

CELL-ASSOCIATED WATER

edited by

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PREFACE

This book was inspired by a Workshop, devoted to "Cell-Associated Water," organized in connection with the First International Congress on Cell Biology (Boston, September 1976). Many of the papers in this volume were read at that Workshop; in addition, a few papers have been solicited from authors unable to attend the meeting.

The state of cellular water continues to be the subject of considerable activity and controversy. For this reason, as Editors, we have stressed the desirability of each author "setting the stage" for his discussion by the presentation of appropriate review material, but apart from that we have urged each author to discuss freely his views and opinions, however controversial. As a result, the views expressed in this volume do not necessarily form a conceptually integrated overview of cell-associated water but rather offer various new insights and, in some cases, novel approaches to the topic. By the same token the Editors disclaim responsibility for the personal opinions expressed by the individual authors. On the other hand, we believe with Dr. Ralph Horne that we "would like to see scientific texts written as if the authors cared about the subject matter, as if they thought it important enough to venture an opinion. Opinions as well as facts *belong* in scientific papers, monographs, and texts." To this we may well add T. H. Huxley's observation, "It is a popular delusion that the scientific enquirer is under an obligation not to go beyond generalization of observed facts—but anyone who is practically acquainted with scientific work is aware that those who refuse to go beyond the facts, rarely get as far."

The material in this volume is primarily ordered according to the complexity of the systems examined, starting with aspects of pure colloid and surface science and ending with topics in zoogeography.

It is our hope that this volume will reach those scientists who are concerned with cell biology in general and those in particular who are concerned with the events in and the role of the aqueous phase of cells. It is also our hope that graduate students

WELCOMING ADDRESS

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The biologists of the XIX century were unable to approach the problem of the living state, being prevented to do so by their cellular prejudice. They were convinced that complex chemical processes could be performed only by complete cells. It was E. Buchner who destroyed this prejudice by showing that fermentation could take place in an assembly of dissolved molecules. Hence, present-day biology is a molecular biology. We have traded in our cellular prejudice for a molecular prejudice. Our body being built of molecules, we believe that all its reactions have to be molecular.

There can be no doubt about the wonderful achievements of the molecular approach. However, the molecular is but one of the three dimensions involved in the mechanisms of life: the molecular, the infra- and the supra-molecular. Life can be understood only by their combination.

While, in theory, there is no dividing line between these dimensions, there is a difference in the tools and methods by which they can be studied. While in the molecular dimension the main tools are chemical, in the infra- and supra-molecular dimensions the main tools are physical, like NMR or ESR. In the everyday life of the scientist there is also another difference between these dimensions. The molecular outlook being the oldest, the physical approach is not sufficiently represented on committees of granting agencies and editorial boards, which favor the molecular approach at the expense of the physical, a difference which has to be corrected urgently.

3. Maisch PA, Calderone RA. *Role of Surface Mannan in the Adherence of Candida albicans to Fibrin-Platelet Clots Formed in vitro.* *Infect Immun* 32:92, 1981.

The results indicate that cell surface mannan may play an important role in the adherence of *Candida* to the fibrin-platelet matrices which form in vivo in the endocardium of heart valves. This mannan can be shown to be an important component of a thick floccular material which is adherent to the external surface of *C. albicans* cell walls.

4. Sobel JD, Myers PG, Kaye D, Levinson ME. *Adherence of Candida albicans to Human Vaginal and Buccal Epithelial Cells.* *J Infect Dis* 143:76, 1981.

This is an in vitro study of adherence. Factors that enhanced germination and viability of the organism enhanced adherence. There were significant differences between adherence with cells of different volunteers, and adherence to buccal cells was slightly greater than to vaginal cells. Pre-incubation of the cells with certain enzymes or sugars inhibited adherence, as did precoating of the epithelial cells with lactobacilli. They believe that adherence of *C. albicans* is enhanced by a surface component of germinated yeast cells, probably a surface protein, that binds to the epithelial receptor, possibly to glycoprotein.

5. Sobel JD, Schneider J, Kaye D, Levinson ME. *Adherence of Bacteria to Vaginal Epithelial Cells at Various Times in the Menstrual Cycle.* *Infect Immun* 32:194, 1981.

They studied ten healthy sexually active medical students for adherence of *E. coli*, lactobacilli, group B streptococci, *Gardnerella vaginalis* and *N. gonorrhoeae* to isolated vaginal epithelial cells at various times during the menstrual cycle. There were no significant differences in adherence for any of the organisms at various times.

6. Lomberg H, Jodal U, Svanborg-Eden C, et al. *P1 Blood Group and Urinary Tract Infection.* *Lancet* i:551 (Letter), 1981.

The severity of a urinary tract infection correlates with the ability of the infecting *E. coli* to adhere to human uroepithelial cells and greater adherence has been recorded for patients especially prone to UTI's. The authors have suggested that glycolipids act as receptors for the organisms on cells, and that the carbohydrate sequence gal-alpha 1-4 gal is recognized by the bacteria. Many individuals carry a P1 antigen on their red blood cells which contains the specific sugar sequence mentioned. The authors considered the question that such patients might have a similar glycolipid composition in other cells such as epithelial

THE PROBLEM OF WATER STRUCTURE
IN BIOLOGICAL SYSTEMS¹

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I. INTRODUCTION

The structure of water has aroused the interest of many investigators since the turn of the century, but its highly complex nature has prevented a good understanding of its physical properties (61,49). The problem becomes even more difficult if water is structurally modified by ions or by proximity to a solid surface. Indeed, the effects of interfaces on water structure is attracting considerable attention as the results of such studies (on both pure water and aqueous solutions) must be applicable to and necessary for an understanding of the state of water in cellular systems. As long as no theoretical model exists which rigorously reflects all bulk properties of water, it appears an overwhelming task to describe water structurally as influenced by the proximity to a solid interface in terms of concrete structural parameters. Efforts made in this direction were summarized excellently by Drost-Hansen (45).

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It is commonly believed that the structure of water near interfaces is different from the bulk, this change originating from the first molecular adsorption layer which, when being attached to an underlying hydrophilic surface, is regarded to be strongly subject to restricted molecular motions. This effect is envisaged to be transmitted over many molecular diameters via hydrogen bonds deep into the bulk phase.

It is still unclear whether there is an abrupt breakdown of molecular surface orientation far from the interface, though some findings support this view (32,29). Moreover, there is a significant amount of evidence that the degree of structural change in vicinal water depends sensitively on whether the interface is more or less hydrophilic or even hydrophobic. Drost-Hansen (44) has adapted a frequently cited model for explaining the "structuring" of aqueous surface zones. In his opinion, the adjacent solid wall induces the formation of some sort of clusters, clathrate cage-like or high-pressure ice polymorphs, all being in equilibrium with monomeric water molecules. Extensive clathrate-like ordering was ascribed to the presence of hydrophobic surfaces. This means that the surface properties of water are also strongly directed into the third dimension and, therefore, display "capillary-II effects" as introduced by Shcherbakov (105). Former work in the field of anomalous behavior of vicinal water was summarized by Henniker (56).

Although it is well-known that water plays a major role in a vast number of biological processes in living tissue, this problem has until recently received little attention. It is quite clear that cells and tissues are very rich in interfaces and these must exert a marked influence on adjacent water structure. The problem of elucidating water structure in living tissue has chiefly been studied by NMR methods which permit non-destructive experiments.

Important early work in this field was carried out by Hazlewood, *et al.* (53) (see also chapter in this volume). By aid of spin-echo decay measurements, they investigated water in skeletal muscles from rats and mice and found that the intracellular water experiences restricted molecular motions. More recent work by Hazlewood, *et al.* (54) revealed that water in cells might be composed of three different fractions of non- (or slowly) exchanging water. The authors believe that the structure of all or a very large part of the cellular water is affected by the presence of

the large number of interfaces encountered within cells. This point, to be sure, is still a subject of active controversy.

Similar studies by Chapman and McLauchlan (14) lead to the observation that two types of proton environments exist in biological tissue. These findings are more or less confirmed by a number of other authors using different tissue specimens (10,18,71,46,20,9,26,79).

The NMR behavior of Na^+ ions in muscle, brain and kidney tissue has been examined by Cope (19). The results indicated that 60-70% of the Na^+ had a response time analogous to that of Na^+ complexed in an ion exchange resin. Thus, Na^+ might be considered to be complexed or associated with charged sites on macromolecules. The rest of the Na^+ ions appear to be found within the structured vicinal water. Further studies on Na^+ were carried out by Czeisler and Swift (23) and Magnuson and Magnuson (72). Corresponding tests with K^+ were made in bacteria (21). It should be mentioned that Cope treated his results in terms of semi-conduction phenomena and solid state physics. The particular importance of the NMR investigations of Damadian (24) lies in his attempts to devise a method by which malignant tumors in tissue can be detected via structured water NMR signals.

