further strengthen this conclusion:

(1) Cenhifugation at similar force and duration of a dialysis sac filled with distilled water rapidly and steadily removed its water contents. When the dialysis sac was tilled not with pure water but with 15% gelatin gel and subjected to the same centrifugation treatment, it rapidly lost only about 20% of its weight of water. Further centrifugation led to loss of water at a more reduced rate (Ling and Zhen, to be published). Taken as a whole, these data are in harmony with our earlier conclusion that some 25% of the water in 18% gelatin gel is free and that centrifugation of the right g-force and duration removes more rapidly this fraction of free water (Ling, 1993).

(2) When ATP in muscle cell is depleted in response to metabolic poisons, the normal ability of the cell water to exclude solutes like sucrose is lost (Ling, 1992, pp. 190–193). According to the AI hypothesis, this loss reflects a change in the degree of cell water polarization (see also Ling, 1992, p. 184). Since cell water depolarized in consequence of ATP depletion is close to being free liquid water, in theory the liberated water should be removable by centrifugation at 1000 g for four minutes,

This is what we found experimentally: Exposure to iodoacetate and 2,4-dinitrophenol led to loss of cell ATP and intense swelling of frog muscle. Cenhifugation at 1000 g for four minutes now no longer removed only water in the extracellular (9% of cell weight), but a total of more than 40% of the muscle weight of water (Ling and Walton, 1976). This observation demonstrates that free water within muscle cells is removable by the centrifugation procedure described. Failure. of similar centrifugation to extract intracellular water indicates no free intracellular water in observable quantity exists in healthy frog muscle cells.

(3) From ultra high frequency dielectric studies, Clegg *et* al. (1984) concluded that there is no free water in another type of living cells, the brine shrimp cyst cells,

In summary, the basic tenet that all or virtually all cell water is polarized is continued.

* A still larger solute PEG-900 (mol. vol. 1055 cc) has a still lower q-value of 0.082. See legend of Table III and footnote for reason far not including this point on Figures 5 and 6.

Alternative explanations for the aberrantly high q-values of sucrose, raffinose and PEG 900

In the q-v plots of the data shown in the Inset A and B of Figure 5, Ling showed that by taking into account all relevant evidence on hand, the data are best interpreted on the assumption that some 25% to 28% of the water in the extrovert model systems are free (Ling, 1993). Since in Figure 6, the fmg muscle system also reveals aberrantly high q-value for the largest solutes (sucrose, raffinose and PEG-900), our first response was that the data would be brought in line by assuming the presence of 10% free water here also. However, independent evidence cited above show that this assumption is incorrect. We now suggest and examine two other alternative interpretations for the aberrantly high q-values of sucrose, raffinose (and PEG).

(I) Exceptionally high surface component of the polarization energy, u_s.

In this interpretation, sucrose, raffinose and PEG 900 have exceptionally high u_s values and are thus similar to ethylene glycol, glycerol, as well as other compounds with cyroprotective activity and exceptionally high q-values. If so, self-consistency demands that sucrose, raffinose and PEG 900 also have cryoprotective activity. Do they?

The answers are divided. On the one hand, Lovelock (1954) found no cryoprotective activity of sucrose or poly(ethylene glycol)-400, at any concentration, against freezing-and-thawing injury of human erythrocytes. On the other hand, the specific protective effect of high concentration of sucrose has long been known in the widely-used procedure for isolating liver mitochondria (Hogeboom, 1955). The cryoprotective activity of high molecular weight poly(ethylene glycol) (PEG-8000) is also well-established (Rall, 1987). In work yet to be published, Ling and Niu have demonstrated strong cryoprotective activity of low-molecular weight poly(ethylene glycol), (PEG-200) against freezing-thawing injury of human erythrocytes. The apparent conflict between Ling and Niu's as yet unpublished finding on PEG-200 and Lovelock's earlier opposite conclusions on sucrose and PEG-400 requires further study.

