The State of Water in Biological Systems

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This paper addresses the issue of how the aqueous cytoplasm is organized on a macroscopic scale. Mitochondria were used as the experimental model, and a unique experimental approach was used to probe the properties of water in the mitochondrial matrix. The results demonstrate aqueous phase separation into two distinct phases with different osmotic activity and different solute partition coefficients. The larger phase, designated "normal water," is osmotically active and behaves in every respect like a bulk, dilute salt solution. The smaller phase, designated "abnormal water," is osmotically inactive and comprises the water of hydration of matrix proteins. It is, nevertheless, solvent water, with highly selective partition coefficients, and behaves like a Lewis base.

KEY WORDS: Water, Mitochondria, Partition coefficient, Osmotic, Membranes, Protein hydration.

I. Introduction*

This review deals with the time-averaged, equilibrium properties of water in cells. In this domain, one is dealing with familiar macroscopic properties such as osmotic activity and solute activity coefficients.

In the early 1970s, I put forward a novel hypothesis for the macroscopic state of water in biological systems: that biological water spontaneously separates into two (or more) phases with distinct solvent properties (Garlid, 1976, 1978, 1979). This hypothesis was both unusual and, it still seems to me, inescapable. Thus, a straightforward application of the scientific method conclusively excluded the alternative hypothesis that biological water com-

* Abbreviations: BSA, bovine serum albumin; CCCP, carbonyl cyanide m-chlorophenylhydrazone; PEG, polyethylene glycol.

prises a single, homogeneous phase. This experimental evidence will be reviewed.

Isolated rat liver mitochondria were used as the experimental model for this investigation. The mitochondrial matrix is a single, membrane-bounded compartment that is very rich in proteins. A thermodynamically complete description of matrix water requires no more than two different phases,[1] which I call "normal" and "abnormal." The *normal* aqueous phase behaves in every respect like a bulk aqueous solution of similar composition. The *abnormal* aqueous phase is osmotically inactive and comprises the water of hydration of mitochondrial membranes and proteins. Nevertheless, the abnormal phase dissolves small solutes, with solute activity coefficients that are very different from those in bulk aqueous solutions.

The decisive results came from a novel, but perfectly straightforward, experimental approach, in which the effects of matrix volume on nonelectrolyte distribution coefficients were compared with distribution coefficients measured at constant volume. It remains surprising to me that this powerful, but conceptually simple, approach has not been applied to other biological systems.

The two-phase hypothesis contains important implications for understanding the aqueous cytoplasm. The proteins of the cell are neither distributed randomly nor do they impart the aqueous phase separation in a manner analogous to that observed with macromolecules in a test tube, as suggested by Walter and Brooks (1995). Rather, they are directed by the biological machinery of the cell to form close associations with each other and the cytoskeleton and membranes of the cell. The association serves the purpose of minimizing the water of hydration associated with all proteins. Based on hydration estimates of serum albumin, it has been suggested that, if it did not occur, all of the water of the cell could be osmotically inactive (Cameron *et al.,* 1997). Proteins are also specifically localized within the cell. This localization not only serves the purpose of mechanical function, as in muscle, but also the purpose of metabolic channeling (Saks *et al.,* 1994). The picture that emerges is that cell proteins exist in a semi-solid, gel-like state, and their water of hydration possesses unique solvent properties as a consequence of its organization. The remainder, comprising the major portion of cell water, is a dilute salt solution, probably containing few proteins. This bulk solution, through which metabolites and signal ligands flow, is dispersed in streams and pockets among the organized matrices of proteins and membranes.

[1] A small fraction of cell water is irrotationally bound to proteins and, therefore, cannot participate as a solvent. Attempts to quantitate this fraction lead to values on the order of 0.1% of total cell water (Cooke and Kuntz, 1974). All of the comments in this article are meant to exclude this irrotationally bound water.

II. Osmotic Behavior of Polar Solutes—Introduction to the Osmotic Intercept

The thermodynamic activity of water in cells, a_w, normally ranges between 0.990 and 0.997. The preferred measure of water activity is the osmolality, ϕ, which avoids the awkwardness of these numbers, giving a range between 166 and 558 mosM. Osmolality of a solution containing a single solute is defined as follows (Kirkwood and Oppenheim, 1961, discussed in Garlid, 1998):

$$\phi = gm = -\frac{1000}{M_w} 1n\ a_w \tag{1}$$

where m is molality of the solute and M_w is the molecular weight of water; g is the osmotic coefficient and is equal to 1 for an ideal solution. Note that osmolality, which can be measured by freezing point depression, osmotic pressure, or vapor pressure lowering, is a thermodynamically rigorous measure of water activity.

Polar solutes exhibit nonideal behavior in aqueous solutions, as illustrated by the osmotic behavior of sucrose and PEG400, plotted in Fig. 1. Both curves deviate strongly from the line of ideal behavior ($g = 1$), but extrapolate to zero at infinite dilution. BSA exhibits similar behavior (not shown).