A number of studies on the structure of intracellular water is based on freezing point changes which, according to the nature of the different intracellular interfaces, are lowered to variable extents. This suggests that at least part of the intracellular water might be "anti-crystalline" in the sense advanced by Ubbelohde (109). Basic work in this field has been done by Mazur (73) and further evidence for anomalous water properties in biological systems comes from intracellular freezing patterns (98), indicating that intracellular water can undergo appreciable super-cooling. The lowering of the freezing point is commonly ascribed to the lack of intracellular nucleators of super-cooled water. Further details are summarized by Drost-Hansen (45).

Electrical conductivity studies by Carpenter, *et al.* (13) using *Aplysia* neurons suggest that the observed reduction in conductivity is somehow connected with the presence of structured water. Fernández-Morán (50) pointed out that structured water might be an important constituent of biological membranes exhibiting a number of functional properties. Particularly, the selective permeability of membranes should be controlled by modified water existing chiefly in membrane

pores. More detailed work in this field has been conducted by Schultz and Asunmaa (103) who have developed molecular models of lipid/water components of the hexagonal subunits found in plasma membranes.

The most interesting finding, however, is the observation of thermal anomalies in a vast number of kinetic processes in living tissue, including enzyme activities. These anomalies, discussed in detail by Drost-Hansen (42,43,45) occur in narrow temperature ranges around 15°, 30°, 45° and 60°C and are ascribed to structural transitions of surface-ordered intracellular water which, in turn, may exert a marked influence on the conformations of enzymes and proteins in the cell. Abrupt changes in enzyme activity near these transition temperatures point to larger energy differences between two forms of the enzyme, both being stabilized by vicinal water structure.

Long-range structuring effects represented by particularly deep surface orientation of water between two fused silica plates with extrema close to the temperatures cited above have been observed by one of the present authors (85, 87). These results support the belief that most of the thermal anomalies occurring in biological systems are due to structural peculiarities of intracellular structured water.

The cooperativity of surface-ordered water is also stressed by Ling (66-69) (see also chapter by Ling in this volume). He has advanced the "association-induction hypothesis" in which intracellular water is considered to exist as polarized multilayers which are functionally linked to a cooperative adsorption of ions on protein sites. In contrast to the classical view which postulates that ion accumulation in cells occurs by the action of ion membrane pumps, Ling provides experimental evidence that structured water is an essential ingredient in this regard by exhibiting changed solvent properties for the different electrolytes and non-electrolytes encountered in biological tissue. Excellent model investigations by Wiggins (116) with inanimate materials have strengthened the view that intracellular water structures play a major role in cellular solute distributions. The serious drawback of all these experiments lies in the fact that biological tissue has an extremely complex structure which makes it very difficult to elucidate the basic characteristics of structured water.

In order to make further progress in this field, it seems worthwhile to use solid/aqueous systems which are much simpler than biological tissue. To familiarize the reader with water research in model systems containing boundary layers, we will present next an account of the most successful methods employed for this purpose.

II. MODEL TESTS IN VICINAL WATER RESEARCH

Without doubt, a variety of measurements using different methods on physico-chemically well defined materials have provided knowledge about some of the details of water structure near interfaces.

Infrared spectroscopic methods have proven to be feasible in the case where the cuvettes used had a separation of their windows of about 10^{-3} cm. Salama and Goring (101) were interested in the temperature dependence of the intensity of the infrared absorption of water at 2100 cm^{-1} and found an inflection point near 30°C which suggests the participation of vicinal water in the absorption process.

More quantitative experiments were made by Metsik, *et al.* (77); these authors measured the infrared absorption of a water layer between mica plates in the range of the stretching vibrations of the molecules. The essential features of the results are the appearance of new vibration frequencies and the shift in the maximum for the stretching vibration band, these effects being created by surface-structured water. Related tests with aqueous copper sulfate solutions in the ultraviolet region were carried out by Peschel and Caffier (12,93) who detected abrupt changes in the temperature shift of the absorption bands near the characteristic temperatures mentioned in the foregoing section.

Nuclear magnetic resonance studies have yielded interpretable results where chemically well defined colloidal particles, suspended in water or aqueous solutions, have been examined. Pethica, *et al.* (60) investigated the T_2 relaxation times in aqueous colloidal systems containing particles of polyvinylacetate and polyvinyltoluene and detected thermal anomalies near 3° and 31°C which suggest the presence of structured water surrounding the particles. NMR results for proton T_1 relaxation times of water near minerals obtained by Woessner, *et al.* (118-122) suggest the existence of preferentially oriented water close to solid surfaces.

Agar gels are also useful model systems for proton NMR spectra, as shown by Abetsedarskaya, *et al.* (2) and Woessner, *et al.* (49); the properties of vicinal water in these cases were found to deviate only slightly from those of pure water.

The polymolecular water film adsorbed on mica sheets exhibits a decreased electrical conductivity as was shown by Metsik and Perevertaev (76); the conductivity decreases with decreasing film thickness (as determined by an optical procedure).

If the intermolecular arrangement of water molecules in boundary layers is altered by the influence of the solid surface the thermal conductivity should likewise display an anomalous behavior. Metsik and Aidanova (75) and Metsik and Timoshchenko (78) presented impressive evidence that water in the gaps between mica sheets, arranged in the form of a stack, has an unexpectedly high thermal conductivity which was considered to be due to facilitated transmission of thermal vibrations within the surface-ordered lamellae.

An ingenious test by Deryagin (27) involving the study of a thin layer of water sandwiched between glass plates showed that surface-structured water has a shear-modulus, thus exhibiting quasi-solid properties. A subsequent improvement of this method was reported by Bazaron, *et al.* (7). They used a quartz crystal vibrating at its resonant frequency in contact with a prism of fused silica. Both bodies were separated by a thin water layer, the thickness of which could be adjusted. The authors' interest was in the shift of the resonant frequency of the crystal as a function of film thickness. The point of interest for us is that this resonant frequency was increased, indicating the presence of some sort of shear elasticity. Dielectric investigations of water between sheets of mica revealed that part of the dipole orientation of water had degenerated (83). Dielectric studies of wet membranes (22) revealed that the apparent thickness of water layers attached to the membrane surfaces might extend to about 4×10^{-4} cm which is, to be sure, an impressively high value. It remains to be seen if all or part of this effect may be due to polarization effects.

More detailed studies utilizing the same system were performed by Metsik (74). He cleaved a "crystal" of mica on which had been deposited measuring and guard electrodes. The sheets were kept separated by an interlayer of water, the thickness of which was measured gravimetrically or by IR absorption. When the film thickness exceeded a value of

1.6×10^{-4} cm the dielectric constant was near 80, but when the thickness was decreased to about 7×10^{-6} cm the dielectric constant was found to be only about 4.5; thus, the orientation polarization had practically faded away. Peschel and Schnorrer (95) developed an experimental device which permits the determination of the dielectric constant of fluids in thin layers between platinum/iridium electrodes. Use of this method revealed that a number of organic liquids show a decrease in their dielectric constant within the same temperature ranges at which long-range ordering of water molecules occurs near solid/liquid interfaces. Unfortunately, tests with thin water layers have not yet been very successful because of the occasional occurrence of dielectric breakdowns at smaller gap widths.

It must be emphasized that the transport properties of liquids are usually highly sensitive to structural changes. It can be shown that the viscosity of structured water (surface viscosity) is markedly enhanced over bulk water. Churaev, *et al.* (16) measured the flux of water through glass capillaries having a radius of only 4×10^{-6} cm. The viscosity of water was elevated by 40% and showed a maximum value at about 30°C. Similar tests with capillaries of 10^{-5} cm diameter were carried out by Deryagin and Krylov (33). Peschel and Adlfinger (2,85,86) attempted to determine the altered viscosity of thin water layers confined by silica plates which were brought together by the action of a known applied external force. The viscosity of the surface-structured water was found to be appreciably enhanced and to exhibit marked extrema at about the temperatures where the disjoining pressure maxima arise.

The action of short-range adsorption forces which, via a cooperative molecular immobilizing effect, might be considered to be long-ranged, has lead to a very important observation (for thin water layers); namely, the existence of a disjoining process. This was detected for the first time by Deryagin and Obukhov (37) when pressing two planar mica plates against each other, immersed in water. The disjoining pressure observed results specifically from the overlap of extended hydration layers. The surface orientation of the water at an interface was observed to have a thickness of up to about 10^{-4} cm. The plate distance was determined via a highly precise micrometer screw.