If high u_s's for sucrose, raffinose and PEG 900 are the correct interpretation for their exceptionally high q-values, the u_s values for each of the three solutes must be even higher (and by different amounts) than that which described the seven aberrant solutes represented by solid circles in Figure 6 (156 cal/mole); none of the q-values of these three solutes fall on, or even near, this theoretical curve.

(2) Hydration water interpretation.

Sucrose, raffinose and PEG-900 and other similar larger solute molecules may have the capability of polarizing and orienting water molecules just as the folly-extended protein chains do. Only the extent of this polarization is probably more limited and the exact dynamic structures of water induced by sucrose, raffinose and PEG-900 may also be different in subtle ways. As a result, each of these larger and more assertive molecules orients a portion of the cell water in a specific way different from the normal cell water. The overall consequence would be to raise their own q-values in the cell water to beyond what its molecular volume predicts. This elevation of the q-value, however, would be entirely limited to the water polarizing (large) solute itself, whereas free water, in contrast, has unity q-value for solutes of all sizes and types. There are also three pieces of supportive evidence for the hydration water-interpretation:

(i) Long ago, Scatchard (1921) demonstrated that in aqueous solution, each sucrose molecule is associated with six water molecules.

(ii) In inset B of Figure 5, the ability of PEO to orient water like gelatin and NaOH-denatured hemoglobin is demonstrated. Now, the molecular formula of PEO, $H(CH_2CH_2O)_nH$ and that of PEG, $H(OCH_2CH_2)_nOH$ are highly similar. It was no surprise that like PEO, solutions of PEG-8000 also exclude solutes like Na^+ salts, and sorbitol (Ling and Ochsenfeld, 1983; 1987). Taking all these relevant facts together, one suspects that PEG-900 also orients and polarizes water.

(iii) Figure 1 contains additional information suggesting hydration-water formation around erythritol in frog muscle. cells. After staying for over 60 hours at one equilibrium level, the q-value of erythritol rather abruptly went up to a higher level and remained unchanging at this higher level for the duration of the observation (168 hours). An all-or-none shift of this kind suggests that erythritol can interact with cell water in two discrete ways, one entailing a higher q-value than the other. The lower one may be primarily determined by the molecular volume of the sugar alcohol and the dynamic structure of the cell water; the higher one may be to some extent at least in response to erytbritol's own need. It should be painted out that the type of behavior demonstrated by erythritol (time-dependent stepwise q-value rise) was seen in other "larger" solutes, including sucrose itself, not only in frog muscle but also in cancer cells (Ling and Fu, to be published).

In summary, of the three explanations offered for the exceptionally high q-values of sucrose, raffinose and PEG-900, the evidence against the free-water concept is the strongest. Of the two other explanations, it may not be a question of either/or. One explanation could explain the high q-value of one solute; the other one could explain that of another one. A combination of both could explain a third. Only future investigation can determine decisively which is which.

Taking these interpretations into account, we are inclined to say that all the available data points in Figure 6 can be adequately explained in terms of the theory.

Establishing the multiplicity of layers of water polarized in living cells

The central postulate of the polarized multilayer theory of cell water is that water in the cell and in suitable model systems assumes the dynamic structure of polarized multilayers. In the past, Ling and his coworkers have already pointed out the theoretical as well as experimental evidence for the fact that multiple layers of water are adsorbed on various *inanimate* model systems (Bradley, 1936; Brunauer *et* al., 1938; Harkins, 1945; Ling, 1992, pp. 71-76). In this section, we will show that in *living frog muscle cells, water is also* polarized in multiple layers.

To avoid unnecessary argument, we begin with the safe position that only 90% of the muscle-cell water is polarized. The total water content of frog muscle cells is 80% of the fresh muscle cell weight. 90% of cell water then amounts to $0.9 \times 0.8 = 0.72$ or 72% of the cell weight. 72% of polarized water is equivalent to $720/18.02 = 39.96$ M of water. The average amino acid-residue weight of most proteins is about 112 (Ling, 1962, p 48). Dividing this number into the total protein weight in a liter of cells (200 g), one obtains an amino-acid-residue concentration of 1.79 M. The average number of polarized water molecules per peptide bond is then $39.96/1.79 = 22.32$ water molecules.