As shown nearly 80 years ago by Scatchard (1921), nonideal behavior of polar solutes can be rationalized by considering their hydration. In solution, sucrose is unlikely to undergo thermal motion as the dry molecule. Rather, it will move as the hydrate, and the water available for freezing or vaporization will be correspondingly reduced by an amount equal to the molal hydration, W_h. A new concentration, m', may thus be defined:

$$m' = \frac{m}{(1 - W_h m)} \tag{2}$$

where W_h is the water of hydration, in kg H_2O/mol solute. We may write

$$\phi = g'm' \tag{3}$$

where g' is the osmotic coefficient of the newly defined solution. To linearize the expression, we take the inverse of Eq. (2) and obtain

$$1/m = W_h + g'/\phi \tag{4}$$

Two aspects of Eq. (4) are noteworthy. First, an entirely analogous relationship between inverse quantities is used for osmotic studies in cells and organelles. Second, the intercepts of such plots represent the approach

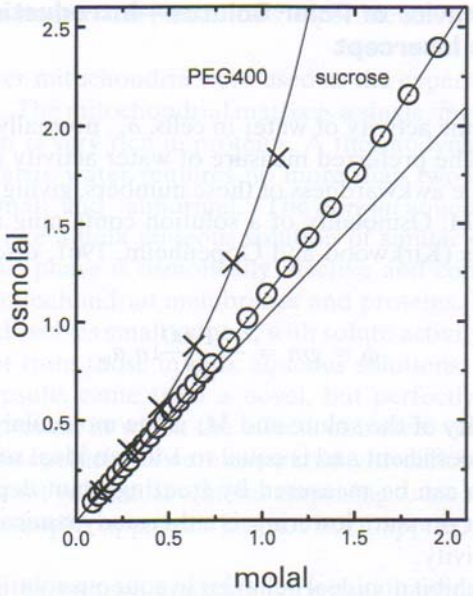

FIG. 1 Osmotic behavior of polar molecules in solution. Osmolality is plotted as a function of molal concentration of PEG400 and sucrose. The straight line, osmolal = molal, is included to demonstrate the behavior of an ideal solute. The values for PEG400 were converted from the data of Parsegian *et al.* (1995). The values for sucrose were converted from the data of Wolf and Brown (1965). The curves drawn through the data points used $\phi = m/(1 - W_h m)$ (see Eq. (2) in text), where W_h is the constant water of hydration. Use of this equation implies that sucrose and PEG400 behave as ideal solutes when hydration is taken into account ($g' = 1.0$ in Eq. (1)).

to infinite concentration, rather than infinite dilution. Thus, *the osmotic intercepts cannot equal zero except for ideal solutions.*

Figure 2 contains data for sucrose, PEG400, and BSA, plotted according to Eq. (4). Note that all three sets of data are linear with positive intercepts, W_h. More impressively, the slopes of the lines equal 1.0 within experimental error. This means that each of these solutes behaves as an *ideal* solute ($g' = 1$) when a constant molar hydration is taken into account. Thus, hydrated sucrose behaves ideally to 2 molal.

The hydration values are instructive. In mol H_2O/mol solute, they are 5 for sucrose, similar to the value obtained by Scatchard (1921), 22.7 for PEG400, and 1.05×10^4 for BSA. The range is still large in g H_2O/g solute: 0.28 for sucrose, 1 for PEG400, and 2.86 for BSA. These numbers indicate that sucrose is hydrated with one water per –OH group, whereas the large surface of BSA permits building up of water multilayers.

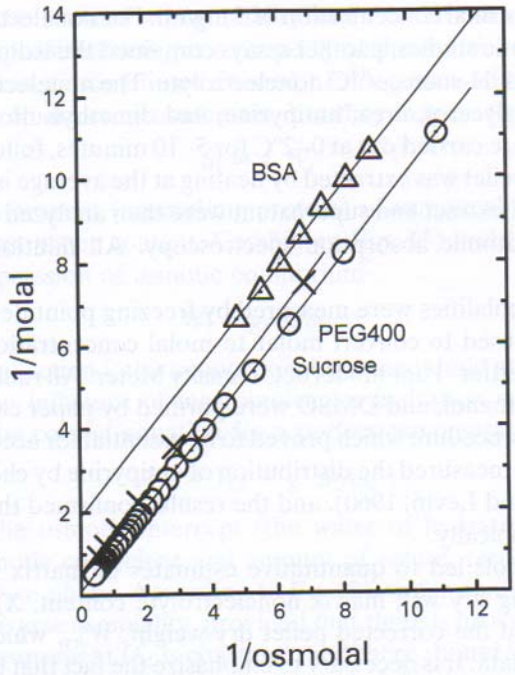

FIG. 2 Ideal osmotic behavior of hydrated polar solutes and macromolecules. Inverse molality is plotted versus inverse osmolality, as in Eq. (4). Molal and osmolal values for BSA were multiplied by 100 to fit in the same figure. The intercepts (W_h) and slopes (g′) were obtained by linear regression, with correlation coefficients > 0.999 for each curve. Sucrose: 0.0956, 1.003; PEG400: 0.4094, 1.043; BSA: 189.48, .989. The values for BSA were converted from those of Zimmerman *et al.* (1995), after correcting observed osmolalities for the Donnan effect (Garlid, 1998).

III. Mitochondria—The Experimental Model

The mitochondrion is an excellent experimental model for studies of biological water. It is structurally simple and contains a single osmotically active compartment. Under controlled experimental conditions, this compartment retains its ionic contents during wide volume changes. Mitochondria behave as osmometers (Tedeschi and Harris, 1955; Bentzel and Solomon, 1967; Beavis *et al.*, 1985; Garlid and Beavis, 1985), and they can undergo very large amplitude swelling without rupture, a consequence of the extensive folding of the inner membrane (Stoner and Sirak, 1969).