In further experiments of this kind one of the two solid/liquid interfaces was replaced by a gas/liquid interface; in other words, a bubble of gas (hydrogen) pressed

against a smooth mica surface submerged in water. In this case also a long-range oriented film of water was observed, having an equilibrium thickness of about 3×10^{-6} cm (34,35). More recent work in this area comes from Deryagin and Zorin (39), who likewise prepared a water film between a gas bubble and a solid surface. They ascribed the disjoining pressure at larger film thicknesses to a so-called β -isotherm, and for smaller film thicknesses, to an α -isotherm. Along with this it was stated that the β -isotherm is largely determined by an ionic-electrostatic disjoining pressure component, dependent on ion concentration. The α -isotherm is due to the presence of surface-structured water forming a contact angle of about 10° with the bulk phase. This problem, part of which is within the framework of our own studies, will be discussed in some detail in the following section.

Precise model investigations of the disjoining pressure were reported by Roberts (99). In order to eliminate the serious problem of the surface roughness of solid plates even through highly polished, he pressed a rubber lens against a planar glass plate, both being immersed in electrolyte solution. In this way, the rather soft rubber surface could adapt to the protrusions of the glass surface. For a separation distance of about 2×10^{-6} cm he found a disjoining pressure of about 10^5 dyne \cdot cm $^{-2}$ which cannot result solely from electrostatic repulsion phenomena.

Recently, Israelachvili and Adams (59) have developed an ingenious device for determining the disjoining pressure of aqueous electrolyte solutions between two crossed cylindrical sheets of molecularly smooth mica. By means of an optical method, the gap width could be measured to within 10^{-8} cm. Thus far, the only results presented confirm the conclusions of the DLVO-electrostatic repulsion theory (Deryagin-Landau-Verway-Overbeek theory), but give no conclusive evidence either way for the existence of structured water.

A system of planar mica sheets separated by a thin layer of aqueous solutions was also used by Korchinsky (63). According to the idea of Deryagin and Obukhov (37) the plate distance was determined by a mechanical method. His results show that the surface orientation of water might extend over a distance of some 10^{-4} cm.

Barclay and Ottewill (6) used a clay suspension (stacks of sodium montmorillonite plates) placed in electrolyte solution, and measured the distance of separation between the plates at different pressures. It follows from their results

that at plate separations shorter than 1.5×10^{-6} cm the experimentally observed pressures were greater than those predicted by the DLVO theory. Although the reason for this effect is still somewhat obscure, a role for structured water between the clay platelets cannot be excluded.

Despite the fact that measuring the disjoining pressure in fluid layers between solid surfaces is extremely difficult, Peschel, *et al.* (4,90) devised an apparatus which allows for the analysis of disjoining pressure in layers between juxtaposed fused silica plates. One plate is planar, the other is spherically formed so that the problem of plane-parallelity is avoided. This method is the basis of a number of studies of water and electrolyte solutions which will be discussed in the following sections and which may help us to understand some new and unanticipated effects observed in experiments on biological tissue.

III. THE DISJOINING PRESSURE IN THIN LAYERS OF WATER BETWEEN IDEALIZED SURFACES

In living systems the surface-to-volume ratio is known to be high and hence, it must be expected that the structure of cellular water is notably affected. Because of the altered physical properties of water in boundary layers, thermodynamic excess quantities must be considered which, unlike the quantities in the Gibbs treatment, are directed in three dimensions and are treated as capillary-II effects (105). The reason for this anomaly is considered to be principally due to a long-range molecular immobilization extending from a solid surface and being transmitted deep into the bulk phase. If, therefore, two solid surfaces submerged in water or aqueous solutions, respectively, are brought together to sufficiently small plate separations, an interpenetration of the structured surface zones occurs and a repulsive force begins to act. This effect is known in the literature as "disjoining pressure" (or "splitting pressure") (28). To be exact, this phenomenon which is attributed solely to special modifications of liquid structure near interfaces is generally characterized as the "structural" component of the total disjoining pressure acting between closely spaced interfaces (31).

A. Theoretical Considerations

Following is outlined a simple physical model of a

structured boundary layer, confined by two solid surfaces. For the sake of simplicity, let us consider in the first instance only the structural component in order to develop some aspects of thin film thermodynamics. Two solid surfaces of unit area, submerged in water (which is assumed to form structured surface zones) are envisaged to be approached from infinity to a plate separation (h) where a repulsion becomes notable. This procedure requires the expense of free excess energy:

$$(\Delta F^E)_{s,h} = - \int_{\infty}^{h} \Pi_s(h') dh' \quad (1)$$

(The superscript "s" stands for "structural"). Unfortunately, this is an integral quantity which does not provide any comprehensive, direct information. A basic improvement is possible by imagining an infinitesimally thick lamella midway between the plates separated by a distance, h . Let us, therefore, decrease the plate distance by a differential amount, dh . The corresponding differential free energy is then given by:

$$d(\Delta F^E)_{s,h} = \frac{\partial (\Delta F^E)_{s,h}}{\partial h} dh \quad (2)$$

If, to a first approximation, the structured layer between the two surfaces is regarded as (ideally) incompressible, then the differential step expressed by equation (2) is accompanied by an extrusion of part of the structured layer into the surrounding bulk phase.

The molecules within the plane midway between the juxtaposed surfaces will be least affected by long-range surface forces. Thus, this molecular layer, having a thickness, Δh , will be expelled from the space between the plates when the gap width is decreased by Δh . For this real case, equation (2) might be rewritten:

$$\Delta(\Delta F^E)_{s,h} = \frac{\partial (\Delta F^E)_{s,h}}{\partial h} \Delta h \quad (3)$$

Hence, $\Delta(\Delta F^E)_{s,h}$ has the character of a differential free excess energy. A closer inspection of our problem reveals that by decreasing the plate separation from h to $h-\Delta h$, a slight structural change of the whole boundary layer might be caused by the resulting stronger mutual influence of the overlapping ordered zones. Strictly speaking, $(\Delta F^E)_{s,h}$ must be split into three terms:

$$\Delta(\Delta F^E)_{s,h} = \Delta(\Delta F^E)'_{s,h} + \Delta(\Delta F^E)''_{s,h} \\ + \Delta(\Delta F^E)'''_{s,h} \quad (4)$$

The first term is associated with the transition of the material from the structured lamella into the surrounding bulk liquid; the second term refers to the structural change in the remaining boundary layer. The last term is a compression term which, as may be proven, can be omitted from any further considerations. For not too close plate separations the condition

$$\Delta(\Delta F^E)'_{s,h} \gg \Delta(\Delta F^E)''_{s,h} \quad (5)$$

is met so that $\Delta(\Delta F^E)''_{s,h}$ might also be neglected. Hence, $\Delta(\Delta F^E)_{s,h}$ represents approximately the free energy which is necessary to transform the structured layer into one having bulk structure.

Conversion into molar quantities requires knowledge of the density of the structured lamella. Van Gils (110) performed experiments to determine the density of water existing as wetting layers on glass powder. His results, to be sure, can only represent crude average values which might be assigned (only) to the first molecular layers near the walls. Yet, it is of much interest that van Gils cites a density value for vicinal water of about 2.4. For the sake of simplicity, however, we use the bulk density when the condition $\Delta h \ll h$ is fulfilled (though an appreciable error might still be imposed by this procedure). In this approximation, $\Delta(\Delta F^E)_{m,s,h}$ is the molar differential free excess energy which must be spent for extruding a lamella of thickness Δh and the area $V_m/\Delta h$, from midway between the plates (having the same surface area) into the bulk. V_m is the molar volume of the liquid investigated.

From equations (1) and (3) we obtain:

$$\Delta(\Delta F^E)_{s,h} = V_m \Pi_s(h) \quad (6)$$

Extending this idea further to other thermodynamic quantities one may write:

$$\Delta(\Delta S^E)_{s,h} = - \frac{\partial \Delta(\Delta F^E)_{s,h}}{\partial T} \quad (7)$$

and

$$\Delta(\Delta U_m^E)_{s,h} = \Delta(\Delta F_m^E)_{s,h} + T\Delta(\Delta S_m^E)_{s,h} \quad (8)$$

$\Delta(\Delta S_m^E)_{s,h}$ is the corresponding molar differential excess entropy and $\Delta(\Delta U_m^E)_{s,h}$ the corresponding differential molar total excess energy for transferring the lamella in question into the bulk phase. Other excess functions can be defined in a similar way. The formulae given above can be used to express, in a rather simple manner, the thermodynamic behavior of structured liquids near interfaces.