As pointed out in the preceding paper (Ling, 1993), through its lone proton, each NH group of a peptide can form an H-bond with one neighboring water molecule. In contrast, each CO group, through its two pairs of lone-pair electrons of the oxygen atom, can form H-bonds with two neighboring water molecules. Thus, three water molecules form the first layer surrounding each peptide group. Now each of the three first-layer water molecules

can, in turn, form H-bonds with three more water molecules, leading to the polarization of nine water molecules in the second polarized layer. More than twelve additional water molecules are left to participate in the formation of a third polarized water layer. Since the word, multiplicity denotes "more than one", the basic concept of the polarized multilayer theory is thus proven.

Actually there are compelling reasons to believe that each of the exposed NHCO groups of the water-polarizing-protein polarizes much more than the 22.32 water molecules cited above. Thus only a fraction of cell proteins can be expected to exist in the fully-extended conformation. If the folly-extended fraction amounts to 20% of the cell-proteins, then as many as four layers of water will be polarized by each NHCO site. If only 10% of the protein chains are fully extended, then the q value vs. molecular volume plot of Figure 6 would in fact inform us that even water molecules in a fifth layer are polarized.

Returning to the two extrovert model systems studied, 18% gelatin gel and 20% NaOH-denatured hemoglobin, where Ling showed that each NHCO group polarizes a first layer of three, a second layer of nine as well as some additional water molecules in a tbii layer surrounding each of the NHCO groups. Thus the major difference between water in living muscle cells and water in inanimate extrovert model systems seems to lie only in the *degree* of water polarization. In the next section, we will discuss this issue in more detail.

The polarization energy of bulk-phase cell water

The present investigation has made it possible to determine, for the first time in history, the exclusion intensity of the bulk-phase muscle cell water (126 cal/mole). This determination is remarkable because in magnitude it amounts to a very small percentage of the total water-to-water interaction energy of the cell water (the heat of vaporization of water is in the neighborhood of 10,000 cal/mole). (See Ling, 1993 for explanation of how this is achieved).

In the preceding paper, it was pointed out that the intensity of water-to-water polarization is given by the term, $\mathcal{U}_{vp}$ (the exclusion intensity), incorporating both the volume component and the entropy (or partition-function-ratio) component of the polarization energy. In Figure 6 we have shown that the majority of the experimental points can be described by a $\mathcal{U}_{vp}$ equal to 126 cal/mole. Comparing this figure with those listed in Table I of the preceding paper (Ling, 1993). we find that of all the systems investigated thus far, including 20% NaOH-denatured bovine hemoglobin (26.6 cal/mole), 18% gelatin gel (16.5 cal/mole), and 15% PEO solution (15.9 cal/mole) as well as sulfonate ion exchange resin loaded with Li^+ *(55.2* cal/mole) or with Rb^+ *(26.6* cal/mole), *water in frog muscle cells has by far the highest polarization intensity,* $\mathcal{U}_{vp}$ (126 cal/mole).

Why do the fully-extended protein chains in living cells exercise a much stronger influence on the intensity of water polarization than those of the inanimate models? We suggest that the main reason lies in the degree of order in the arrangement of the (folly-extended) protein chains: highly ordered in living cells; poorly ordered in the extrovert models. There are evidence for this contention.

Stirring, which increases the order of arrangement of linear polymers, significantly decreases the apparent equilibrium distribution coefficient or p-value of sodium sulfate (and sodium citrate) in solutions of the extrovert model, polyvinylpyrrolidone (PVP) (Ling *et al.*, 1980a, 1980b). Since the most likely cause for a decrease of the q- or p-value is an increase of the intensity of polarization, enhancing the ordering of water-polarizing models by stirring elevates tbe intensity of water polarization.