Distributions of water and nonelectrolytes in isolated rat liver mitochondria at 4°C were measured according to exacting protocols, which are described elsewhere (Garlid, 1998). Briefly, mitochondria were added to

assay media at a final concentration of 5 mg/ml. For nonelectrolyte distributions and osmotic studies, parallel assays contained the isotope pairs ^3H_2O/^{14}C-sucrose and ^{3}H-sucrose/^{14}C-nonelectrolyte. The nonelectrolytes studied were ethanol, glycerol, urea, antipyrine, and dimethylsulfoxide (DMSO). Incubations were carried out at 0–2°C for 5–10 minutes, followed by centrifugation. The pellet was extracted by heating at the average isoelectric point (pH 4.6). Pellet extract and supernatant were then analyzed by scintillation counting and atomic absorption spectroscopy. All dilutions were gravimetric.

Solution osmolalities were measured by freezing point depression. Solution densities, used to convert molar to molal concentrations, were measured with a Mettler–Paar model 02C Density Meter. All radioactive probes except THO, ethanol, and DMSO were purified by paper chromatography prior to use, a procedure which proved to be essential for accurate measurements. We also measured the distribution of antipyrine by chemical analysis (Mendelsohn and Levin, 1960), and the results confirmed the distributions measured isotopically.

These protocols led to quantitative estimates of matrix water content, W_i (mg H_2O/mg dry wt); matrix nonelectrolyte content, X_i (nmol solute/mg dry wt); and the corrected pellet dry weight, W^o_{dry}, which was used to normalize the data. It is necessary to emphasize the fact that these protocols measure matrix *water* content, and not matrix *volume*.

Over a period of years, rat liver mitochondria proved to be highly reproducible. For example, total K^+ and Mg^{2+} in the stock suspension were almost invariant over 6 years of measurement, at 153 ± 1 nmol/mg and 40 ± 0.3 nmol/mg (n = 20), respectively. Some K^+ was predictably lost upon dilution, and matrix values for K^+ and Mg^{2+} were 125 ± 8 nmol/mg and 38 ± 0.3 nmol/mg, respectively. Matrix Mg^{2+} was unaffected by osmotic swelling at 0°C.

IV. Osmotic Equilibria in Mitochondria

A. Theory

Osmotic equilibrium is the state in which, in the absence of pressure gradients, water activity, a_w, is equal in the two phases. Thus, osmolality, ϕ, is equal in the two phases, and

$$g_i\, s_i = \phi_0 \tag{5}$$

where s_i designates the ideal osmolality of the internal phase; $s_i = \Sigma \nu_j\, m_j$ where ν_j = the number of particles into which solute j dissociates. The

variable ϕ_0 is the (measured) osmolality and g is the osmotic coefficient. (It must be emphasized that g is a property of the *solution* and not of the individual solutes, as sometimes claimed.) Endogenous matrix solutes are not susceptible to direct measurement, but they may be written

$$S_i \equiv s_i W_i \tag{6}$$

where S_i is the amount (nosmol/mg protein) of osmotically active solute, and W_i is matrix water content. Combining Eqs. (5) and (6), we achieve the general expression of osmotic equilibrium

$$W_i = g_i S_i/\phi_0. \tag{7}$$

As discussed in Section I, this proportionality cannot hold at infinite osmolality, where the influence of macromolecular hydration will be most extreme. Thus, the correct equation for a perfect osmometer is

$$W_i = W_2 + g' S_1/\phi_0 \tag{8}$$

where W_2 is the osmotic intercept (the water of hydration) and g' and S_1 are the osmotic coefficient and amount of solute, respectively, in the osmotically active phase. According to Eq. (8), water content should vary linearly with inverse osmolality, provided that there is no solute movement during the measurement (S_1 is constant), and there should be a positive intercept.

B. Osmotic Swelling in Mitochondria

Figure 3 contains plots of matrix water content, W_i, vs inverse osmolality. The data, which cover a 9-fold range of volume, are in complete agreement with the predictions of Eq. (8). Each curve is linear, and the slopes increase with increasing solute content. The curves have a common, nonzero intercept (W_2), equal to 0.28 mg H_2O/mg mitochondrial protein. Thus, when hydration is taken into account, the mitochondrion behaves as a perfect osmometer.

These results indicate that matrix water partitions into two aqueous phases, an osmotically active phase, W_1, and an osmotically inactive phase, W_2. The question whether W_2 behaves as a solvent or represents "bound," nonsolvent water will be addressed in the section on nonelectrolyte distributions.

C. Osmotically Active Matrix Solutes and the Osmotic Slope

The major solutes of freshly isolated mitochondria are potassium salts of phosphate and organic anions (Gamble and Hess, 1966). Experimentally,