One of the essential points that one must take into consideration is that in real cases the solid surfaces (used in experiments) are not smooth in the mathematical sense, and that the method outlined above does not cover this particular case. Hence, the next stage of approximation is a discussion of the rugosity (roughness) of real solid surfaces and the effects of surface rugosity when using equations (1) - (8).

B. The Disjoining Pressure in Thin Liquid Layers Between Real Solid Surfaces

Real solid surfaces, even though carefully polished, will always show a rather deep roughness on the molecular scale. The critical question in the present context is how the model of two juxtaposed solid surfaces, exhibiting a pattern of irregular protrusions, can be idealized to a simple geometric scheme which allows reasonable calculations. One of the present authors has shown in some detailed considerations (4,88) that the contact of two real surfaces - one planar, the other spherically formed - may be replaced by the contact of a plane and a cone. Introducing the distance-dependence of the structural component, Π_s , of the total disjoining pressure to be of the form

$$\Pi_s(\xi) = C_s e^{-n_s \xi} \quad (9)$$

we assume an exponential decay of the anomalous surface zone properties as advocated by Garbatsky and Folman (51). ξ is the distance between two surface elements facing each other; n_s and C_s are parameters to be determined from experiment. A solution was found for the structural disjoining force, dK_s :

$$\begin{aligned}
 d_{K_S} = & 2\pi C_s e^{-n_s h} \left[\frac{e^{-n_s f \cdot r_f}}{n_s^2 f^2} \right. \\
 & + \frac{R}{n_s} \exp. \{-n_s (r_g^2/2R + 2R_b)\} \\
 & \left. - \frac{1+n_s f r_g}{n_s^2 f^2} \exp. \{-n_s f (r_g + r_f)\} \right] \quad (10)
 \end{aligned}$$

In this model, h is the smallest plate separation distance in the spherical/planar system, R_b is a measure of the average surface roughness; f, r_f and r_g are parameters of the interlocking surface asperities, and R is the radius of curvature of the convex plate. By plotting $\ln \cdot k_{K_S}$ against h , a fairly straight line is obtained (4) which renders possible the evaluation of n_s and C_s and, hence, of Π_s . This quantity, in turn, is the basis for utilizing equations (1) - (8) and for deriving the thermodynamic excess data from experiment. To be sure, our method will introduce a number of errors into the calculations, but at least it allows one to obtain the right order of magnitude for the disjoining process.

C. The Apparatus

In the contacting planar/spherical plate system the load bearing area between the closely spaced plates is exceedingly small and, as a result, the experimental device for determining the disjoining pressure has to be highly sensitive in order to provide fairly reproducible results.

The main part of the instrument is depicted in Figure 1. The planar plate is fixed on the bottom of a container which can be filled with the liquid to be studied. The upper, curved plate is attached to the one end of a balance system which can be deflected by use of an electromagnetic coil. The plate separations can be measured by a displacement transducer (on the balance system) connected with a strain-gauge measuring bridge, and one side of an XY-recorder. To the other side of the recorder is applied a voltage which is proportional to the force by which the plates are pressed against each other.

The whole set-up is placed in a large thermostated vacuum chamber, into which the liquid under investigation can be sucked over a membrane filter which retains dispersed dust and colloidal particles.

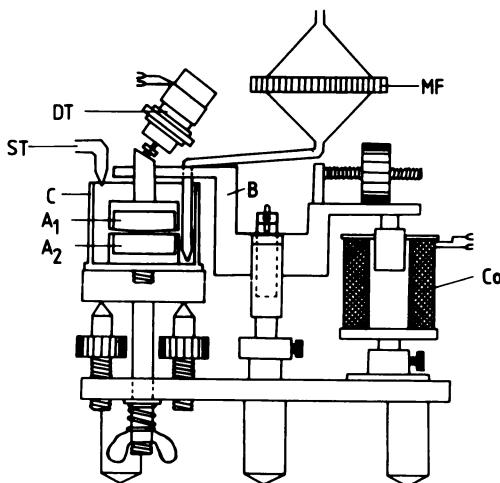


FIGURE 1. Diagram of the unit for investigating the disjoining pressure in thin liquid layers. A₁ - spherically formed silica plate; A₂ - planar silica plate; C - vessel filled with the liquid to be tested; B - balance system; Co - electromagnetic coil; DT - displacement transducer; MF - membrane filter; ST - sucking-off tube.

In this study, fused silica plates were used with a surface roughness of about $R_b = 2.5 \cdot 10^{-6}$ cm. The specific surface parameters were: $f = 4 \cdot 10^{-4}$, $r_f = -10^{-3}$, $r_g = 1.0 \times 10^{-2}$ and $R = 100$ cm.

D. Experimental Results

The disjoining forces, d_{K_s} , and the corresponding plate separations, h , were determined for pure water between fully hydroxylated, fused silica plates over the temperature range 0–75°C. The electrostatic repulsive as well as the van der Waals long-range attractive pressure component, which will be dealt with in the following section, are of minor importance in the present case and can be neglected. Π_s shows an extraordinarily complex temperature behavior, as illustrated in Figure 2, for a plate distance of 5×10^{-6} cm (87). Pronounced extrema were found at about the temperatures 15°, 32°, 45°, and 61°C just where a large number of important biological processes show distinct thermal anomalies as described by Drost-Hansen (42–45).

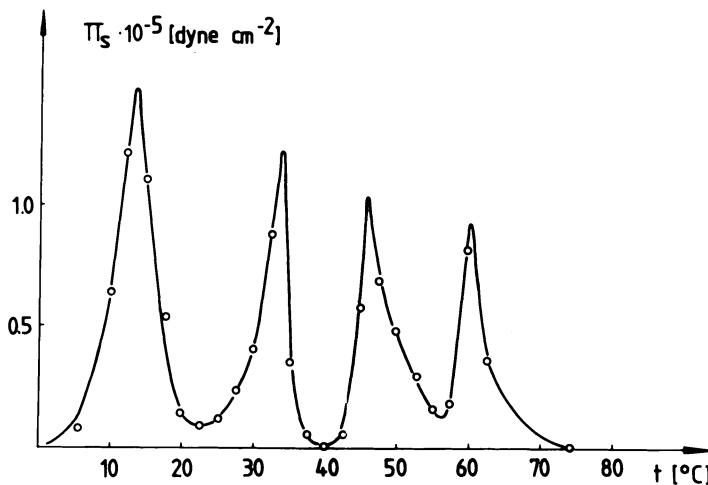


FIGURE 2. Structural disjoining pressure of pure water in thin layers between fully hydroxylated fused silica plates. $h = 5 \times 10^{-6}$ cm.

The abrupt temperature responses according to Figure 2 point to strongly cooperative phenomena in vicinal water structure. Drost-Hansen (44,45) has discussed this effect in detail and explained it as an order-disorder phenomenon which suggests the existence of structured elements of a certain size in thin aqueous layers. There are many qualitative arguments for this belief, but no simple experiment has until now substantiated this view. In order to unravel some of the problems associated with the thermal anomalies, a few additional comments will be made on the analogous behavior of other more simple liquid systems.

One of the present authors has examined the disjoining pressure (94) and the viscous behavior (89) of some benzene-halides in thin layers between fused silica plates. It is of utmost interest in connection with the extrema shown in Figure 2 that the halogen-benzenes exhibit structurally created disjoining pressure maxima over only a small temperature range, about 30–40°C above the melting point (Figure 3). These have been recognized in other experiments as ranges of structural transitions of higher order (8). Additional evidence obtained by measuring the dielectric constant of the benzene-halides in thin layers between

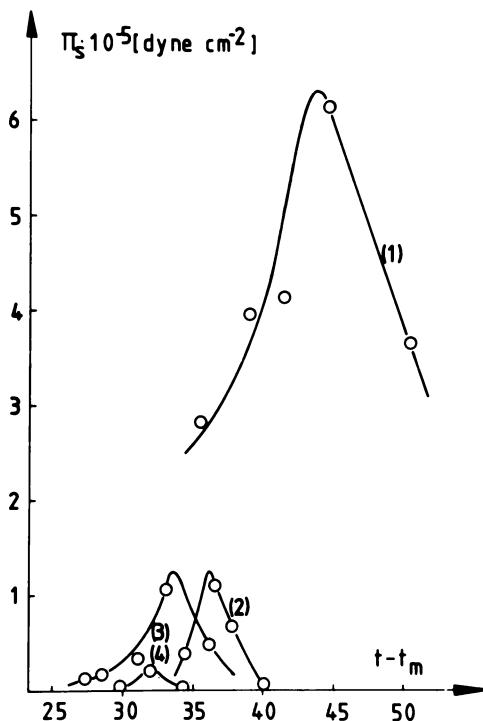


FIGURE 3. Structural disjoining pressure of (1) fluorobenzene, (2) chlorobenzene, (3) bromobenzene, (4) iodobenzene in thin layers between fully hydroxylated fused silica plates. $h = 10^{-6}$ cm; t_m melting temperature (uncorrected for the van der Waals attraction).

iridium/platinum plates suggests that on the lower temperature side of the transition region, the halogen-benzenes show a molecular long-range orientation or immobilization, respectively, near solid surfaces (95). It is assumed that the bulk phase at these temperatures is also restricted in its molecular motions so that the difference in the chemical potentials between the surface-ordered and the bulk phase is of minor importance which, therefore, leads to a negligible disjoining pressure. By further elevating the temperature the structured surface zone suffers destruction and the disjoining pressure vanishes.