Concluding remarks on the polarized multilayer theory and its confirmation

As mentioned in the opening paragraph of this paper, asymmetrical solute distribution in living cells-in particular the maintained low levels of Na^+, sugars and other solutes-has played a major role in the formulation of all proposed major theories of living cells.

To maintain unequal concentrations of a substance in two contiguous spaces, there are altogether only three basic mechanisms known (Ling, 1992, pp. l-2). Two of these mechanisms (permanent energy barrier or sieve idea; pumping) have been ruled out (for details, see Ling, 1992, Chapters 1 and 2). The third and only remaining mechanism is shared by Troshin's sorption theory and Ling's association-induction hypothesis: the cell interior represents a physico-chemical environment different from that in the surrounding medium. For the reduced level of solutes in the cell, this means that the cell water has reduced solvency for these solutes.

Troshin's sorption theory offers no molecular mechanism *how* cell water is different from normal water nor *why* cell water has reduced solubility for some solutes but not for others. In contrast, the association-induction hypothesis offers explicit answers to both questions.

Put in the simplest way, cell water is different because it assumes the dynamic structure of polarized multilayers; the enhanced water-to-water interaction energy creates the phenomenon of size-dependent solute exclusion. Climaxing more than thirty years of research, the data presented in the present paper have confirmed most, if not all, the major postulates and predictions of this theory of solute distribution in cell water.

Reaching back to important but unanswered questions and almost forgotten wisdom of earlier times

Many contemporary biomedical scientists have noted with alarm the continuing fragmentation of an inherently indivisible biomedical science. The causes for this illness are multiple. The lack of a widely accepted, coherent theory of the living cell is unquestionably a major contributing factor to this loss of coherence-both laterally with other contemporary scientists and longitudinally with scientists who have passed away.

With the polarized multilayer theory (and other aspects of the association-induction hypothesis) confirmed (Ling, 1992), we believe that it is high time to begin an effort at seeking and, if possible, weaving more coherence into the biomedical sciences. As a beginner, we shall respond to some important comments and ideas of great scientists of the past. Like us, they too had a keen interest in the water of living cells.

(1) Carl Ludwig's quest:

Carl Ludwig, sometimes referred to as the father of modem physiology, published in 1849 his finding on the selective uptake of water over sodium sulfate by dried pig's bladder (Ludwig, 1849). In this article, he wrote: "Die kleinsten Theilchen der Membran haben eine ausgesprochene Verwandschaft—ob chemische oder adhäsive wird uns die Chemie erläutern, wenn sie sich aus ihrem theoretische Elend erhoben hat—zu dem wasser "* (p. 22).

Twelve years later, Thomas Graham of England coined the word, colloid, from the Greek word for glue or gelatin (κολλα) (Graham, 1861). Another 73 years went by before Hol-

* "The smallest part of the membrane has an outstanding affinity—whether chemical or adhesive, chemistry will explain to us, when it has arisen out of its theoretical misery-for water.. "

leman *et* al. (1934) demonstrated that like Ludwig's pig bladder, gelatin also selectively takes up water over sodium sulfate. Nearly a century went by before Troshin picked up this train of thought and pointed out that like gelatin, living cells also selectively take up water over sodium sulfate (and nonelectrolytes like sucrose)

Not too long after the introduction of the PM theory, a new definition of "colloid" was introduced (see Ling, 1992, p. 85): *a system* ***containing*** *water* (or another suitable polar liquid) ***in the*** *state* ***of*** ***polarized*** *multilayers* ***in*** *consequence* ***of interaction with a polar solid*** component also present ***in the*** system, e.g., gelatin. Gelatin polarizes water in multilayers because it contains a majority of amino acid residues that are either not able (proline, hydmxyproline) or not inclined to form a-helical and P-pleated sheet conformation (e.g., glycine). As a result, a major share of the NHCO groups are directly exposed to, and polarize in multilayers, the surrounding water.