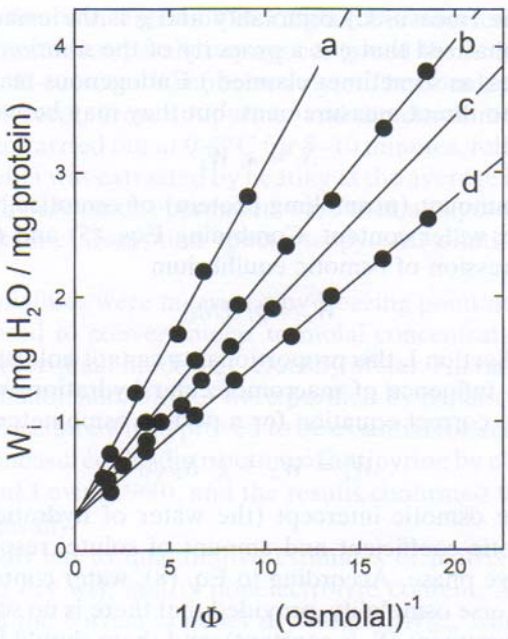

FIG. 3 Osmotic swelling in mitochondria. Matrix water content, W_i (mg H_2O/mg dry weight), is plotted versus inverse osmolality, ϕ^{-1}. The data shown were obtained on four separate preparations with different matrix K^+ contents. Each preparation began with mitochondria suspended in 0.25 M (0.272 osmolal) sucrose. **Curve a,** mitochondria were preincubated at 25°C in the presence of substrate, O_2 and K^+. Prior to the assay, the preparation was treated with rotenone and subjected to a 5-min 0°C wash in K^+ malate to block the K^+/H^+ antiporter. They were resuspended in sucrose following a large volume wash in 0.25 M sucrose. K^+ content was 165 nmol/mg dry weight. **Curve b,** no pretreatment. K^+ content was 125 nmol/mg dry weight. In order to maintain constant K^+, aliquots were incubated for 10 s prior to centrifugation. Longer incubations cause curvature due to swelling-induced K^+ loss (Garlid and Beavis, 1985). **Curve c,** mitochondria were incubated in 80 mosmolal sucrose for 15 min, during which they lost K^+ via the K^+/H^+ antiporter. They were then reisolated and suspended in 0.25 M sucrose. K^+ content was 82 nmol/mg dry wt. **Curve d,** mitochondria were added to 54 mosmolal sucrose for 15 min, reisolated and suspended in 0.25 M sucrose. K^+ content was 75 nmol/mg dry weight. These results are representative of 10 osmotic swelling curves. In each case, the slopes depended on K^+ content and the curves were linear if matrix K^+ was constant.

it is a simple matter to alter the amount of K^+ and the amount and type of anion in respiring mitochondria. It was essential to maintain constant amounts of endogenous solutes through subsequent volume changes, so that the quantity S_1 remained constant. By manipulating the Mg^{2+}-regulated K^+/H^+ antiporter (Garlid, 1980, 1988), we succeeded in preparing mitochondria which contained widely varying amounts of K^+ salts and which did not lose these solutes during swelling.

Figure 4 contains a plot of the osmotic slopes, S_1, versus matrix K^+, using the data in Fig. 3. The slope of the line is 1.52 nosmol/nmol K^+, and the intercept is 15 nosmol/mg. Uptake and loss of K^+ salts constituted the only significant change in matrix solutes in these experiments, and any net change in matrix K^+ was accompanied by anions of average valence, z_a. The relationship between osmotic slope and K^+ may be written

$$S_1 = g'\{s_1^0 + (1 + 1/z_a)\, K_i^+\} \tag{9}$$

where s_1^0 represents internal solutes not included in the second term in the equation. We have estimated g', the osmotic coefficient of the "normal" phase, to be 0.94, so the slope of 1.52 yields an estimated anion valency, z_a, of 1.62. This is in good agreement with expectations, since the labile anions are primarily phosphate and divalent anions under these conditions. This is an approximate analysis, because the distribution of K^+ between phases 1 and 2 is unknown.

D. Osmotically Inactive Matrix Water and the Osmotic Intercept

Four facts can be noted about the osmotic intercept: (1) W_2 represents osmotically inactive *water,* as should be clear from the way the measurements were carried out. The suggestion frequently made that the osmotic

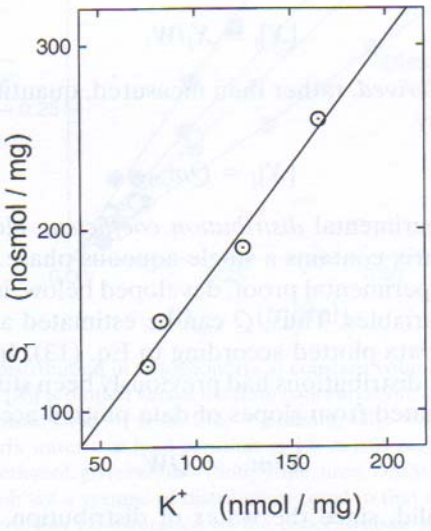

FIG. 4 The effect of matrix K^+ on the osmotic slope. The osmotic slope, S_1, is plotted versus matrix K^+ content. The values plotted correspond to the four curves in Fig. 1.

intercept represents proteins and lipids is incorrect; (2) W_2 is unaffected by changes in matrix solute concentrations; (3) A positive osmotic intercept is characteristic of all biological systems, as well as of most inanimate systems, ranging from small nonelectrolytes to gels and coacervates; (4) The size of W_2 is about an order of magnitude less than the water of hydration of proteins in solution (compare W_2 with W_h for BSA).

V. Nonelectrolyte Distributions in Mitochondria

A. Theory

When solute x is at equilibrium between two homogeneous phases, the following relationship holds:

$$m_{x1} = f\, m_{x0} \tag{10}$$

where f is the thermodynamic *partition coefficient,* given by

$$f \equiv Y_{x0}/Y_{x1} \tag{11}$$

where the Ys are molal activity coefficients. The internal concentration, m_{x1}, cannot be measured directly. Instead, we know internal solute content, X_i, and total matrix water content, W_i, which leads to an average concentration, $[X_i]$:

$$[X]_i \equiv X_i/W_i \tag{12}$$

Note that $[X]_i$ is a *derived,* rather than measured, quantity. Solute distribution is then given by

$$[X]_i = Qm_{x0} \tag{13}$$

where Q is the experimental *distribution coefficient.* Note that $Q = f$, if, and only if, the matrix contains a single aqueous phase.