Attention should be called to the fact that rotational transition in the bulk liquid phase in a distinct temperature range creates some molecular disorder so that the underlying surface can exert its influence deep into the adjacent phase. It should be emphasized that in this case the disjoining pressure extrema occur over temperature ranges in which the bulk phase likewise displays anomalies. Plotting the logarithm of the bulk viscosity against the inverse of temperature yields in each case two straight-line segments of different slope which intersect at the temperatures in question (8). In other words, there is an abrupt change in the activation energy for flow when a rotational transition occurs.

Returning to the vicinal water problem one may then attribute the disjoining pressure extrema to some sort of structural transition in the bulk phase. But precise studies carried out by Rushe and Good (100), Cini, *et al.* (17), Korson, *et al.* (64), and Senghaphan, *et al.* (104) have revealed the absence of any anomalies in the bulk phase at the characteristic temperatures. These results have seriously hampered straightforward interpretations of the thermal anomalies in vicinal water structure. Thus, there seems to remain only the concept that a solid surface influences the adjacent water structure to such an extent that a structurally changed surface phase is generated which consists of molecular entities undergoing structural transitions at about the characteristic temperatures. By this process some molecular disorder is created which, in turn, facilitates the particularly far ranging influence of the surface by decoupling the specific structure of ordinary water or aqueous solutions, respectively, in favor of forming a modified surface phase. This view, which has also been advocated by Drost-Hansen (45), is supported by the fact that in comparison to simple organic liquids the structured surface zone of water is rather thick which points to the molecular alignment of larger structural units. The essential point, and one of great importance, is that small energy changes are sufficient to lead to notable changes in water structure.

A brief comment should be made on the term "surface-ordered" water by which the reader is referred to the concept of layers of lower entropy. By inspection of Figure 4 in which the molar differential excess entropy of a lamella (from equation [7]) is plotted against temperature, one notes that in the temperature range from 15 to 23°, $\Delta(\Delta S_m^E)_{s,h}$ is positive, but in the range from 23 to 32°, it is negative. That means that in the former case the surface zone has a lower, and in the latter case, a higher entropy. Regarding the

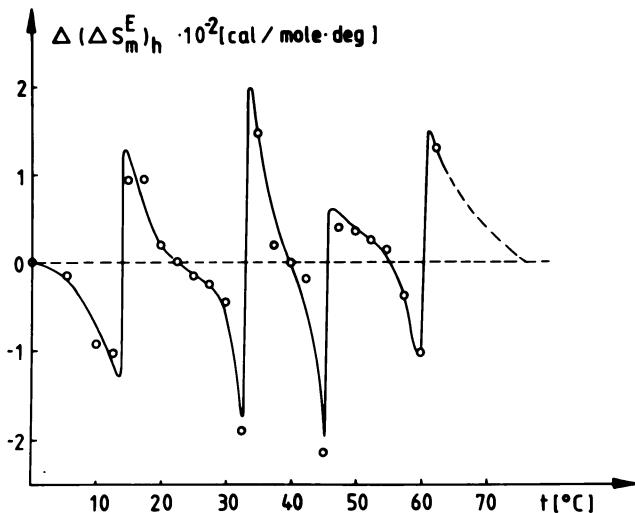


FIGURE 4. Differential molar excess entropies of pure water in a structured lamella midway between fully hydroxylated fused silica plates. $h = 5 \times 10^{-6}$ cm.

whole temperature region under consideration, ranges of lower and higher entropy alternate which might suggest it is desirable to replace the misleading term "ordered" by the more neutral term "structured".

Choosing a plate separation 5×10^{-6} cm, the values for $\Delta(\Delta U_m^E)_s,h$ of the structured lamella lie well below the mean thermal energy, RT, and attain values of the order of 10^2 cal/mole, for $h = 10^{-6}$ cm.

IV. THE DISJOINING PRESSURE IN THIN AQUEOUS ELECTROLYTE LAYERS

In these studies, we have found the disjoining pressure in thin aqueous electrolyte layers between silica surfaces to be a quantity which commonly consists of an electrostatic, a steric and a long-range van der Waals contribution. The first two components are repulsive, the latter is attractive.

A. Theoretical Considerations

When placed in aqueous electrolyte solutions, solid plates commonly attain a surface charge which, on the solution side, is faced by a diffuse layer of counter ions (82, 106,112). Hence, if two solid surfaces with an intervening electrolyte layer are pressed against each other, the diffuse layers will overlap and generate a repulsion pressure which, according to Verway and Overbeek (112) is given in the simple form

$$\Pi_{el} = 2 nkT (\cos hu - 1) \quad (11)$$

with

$$u = \frac{v e \psi h / Z}{kT}$$

where n is the number of ions per ml, k is the Boltzmann constant, v the ion valency, and $\psi h / Z$ the electric potential midway between the plates. The plate distance then takes the following form

$$h = - \frac{2}{\chi} \int_z^u \frac{dy}{[2(\cos hy - \cos hu)]^{1/2}} \quad (12)$$

with

$$y = \frac{v e \psi}{kT}; \quad Z = \frac{v e \psi_0}{kT}$$

ψ_0 is the surface potential and ψ the potential far from the surface. is the well known fundamental Huckel parameter:

$$\chi = \left(\frac{8 \pi n e^2 v^2}{\epsilon kT} \right)^{1/2} \quad (13)$$

in which ϵ is dielectric constant of the medium. This theory was improved by Stern (107) who took into account specific adsorption of ions at the solid surfaces.

Another formula for the electrostatic disjoining pressure component, which is likewise well applicable, was discussed by Deryagin and Landau (36). It was derived for a symmetrical electrolyte and surface potential, $\psi_0 > 100$ mV. However, this equation,

$$\Pi_{el} = \frac{\pi e}{8} \left(\frac{RT}{eZ} \right) \cdot \frac{1}{h^2} \quad (14)$$

proved to be less useful than equation (11) when a quantitative splitting of the different pressure components becomes necessary. Therefore, equation (11) might be preferred in our subsequent calculations.

The long-range van der Waals interaction between solid plates whose separation distance is much smaller than their thickness manifests itself as an attractive pressure given by

$$\Pi_{vw} = - \frac{A}{6\pi h^3} \quad (15)$$

if for not too large plate separations electromagnetic retardation effects are neglected. "A" is known as the Hamaker constant which is sensitively dependent on the plate material and the intermediate layer and can be derived from literature data. For precise computations the non-uniform physical properties in structured layers have to be taken into account. Initial attempts in this field were reported by Vold (113) and Churaev (15).

The steric or solvation component, Π_s , of the total disjoining pressure is still a matter of controversy. Details are summarized by Ottewill (80). The existence of a "solvation pressure" in aqueous electrolyte layers seems to be established, but evidence is not yet unequivocal. Undoubtedly a great number of tests will be necessary to substantiate the view that surface-structured water is also encountered in electrolyte systems despite the strong influence of ions on water structure. According to equations (9), (11), and (15), superposition of all three pressure components gives the total disjoining pressure in thin electrolyte layers:

$$\Pi_t = 2n KT (\cos hu - 1) + C_s e^{-n_sh} - \frac{A}{6\pi h^3} \quad (16)$$

Particular interest attaches to the separation of all three components and, above all, the quantization of the solvation pressure. Evidence given by Barclay, *et al.* (5) and our precise tests permit the conclusion that for low electrolyte concentrations, electrostatic repulsion effects become already notable at relatively large gap widths, while steric repulsion due to surface-structured water arises steeply at much smaller plate separations.