Thus at long last, we now can offer an explanation for Carl Ludwig's original observation on pig's bladder: water in the swollen pig's bladder also assumes the dynamic structure of polarized multilayers. And on account of molecular mechanisms described in detail in the preceding paper (Ling, 1993), this water imbibed by the bladder partially excludes (large) hydrated Na^+ and sulfate ions.

(2) Erwin Schrödinger's negative entropy

In his delightful little book, "What is Life?" (Schrödinger, 1945), physicist E. Schrödinger argued that life is a system which decreases entropy. By inference, death accompanies a gain of entropy.

Now the basic unit of all life is a living cell. Life and death are therefore ultimately life and death of the living cells. A living cell is primarily an assembly of water, proteins and K^+ ions (Ling, 1992, p. 31). In the conventional membrane-pump theory, virtually all the cell water and cell K^+ are free. Therefore, in this model of the living cell, there can be no entropy gain on cell death from either water or K^+. Cell water and cell K^+ cannot become freer than free. This elimination leaves proteins as the only possible source of entropy gain. However, proteins of dead cells do not necessarily become freer and more randomly distributed. On the contrary they tend to be more rigid and less free to move around as the term "rigor mortis" portrays. So where is the source of entropy gain during cell death? Was Schrödinger wrong?

In number, the most abundant component of the living cell is water, the next most abundant is K^+ (Ling, 1992, p. 31). According to the association-induction hypothesis, being alive signifies the existence of the living cell in a high (negative) energy-low entropy state, called the living state. As part and parcel of this living state, virtually all the cell water and cell K^+ are adsorbed: cell K^+ singly on β- and γ- carboxyl groups and water in multilayers on fully-extended protein chains. Adsorption of either kind inevitably reduces their entropy.

In previously published work, we have presented overwhelming evidence that cell K^+ is indeed adsorbed on β- and γ- carboxyl groups (Ling, 1992, Chapter 4). The present paper provides what we believe to be definitive evidence for the multilayer adsorbed state of the cell water on some fully-extended cell protein(s).

Thus, one can now say with confidence that Schrödinger was right. The negative entropy conserved in the living cell lies largely in the adsorbed ions and above all adsorbed water molecules. The entropy gain on death is largely from the liberation of cell water and cell K^+ from the adsorbed state to the free state which inevitably accompanies cell death. Indeed, Ling has suggested that death is an entropy-driven process (Ling, 1992, p. 182).

(3) Albert Szent-Györgyi's dancing water

Twenty years ago, Albert Szent-Györgyi wrote: "Life is water dancing to the tune of solids" (Szent-Györgyi, 1972, p. 9). As mentioned above, in the conventional textbook version of the theory of living cells, cell water is free. It is hard to imagine how such "independent-minded" free water can dance to the tune of anything. Was Szent-Györgyi wrong?

Szent-Györgyi was not wrong. The conventional theory of the living cell is at fault.

Most of the solids in living cells, especially those devoid of a nucleus like adult human erythrocytes, are in the form of proteins. For water in the living cells to dance to the tune of proteins, there must be effective communication and interaction between them. The PM theory provides such an effective interaction and communication in the form of propagated electrical polarization and spatial orientation among cell water molecules by the NH and CO groups of the involved proteins.

However, in life, the "tune" to which water dances, does not *originate from* the proteins, which are closer to being the instruments of music. Rather, these instruments are being played on by "cardinal adsorbents", of which ATF is a prime example. When ATP is adsorbed on the key sites (the cardinal sites), cell water and K^+ are adsorbed, when ATP is enzymatically removed, cell water and K^+ may be transiently liberated. Rapid to and fro transitions of cell water between the polarized and the depolarized state may then underlie the unit physiological activity of the cell pmtoplasm, or in Szent-Györgyi's poetic vision, the water dance of life.

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