The key to the experimental proof, developed below, is that $[X_i]$ contains two independent variables. Thus, Q can be estimated at constant volume from the slopes of data plotted according to Eq. (13). In fact, this was the only way that solute distributions had previously been studied. The variable Q can also be estimated from slopes of data plotted according to Eq. (14)

$$X_i/m_{x0} = QW_i \tag{14}$$

which is equally valid, since the water of distribution, X_i/m_{x0}, is a measured quantity.

B. Nonideal Distribution Coefficients for Simple Nonelectrolytes

In the 1930s, there was considerable controversy over the extent of normal and bound water in cells. In a very nice review entitled, "Water, Free and Bound," Blanchard (1940) suggested that permeant nonelectrolytes should be useful as probes of "free" water, since one would not expect them to penetrate "bound" water. In particular, polar nonelectrolytes should all have equal volumes of distribution and, if all water is normal, their distribution coefficients should be close to 1.0.

These expectations are strikingly unfulfilled in mitochondria. This is demonstrated by the constant volume data in Fig. 5, plotted according to Eq. (13). Note that Q for each solute is independent of concentration up to 0.5 M, the highest level measured. The results in Fig. 5 are qualitatively

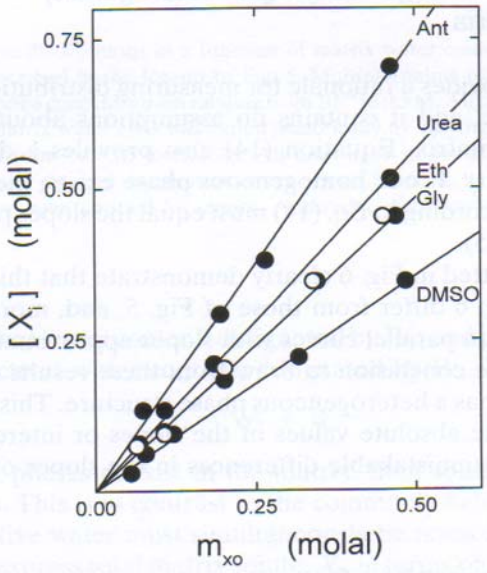

FIG. 5 Nonelectrolyte distribution in mitochondria at constant volume. Apparent molal concentration in the matrix $[X_i]$ is plotted versus medium concentration, m_{xo}, for various nonelectrolytes: Ant. = antipyrine; Urea = urea; Eth. = ethanol; Gly. = glycerol; and DMSO = dimethylsulfoxide. Matrix water was held constant at 1.0 ± 0.05 mg/mg dry weight. Results are representative of 3 (ethanol, glycerol) or 4 (antipyrine, urea, DMSO) experiments. Ethanol and glycerol always exhibited a volume of distribution equal to that of water. These are true phase distributions; there was no saturation in the concentration range between 10^{-4} and 10^{-1} M.

similar to the results of Ling (1970) and Troshin (1966) in various gels, tissues, and cells.

The results with the waterlike solutes, ethanol and glycerol, which have distribution coefficients of 1.0, strongly indicate that all of matrix water is solvent, including the hydration water, W_2.

The other probes confound expectations in that they differ from 1.0 and differ from each other. From such results, Ling (1970) and Troshin (1966) were led to conclude that all of biological water is abnormal. The logical fallacy of this conclusion lies in the implicit assumption that all of matrix water constitutes a single phase. To get around this problem, it is necessary to perform experiments in a different way, as shown in the next section.

VI. Solute Distribution as a Function of Matrix Volume

A. Experimental Evidence for Phase Heterogeneity in Mitochondria

Equation (14) provides a rationale for measuring distributions as a function of matrix volume, and it contains no assumptions about aqueous phase structure in the matrix. Equation (14) also provides a direct test of the single-phase theory. If one homogeneous phase exists, then the slopes, Q, of data plotted according to Eq. (14) must equal the slopes plotted according to Eq. (13) (Fig. 5).

The results plotted in Fig. 6 clearly demonstrate that this is not the case. The slopes of Fig. 6 differ from those of Fig. 5, and, moreover, all solute distributions fall on parallel curves with slopes approximately equal to 1.0.

The inescapable conclusion to draw from these results is that the mitochondrial matrix has a heterogeneous phase structure. This conclusion does not depend on the absolute values of the slopes or intercepts, but rather on the large and unmistakable differences in the slopes of Figs. 5 and 6.

B. Phase Heterogeneity—Theory

The general expression for multiphase solute distribution is

$$X_i/m_{x0} = \Sigma f_j W_j \tag{15}$$

where f_j are the thermodynamic partition coefficients in each phase. It is sufficient to define two hypothetical classes of water: Phases of variable

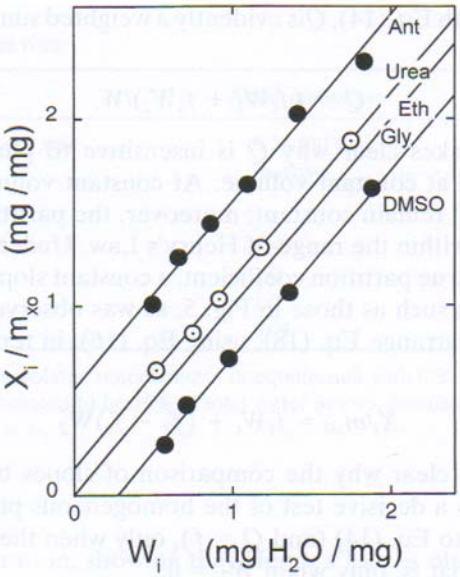

FIG. 6 Nonelectrolyte distributions as a function of matrix water content. The symbols and conditions are as described in the legend to Fig. 5. Multiple points for urea represent data obtained at different urea concentrations ranging from 10^{-4} to 0.5 M. All other probe concentrations were 50 mM. Matrix water (W_i) was varied osmotically by varying medium sucrose plus buffer concentration from 0.08 M to 0.33 M. All lines have slopes of 1.0 ± 0.04 by linear regression. Ethanol and glycerol distributions were always found to be within 4% of W_i, and their data points have been omitted for clarity. Antipyrine distributions were confirmed by chemical analysis.