In order to attack the problem of term separation (90) we have to realize that for not too close plate distances the potential, $\psi h/2$ midway between the plates will be small. For this case, equation (11) simplifies to the exponential form

$$\Pi_{el} = 64 n KT \gamma^2 e^{-\chi h} \quad (17)$$

with

$$\gamma = \frac{e^{Z/2} - 1}{e^{Z/2} + 1}$$

which, analogous to equation (9), can be given the form

$$\Pi_{el} = C_{el} e^{-n_{el} h} \quad (18)$$

with

$$C_{el} = 64 n KT \gamma^2 \text{ and } n_{el} = \chi$$

More detailed considerations about this point will be found in a separate paper (91).

For sufficiently low electrolyte concentrations the relation $n_{el} < n_s$ or even $n_{el} \ll n_s$ will hold. In other words, for large plate separations, Π_{el} is predominant, while Π_s dominates at rather close distances. The attractive forces acting between the plates reach large values only at very close plate separations so that in practical cases dealing with larger plate separations, Π_{vw} might be neglected.

B. The Separation of the Disjoining Pressure Components

If, according to equation (10), the total disjoining pressure force, dK_t , for aqueous NaCl solutions at different concentrations is plotted against a distance coordinate along the axis of the two approaching plates, the graph depicted in Figure 5 results. The concentration was varied between 10^{-4} and 10^{-2} m. In each case, two linear portions of the plots are notable. The more flat branches refer to the electrostatic, the steep branches to the solvation pressure component. From these plots numerical values for Π_{el} and Π_s can be deduced.

A related problem has been discussed by Deryagin and Churaev (30). Their considerations are based on previous experiments (35,39) in which an air bubble was pressed against a solid surface submerged in aqueous electrolyte solutions. As stated in the foregoing, by measuring the total disjoining pressure, a β -isotherm was obtained for larger film thicknesses, and an α -isotherm for sufficiently thin films. The corresponding diagram is shown in Figure 6. Calculations

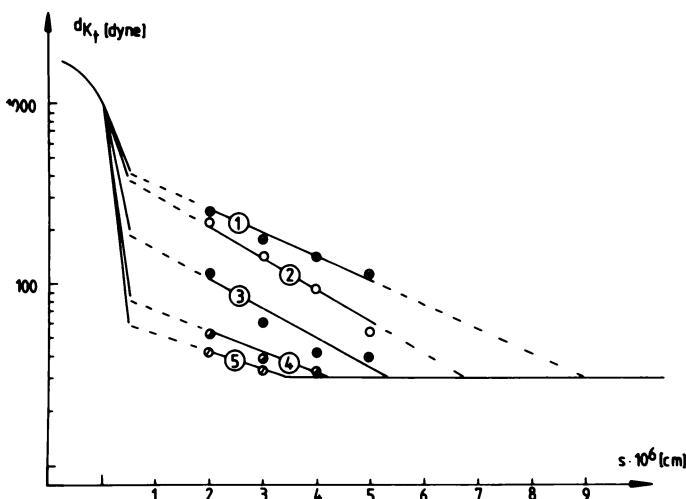


FIGURE 5. Total disjoining force in thin layers of aqueous NaCl solutions between partially hydroxylated fused silica plates plotted against an arbitrary distance coordinate. [1] 10^{-4} m; [2] 3×10^{-4} cm; [3] 10^{-3} m; [4] 3×10^{-3} m; [5] 10^{-2} m; $t = 20^\circ C$.

lead to the conclusion that the stability of thin water films ($h < 1.1 \times 10^{-6}$ cm) can only be determined by a structural pressure component, Π_s , which is ascribed to the α -isotherm. This makes it necessary to associate the flat branches in Figure 5 with β -isotherms, and the steeper ones with α -isotherms. But there is need for further studies to substantiate this view.

It must be emphasized that the data for the electrostatic component are in accord with results computed by aid of the electrostatic theory, when setting $\psi_0 = -150$ mV.

C. Experimental Results for Different Aqueous Electrolyte Solutions

The total disjoining pressure in thin layers of a number of different aqueous electrolyte solutions between fully or partially hydrated, fused silica plates was measured over the concentration range 10^{-4} m to 1 m. The most instructive results were obtained with solutions of the alkali metal halides.

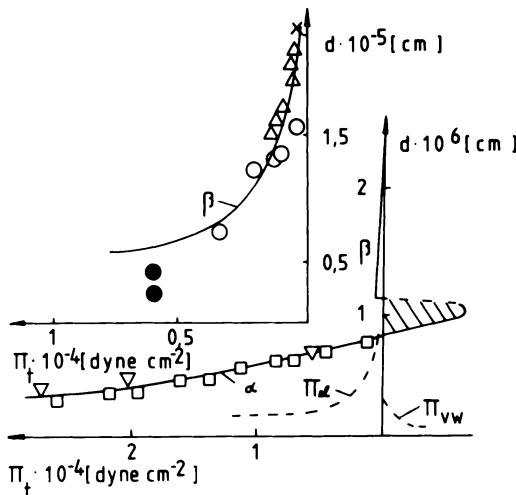


FIGURE 6. Isotherm of the total disjoining pressure for a water film on a glass and a quartz surface. O water on fused glass; Δ 10^{-4} m NaCl on glass; O 2×10^{-5} m KCl on quartz; ∇ water on K-8 glass; [] and X water on quartz. d = film thickness, $t = 20-25^\circ\text{C}$. (From Deryagin and Churaev, [30]).

1. *The Structural Disjoining Pressure Component in Thin Layers of Alkali Metal Halide Solutions.* By using the device illustrated in Figure 1 the structural disjoining pressure component in thin layers of aqueous solutions of LiCl, NaCl, KCl, LiBr, NaBr, KBr, and LiI, NaI, KI was derived from experimental data as depicted in Figures 7-9. All measurements were done with fully hydroxylated fused silica plates. Attention is called to the concentration profiles of Π_s in the different graphs. Generally, three extrema arise which cannot yet be treated by any comprehensive theory.

An obviously reasonable approach to the problem seems to be possible from the work of Vaslow (111). For a number of alkali metal chloride solutions Vaslow found a break in the concentration dependence of the partial molal volumes of these salts in the concentration range 0.1-1 m, just where we have detected disjoining pressure maxima. To be exact, the breaks varied from 0.81 m in KCl solution to 1.11 m in LiCl solution.

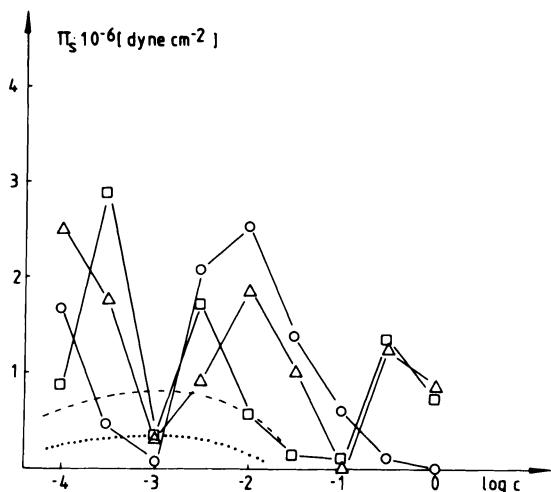


FIGURE 7. Structural disjoining pressure in thin layers of aqueous alkali metal chloride solutions between fully hydroxylated fused silica plates. \circ LiCl; Δ NaCl; $[\square]$ KCl; (----) calculated electrostatic component; (....) improved calculation according to Stern (107). $h = 10^{-6}$ cm, $t = 20^\circ\text{C}$.

Unfortunately, our disjoining pressure data are not sufficiently closely spaced for precise correlation.

The nature of these transitions is not yet fully understood. There is little doubt that extended hydration spheres in form of clusters imbedding the ion suffer a structural change via some cooperative mechanisms, if the average distance between the ions approaches a critical value corresponding to a critical concentration.