(osmotically active) water content, designated by W_1, and phases of constant (osmotically inactive) water content, designated by W_2. Thus,

$$W_i = W_1 + W_2 \tag{16}$$

If two solvent phases coexist in the matrix, then solutes must partition into both phases. This is in contrast to the commonly held assumption that osmotically inactive water must simultaneously be nonsolvent (Ling, 1972; Dick, 1971). To express total matrix solute, X_i, in terms of measured quantities, we begin with the following:

$$X_i = W_1 m_{x1} + W_2 m_{x2} \tag{17}$$

Introducing thermodynamic partition coefficients $f_1 = m_{x1}/m_{x0}$ and $f_2 = m_{x2}/m_{x0}$, we obtain

$$X_i = (f_1 W_1 + f_2 W_2) m_{x0} \tag{18}$$

By comparison with Eq. (14), $Q$is evidently a weighted sum of partition coefficients:

$$Q = (f_1W_1 + f_2W_2)/W_i \tag{19}$$

Equation (19) makes clear why Q is insensitive to phase heterogeneity when determined at constant volume. At constant volume, compartment sizes (W_1, W_2, W_i) remain constant; moreover, the partition coefficients, f, remain constant within the range of Henry's Law. Under these conditions, even if Q is not a true partition coefficient, a constant slope will be obtained from experiments such as those in Fig. 5, as was observed.

We can now rearrange Eq. (18), using Eq. (16), in terms of experimental variables:

$$X_i/m_{x0} = f_1W_i + (f_2 - f_1)\mathrm{W}_2 \tag{20}$$

It should now be clear why the comparison of slopes between Eqs. (13) and (14) provides a decisive test of the homogeneous phase model. Thus, Eq. (20) reduces to Eq. (14) (and $Q = f$), only when the matrix is a single aqueous phase; that is, only when $W_2 = 0$.

The separation into two phases, W_1 and W_2, represents what we are *capable* of knowing about the compartmentation of water and solute. This separation is thermodynamically complete, because it accounts for all phase types that can be distinguished experimentally.

C. Phase Heterogeneity—Quantitative Results

Table I contains a summary of the results of the nonelectrolyte experiments, interpreted using Eq. (20). Some additional inferences may be drawn from these data:

1. The theoretical development does not preclude the possibility of water shifts between phases; however, the fact that the data conform to the linear relationship of Eq. (20) suggests that such shifts do not occur to any significant extent.
2. The results are independent of the means used to vary W_i. The variable W_i may be changed by experimental alterations in two independent parameters: internal solute content and medium concentration of impermeant solute. Solute distributions are dependent on W_i and independent of the parameter or parameters used to achieve a given matrix volume (data not shown).
3. $f_1 = 1.00 \pm 0.02$ for all nonelectrolytes studied and remained constant over a 5-fold range of matrix water content. The variable f_1 is independent

TABLE I
Properties of Mitochondrial Water

	Abnormal phase	Normal phase
Extent (mg H_2O/mg dry wt)	0.28 (30%)	0.68 (70%)[a]
Osmotic coefficient	inactive	0.94[b]
Partition coefficients		
antipyrine	3.0	1.0
urea	1.5	1.0
glycerol	1.0	1.0
ethanol	1.0	1.0
dimethyl sulfoxide	0.04	1.0

[a] Value given is for isolated mitochondria in equilibrium with 0.272 osmolal sucrose. The abnormal phase is estimated to be 15% of total water *in vivo*, because total water content is about 1.93 mg/mg *in vivo*.

[b] By inference.

of probe concentration, showing that Henry's Law is obeyed. Phase I may therefore be considered to be a normal phase indistinguishable in its solvent properties from the medium.

4. DMSO, antipyrine, and urea have nonzero intercepts. From Eq. (20), it may be concluded that W_2 is different from zero. That is, mitochondria contain a fraction of abnormal water that is also osmotically inactive. It is noteworthy that this finding is deduced without consideration of osmotic swelling curves.

5. We may also conclude that partition coefficients in W_2 differ for urea, DMSO, and antipyrine. The f_2s are independent of concentration, so solutes in phase 2 also obey Henry's Law.

6. Whereas the f_1s are the same for all solutes, the f_2s are solute specific. This behavior, together with osmotic inactivity, characterizes phase 2 as an abnormal aqueous phase.

7. The quantitative distribution of water between normal and abnormal phases cannot be resolved by nonelectrolyte probes; however, limits can be deduced from Fig. 6. DMSO is evidently excluded, to a considerable extent, from W_2. If f_2 were equal to zero for DMSO, W_2 would equal the negative intercept of the DMSO curve, since $f_1 = 1$. This value is 0.27 g H_2O/g dry wt, representing the *minimum* value of W_2. This is very close to the independent estimate of W_2 (0.28 g H_2O/g dry wt) from the osmotic studies of Fig. 1.

8. A further implication of these results is that matrix solutes, as well as matrix water, must be divided into osmotically inactive and active portions. That is, solutes in W_2 do not contribute osmotically to phase 1.

VII. Discussion

A. The Evidence for Aqueous Phase Separation in the Mitochondrial Matrix

The hypothesis that biological water separates into two distinct phases was introduced more than 20 years ago (Garlid, 1976, 1978, 1979) and has recently been revived (Walter and Brooks, 1995). Despite an extensive literature of studies on cell water, the experimental approach used to test the hypothesis remains unique. This approach is also powerful, because it provides decisive evidence. It was possible to exclude the alternative hypothesis that matrix water comprises a single phase, and thus prove, in a Popperian sense, that phase separation occurs. The evidence is simple and straightforward:

First, mitochondria behave as perfect osmometers, including a modest osmotic intercept (Fig. 3).

Second, the aqueous distributions of simple nonelectrolytes exhibit strikingly nonideal behavior (Fig. 5).

Third, the *combination* of osmotic swelling and solute distribution excludes a single-phase system (Fig. 6). In a single-phase model of matrix water, the slopes of Figs. 5 and 6 must be identical. They are not, and this simple fact is proof of aqueous phase heterogeneity in the mitochondrial matrix.

As a consequence of this evidence, the osmotic intercept, which constitutes a separate, osmotically inactive fraction of matrix water, may be identified as being responsible for the abnormal solute distributions.

B. The Nature of the Normal Aqueous Phase in Mitochondria

The normal phase exhibits all the characteristics of a dilute solution of potassium salts. It is a perfect osmometer, and the osmotic coefficient appears to be constant over the rather wide range studied, which is typical of a dilute salt solution. It expands and contracts with transport of salts across the inner membrane (Fig. 4). It dissolves nonelectrolytes with partition coefficients of 1.0. This behavior leads me to speculate that the normal phase contains no proteins, and that most matrix proteins are sequestered in the abnormal phase.

C. The Nature of the Abnormal Aqueous Phase in Mitochondria

1. Interpretation of Osmotic Curves and the Osmotic Intercept

Osmotic swelling curves, such as those in Figs. 2 and 3, must necessarily break down toward the origin as external osmolality approaches infinity

(dehydration). Apropos the discussion of polar solutes in solution, we infer that hydration begins at extremely high osmolalities and achieves a constant saturation value at water activities much lower than those of dilute solutions. There is considerable evidence that this is an accurate description.

Ingenious studies on hemoglobin crystals by Perutz (1946) strongly suggest the presence of "bound" water at low water activities. The quantitative relationship of this fraction to the osmotic intercept in red cells was demonstrated by Drabkin (1950). Thus, the value for bound water in hemoglobin suggests that the linear part of the osmotic curve extends very close to the y axis ($1/\phi_0 \rightarrow 0$). This is also seen in studies of red cell membrane preparations in which hydration saturates at about 0.70 g H_2O/g dry membrane (Schneider and Schneider, 1972). This works out to a layer of water with an average thickness of 50 Å, very close to the minimum value for abnormal water associated with the mitochondrial membrane, as will be discussed.

One of the most thoroughly studied systems is that of the brine shrimp, *Artemia salina,* and this brief summary cannot do justice to the valuable work of Clegg and coworkers (Clegg, 1978, 1979, 1992), who have characterized the hydration state of *Artemia* using metabolic studies, nmr, differential scanning calorimetry, and dielectric measurements. Encysted brine shrimp gastrula commonly undergo complete desiccation during their development, and the concomitant cessation of metabolism is completely reversed by rehydration. Up to 0.3 g H_2O/g dry weight, all of the water entering the desiccated cyst is used in primary hydration of proteins and membranes. Below this value, no bulk water exists. Remarkably, several metabolic pathways become active at 0.3 g/g, indicating that metabolites can diffuse within this limited water phase. Sufficient water to support full-blown metabolism is only achieved at 0.6 g H_2O/g dry weight after which further hydration has little effect.

2. Solvent Properties of the Abnormal Phase

The abnormal aqueous phase of the mitochondrial matrix exhibits widely varying solute activity coefficients (Table I). The pattern that emerges from the limited number of solutes studied is that the abnormal phase preferentially attracts solutes with excess hydrogen bond donor groups (urea, antipyrine) and excludes aprotic solutes (DMSO). Thus, abnormal water behaves as if it contains excess electron acceptor groups relative to bulk water, that is, it has properties of a Lewis base. It follows that this phase should exclude other Lewis bases, such as anions of weak acids, and should concentrate Lewis acids, including protonated acids and amines, perhaps including tetraphenylphosphonium and other probes of membrane potential.

How does a solute such as urea, which is already extensively hydrogen-bonded in the normal phase, preferentially partition into abnormal water? Transfer to a region of more structured water would be energetically unfavorable, so it is unlikely that abnormal water is icelike and extensively hydrogen-bonded. If water structure begins with hydration of hydrogen-bond acceptor groups on membranes and proteins, subsequent layers of water would have the same polarization, inhibiting formation of three-dimensional clusters. Such a structure may well have a higher entropy than that of bulk water and would tend to exhibit properties of a Lewis base, as observed. Some of these predictions could be tested by studying the effects of temperature on the nonelectrolyte distributions.

3. Location of the Abnormal Phase and Distribution of Proteins

"Abnormal water" is a phenomenological term, introduced to describe physicochemical solution properties (Garlid, 1979). "Vicinal water" is a morphological term, introduced to describe water near surfaces (Drost-Hansen, 1969). I have suggested that abnormal matrix water and its associated proteins are located next to the inner membrane (Garlid, 1976). Srere (1982, 1985) has also reviewed a considerable body of evidence indicating that matrix proteins are adjacent to the inner membrane.