It is well known that Frank and Wen (47) proposed a two-zone hydration model for cations in aqueous solution. The inner hydration sheath is composed of oriented and, hence, immobilized water molecules, while the outer hydration envelope is believed to have a broken structure because of the misfit between the first hydration sphere and surrounding bulk water structure. But this method does not cover some interesting findings. Because of the strong pressure dependence of electrolytic conductivity, Horne and Birkett (58) have postulated a three-zone hydration atmosphere surrounding cations (Figure 10), whereby the inner structure-enhanced

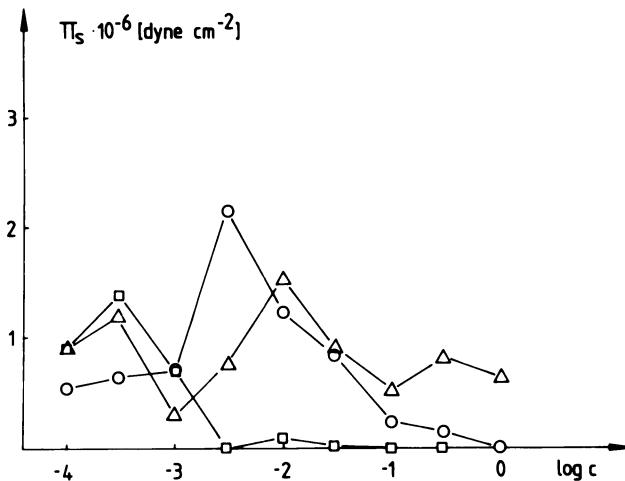


FIGURE 8. Structural disjoining pressure in thin layers of aqueous alkali metal bromide solutions between fully hydroxylated fused silica plates. \circ LiBr; Δ NaBr; \square KBr; (----) calculated electrostatic component.
 $h = 10^{-6}$ cm, $t = 20^\circ\text{C}$. (Uncorrected for the van der Waals attraction).

hydration envelope is composed of two subzones. In their concept the inner hydration region is, as in the Frank-Wen case, electrostricted but the subsequent zone presents a cluster of hydrogen bonded water molecules with a higher specific volume.

Inspection of the hydration behavior of Na^+ showed that the number of water molecules in the total structure-enhanced hydration-envelope is unexpectedly high and approaches that necessary to form a Frank-Wen cluster in pure water at the same temperature. From tabulated data (100) one notes that the structure-enhanced sheath of Na^+ -ions contains as many as 52 H_2O molecules at 5°C and 34 H_2O molecules at 20°C . To be sure, the hydration number for the Na^+ ion varies in the literature from 2 to 70 according to the crudeness of the actual method applied.

The view of Horne and Birkett (58) fits excellently into the concept of Vassilow which requires rather large ionic hydration sheets. When with increasing ion concentration the hydrogen atmospheres get overlapped it seems reasonable to

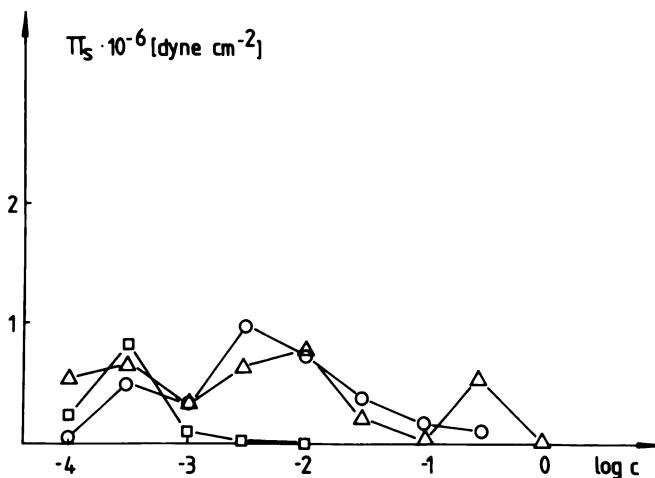


FIGURE 9. Structural disjoining pressure in thin layers of aqueous alkali metal iodide solutions between fully hydroxylated fused silica plates. \circ LiCl; Δ NaI; \square KI; (----) calculated electrostatic component. $h = 10^{-6}$ cm, $t = 20^\circ\text{C}$. (Uncorrected for the van der Waals attraction).

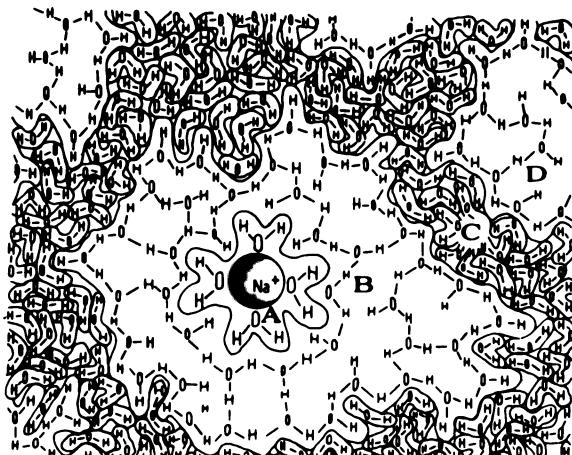


FIGURE 10. Water structure near a sodium ion. (Reprinted with permission from Horne, R. A., In: "Water and Aqueous Solutions", R. A. Horne (ed.), p. 259, J. Wiley & Sons, 1972).

assume the occurrence of some molecular disorder which, according to our findings for a number of organic dipolar liquids (3,94) should support the formation of a long-range structured surface zone. Inspection of Figures 7-9 shows that this is actually the case in the concentration range around 0.5 m. K^+ is shown to form less stable hydration clusters and, therefore, apparently produces the greatest molecular disorder and most extended structured surface zone. Investigating this point by thermodynamic-statistical methods Outhwaite (81) assumes a structural transition to take place in electrolyte solutions in the concentration range around 1 m.

Regarding the ions used in our tests the ordering influence of the cations on water structure predominates over that of the anions.

The extrema around 10^{-2} m need no introduction of new mechanisms for their interpretation. In the present case the fused silica surfaces were negatively charged (all electrolyte solutions investigated having neutral pH values). The zero point of charge for silica lies at low pH values of 2 to 3 (84).

The negatively charged silica surface exerts an electrostatic attraction on the cations within the adjacent solution. This effect leads, as is commonly known, to the formation of an electric double layer (112). But our special interest lies in the fact that cations get particularly concentrated in the "outer Helmholtz layer" (OHP). Calculations utilizing the Boltzmann distribution law show that the critical concentration discussed in the foregoing can be attained in the OHP, far from the surface, when an electrolyte concentration of about 10^{-2} m prevails. Under these conditions most of the water in the region of the OHP is associated with adsorbed, and thereby stabilized, hydration clusters which might serve as a matrix for further immobilization of water molecules deep into the adjacent fluid phase.

But this idea has a sound basis only if the extrema present reflect, at the same time, the structure changing properties of the ions solved. The lithium ions, which strongly enhance the surrounding water structure, actually show the highest extrema at about 10^{-2} m when the species of anion is kept constant. In contrast to lithium or sodium ions, potassium ions break water structure and, therefore, create a less stable matrix in the region of the OHP and a minor disjoining pressure, respectively. Theoretical considerations

related to the present problem stem from Plesner (96) who computed the concentration of ions adsorbed on the surface as a function of the electrostatic potential applied to the surface. He postulates the occurrence of an abrupt phase transition in the electric double layer which is accompanied by a closer packing of the counterions.

At very low electrolyte concentrations some uncommon effects which also do not conform to the electrostatic DLVO theory remain to be explained. The disjoining pressure for water is about 3×10^5 dyne cm $^{-2}$ for $h = 10^{-6}$ cm and $t = 20^\circ\text{C}$ when fully hydrated fused silica surfaces are used. If, for example, KCl is added (Figure 7) the disjoining pressure rises and falls off again at about 3×10^{-4} m. This effect is less pronounced for NaCl and is not noticeable for LiCl. The occurrence of the maximum at very low concentrations might be caused by the superposition of two functional dependencies. On the one hand, addition of electrolyte by disturbing the water structure could facilitate the influence of the underlying solid surface on vicinal water structure; on the other hand, by increasing electrolyte concentration the structuredness of the boundary layer is diminished since it is affected by the hydrating action of the ions present.

The uncommon splitting effect found at about 0.5 m is also indicated from the work of Deryagin and Rabinovich (38) who by pressing two crossed platinum and gold wires submerged in electrolyte solution against each other determined the force barrier for getting metallic contact. The barrier turned out to be strengthened on the order $\text{Li}^+ < \text{Na}^+ < \text{K}^+$.

In Figure 11, additional results for LiCl, NaCl, and KCl solutions are depicted. These were obtained by using fused silica plates partially hydroxylated. All disjoining pressure effects are markedly smaller, but the concentration profiles agree with those of the former tests.

An excellent proof of our concept is that the extremum at about 10^{-2} m is shifted to the higher concentration side if the silica surface is partially discharged by lowering the pH (92).