Studies on a variety of systems indicate that water hydrates the surfaces of proteins and membranes, leading to rapid saturation at low vapor pressures (high osmolalities). Further increases in water activity have little further effect on this surface phase. Thus, it seems reasonable to postulate that abnormal water is vicinal water—hydration water whose structure is induced by macromolecular surfaces.

The observation that proteins are extensively hydrated in solution, and that W_h is osmotically inactive, requires that the abnormal phase contain proteins. The question is, how much? What fraction of matrix proteins associates with the abnormal phase of mitochondria?

The answer to these questions may be found in the 10-fold disparity between hydration of macromolecules in solution and hydration of cells and organelles, as revealed by osmotic behavior. If cellular proteins were randomly located in the cytosol, then hydration water could occupy all of the cell, as pointed out by Cameron *et al.* (1997). The simplest explanation for this disparity is that cellular proteins are not in solution, but rather in a quasi-solid gel phase. Thus, soluble enzymes and other proteins preferentially associate with each other, presumably because protein–protein interactions are more favorable than water–protein interactions. I favor the view that virtually all proteins in the mitochondrial matrix are associated with this phase. Similarly, virtually all proteins in the cytosol, and their

associated water, are associated with internal membrane surfaces and cytoskeleton. The effect of these interactions will be to minimize the water of hydration in cells and, thus, to minimize the osmotic intercept.

Because the hydration water behaves as a Lewis base, its time-averaged structure must differ from that of bulk water. Accordingly, the phase boundary is likely to be sharp rather than gradual.

4. The Extent of Abnormal Water in the Matrix

Taking the value of abnormal water at 0.28 g H_2O/g dry weight, I have calculated the thickness of this phase, assuming it forms a layer along the inner surface of the inner mitochondrial membrane (Garlid, 1998). I obtained two values, depending on the amount of matrix protein in this phase. The average thickness of the abnormal phase layer would be about 59 Å if it were protein-free. Its thickness would be about 189 Å if it were to contain all of matrix proteins. Because I believe that most of the proteins are associated with the abnormal phase, I favor estimates near the latter value.

D. Biological Consequences of Aqueous Phase Heterogeneity

A theme of this analysis is that proteins are segregated into a quasi-solid gel. This results in a minimization of the water of hydration, which would otherwise occupy all of the cell. There are now instances in biology which suggest that minimization of the hydration water may be variable and under regulation. Kelly *et al.* (1995) have shown that the immature frog oocyte exhibits osmotic behavior typical of most cells. The mature egg, however, is osmotically unresponsive, despite having a normal permeability to water and ions. Since the mature egg must survive in distilled (fresh) water, this is a marvelous adaptive mechanism. Having excluded other alternatives, Kelly *et al.* (1995) postulate that, during maturation, increased cross-linking of cytoskeletal polymers acts to increase the degree of cytoplasmic gelation. It is interesting to speculate that the opposite occurs; that the cytoskeleton *releases* proteins from the gel domain. This exposure of hydration surfaces would have the effect of converting all the bulk water to osmotically inactive hydration water, making the cell osmotically unresponsive and able to survive in pure water.

Similar regulatory changes appear to occur with single proteins that exist in different conformations, such as hemoglobin and the delayed rectifier K^+ channel of squid axon (Zimmerberg *et al.*, 1990; Colombo *et al.*, 1992). It is to be expected that the two conformations may have different hydration values, and these authors have devised methods for quantitating the differ-

ence. Parsegian and Rau (1984) propose that protein–water interactions (hydration) play a major role in both biological mechanisms and intracellular protein associations.

There are preliminary indications that the extent of abnormal water in mitochondria depends on the redox state of the electron transport chain. Although W_2 is normally invariant, I have observed that the uncoupler, CCCP causes a significant and reproducible reduction from 0.28 to 0.19 mg H_2O/mg protein, without changing the osmotic slope (Garlid, 1998). It would be interesting to know the effects of other membrane-localizing drugs on the osmotic intercept.

VIII. Concluding Remarks

Walter and Brooks (1995) have suggested that cellular proteins segregate into two or more phases, much like macromolecules in the test tube, as described by Albertsson (1971, 1986). Similar forces may be involved in cells, but I do not believe that proteins segregate randomly, by a purely physicochemical process. Rather, I believe that proteins undergo "channeling" of their own, on their passage from the ribosomes to their target destinations. In this view, the overwhelming majority of proteins are never "dissolved" in the bulk water of cytoplasm, but rather form a segregated domain more akin to a hydrated solid, or gel. In cytosol, this domain is associated with endoplasmic reticulum and cytoskeletal filaments. In the mitochondrial matrix, this domain is concentrated along the inner surface of the inner membrane.

Water in the hydration phase is osmotically inactive and separate from bulk cellular water. Both phases dissolve small solutes, but the partition coefficients may differ appreciably from one.

Localization of cellular proteins must be organized if bulk water is to be present in the cell. Protein localization is also a prerequisite for metabolic channeling, which appears to be ubiquitous in nature. The metabolic consequences of such microcompartmentation have been discussed extensively by Srere (1985) and Clegg (1978, 1979, 1992), and the present analysis supports Clegg's model of enzyme segregation (1978). Clegg (1992) has raised the additional questions of whether proteins are specifically localized and, if so, what determines this localization. In my view, the existence of organized enzyme complexes within the cell (Srere, 1987; Saks *et al.*, 1994) is now well established. Such assemblies would indeed require biological machinery, beyond the physicochemical forces causing aqueous phase separation, to provide the spatial organization and localization of proteins necessary for metabolic channeling.

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