We have to emphasize that the excess enthalpy or excess energy, respectively, of structured boundary layers near interfaces is only low when being compared with RT so that it is far too rigorous and might therefore hamper our understanding of the solvation phenomena near surfaces. It seems reasonable, however, to make use of the average thickness,

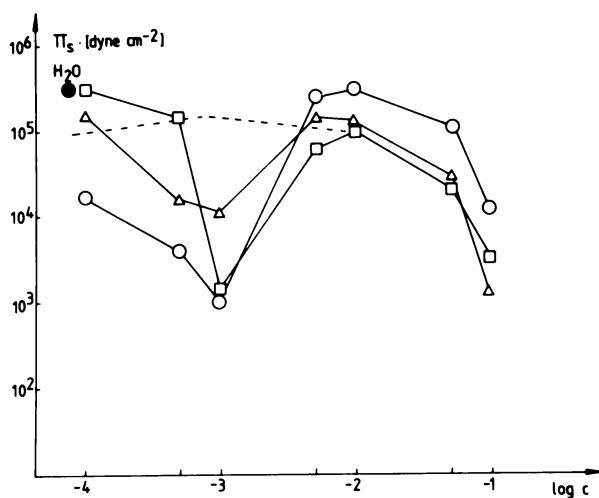


FIGURE 11. Structural disjoining pressure in thin layers of aqueous alkali metal chloride solutions between partially hydroxylated fused silica plates. \circ $LiCl$; Δ $NaCl$; $[\square]$ KCl ; (----) calculated electrostatic component. $h = 10^{-6} \text{ cm}$, $t = 20^\circ\text{C}$. (Uncorrected for the van der Waals attraction).

$1/n_s$, of the solvation layer between two juxtaposed surfaces. This quantity turned out to lie on the order of about 30–80 Å for pure water, for aqueous electrolyte solutions still lower values on the order of 20–25 Å were found. This corresponds to about 3–4 molecular layers of water per one surface which is in excellent agreement with the view of many specialists in the field of electrolyte double layer structure.

2. *The Disjoining Pressure in Thin Layers of Aqueous Ammonium and Tetraalkylammonium Halide Solutions.* Analogous tests as described in the foregoing were carried out with a number of aqueous ammonium and tetraalkylammonium halide solutions in thin layers between fully hydroxylated fused silica surfaces. The observations are depicted in Figures 12–14.

The NH_4^+ ion shows structure breaking properties similar to those of the K^+ ion. As a consequence, the disjoining pressure values agree closely with those of the KCl solution. F^- is a strongly structure enhancing ion; hence, it produces

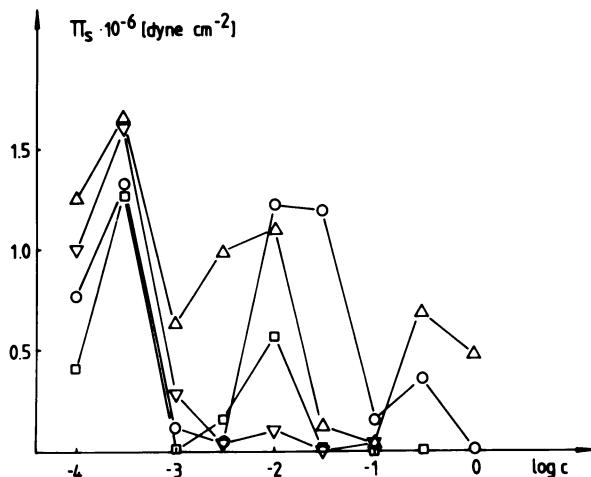


FIGURE 12. Structural disjoining pressure in thin layers of aqueous ammonium halide solutions between fully hydroxylated fused silica plates. \circ NH_4F ; Δ NH_4Cl ; \square NH_4Br ; ∇ NH_4I . $h = 10^{-6} \text{ cm}$, $t = 200^\circ\text{C}$. (Uncorrected for the van der Waals attraction).

the highest extremum at about 10^{-2} m . Essential from the standpoint of our concept is the inversion of the disjoining effects for NH_4Br and NH_4I .

At this point it seems certain that the peculiar hydration behavior of ions present in the solution must be associated with this problem. An explanation for this unexpected phenomenon can be derived from knowledge of the specific mobilities of water molecules near ions as studied in detail by Samoilov (102). His approach is based on two quantities affected by the motion of the closest water molecules in the electrolyte solutions. The time, t_i , denotes the average time that a water molecule spends in the space near the ion. Imagining a water molecule in pure water and regarding analogous motions we are faced with the self-diffusion phenomenon with the relevant quantities t and E , the latter being the energy of activation for the translational step. Samoilov assigns special interest to the quantities t_i/t and ΔE_i with $E_i = E + \Delta E_i$. According to his model two cases are possible. For structuring enhancing ions we have $E_i > 0$ and $t_i/t > 1$; for structure breaking ions there is $E_i < 0$ and $t_i/t < 1$.

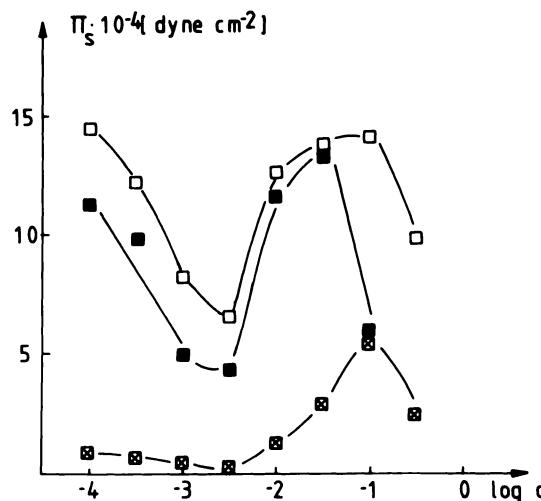


FIGURE 13. Structural disjoining pressure in thin layers of aqueous tetramethylammonium halide solutions between fully hydroxylated fused silica plates. [□] tetramethylammonium chloride; [■] tetramethylammonium bromide; [●] tetramethylammonium iodide; $h = 10^{-6} \text{ cm}$, $t = 20^\circ\text{C}$. (Uncorrected for the van der Waals attraction).

For the halogen-anions, Samoilov presents the following results:

TABLE I. Values of ΔE_i ; and t_i/t at 21.5°C

<i>Ion</i>	ΔE_i (kcal/mole)	t_i/t
Cl^-	-0.21	0.70
Br^-	-0.89	0.51
I^-	-0.24	0.66

Actually, Br^- and I^- hydrate negatively and show a reverse order in their hydration properties. Regarding our findings this is likewise manifested by NH_4Br and NH_4I in the concentration range 10^{-4} to 10^{-3} m . It is hardly possible to present a satisfactory explanation for this observation which is unlike that found for the alkali metal ions. Possibly, when choosing a strongly structure breaking cation (e.g., the

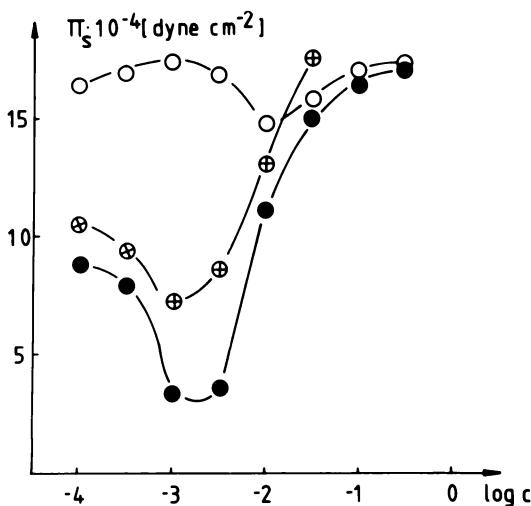


FIGURE 14. Structural disjoining pressure in thin layers of aqueous tetraethylammonium halide solutions between fully hydroxylated fused silica plates. O tetraethylammonium chloride; \bullet tetraethylammonium bromide; \bullet tetraethylammonium iodide. $h = 10^{-6} \text{ cm}$, $t = 20^\circ\text{C}$. (Uncorrected for the van der Waals attraction).

ammonium ion) the specific hydration behavior of the anions might become predominant.

This view is corroborated by the results found for tetramethyl- and tetraethylammonium halides. For the tetraalkylammonium halides the disjoining pressure values follow the normal structure breaking order $\text{Cl}^- < \text{Br}^- < \text{I}^-$. For tetramethylammonium ions the inversion with respect to the bromides and iodides becomes essential (Figure 13). It is not at all clear what has happened in this case, although there is overwhelming evidence that the $(\text{CH}_3)_4\text{N}^+$ ion is a structure breaker. However, for $(\text{C}_2\text{H}_5)_4\text{N}^+$ ions, the structure making and breaking properties cancel (11,62,65,123) so that the arguments presented above might hold. These findings moreover, implied that we were faced with a range of structural transitions around 1m which, however, had not yet been a subject of investigation. In order for such a transition to take place, we had to take for granted that the alkylammonium cations possess larger hydration spheres. In this regard, Pottel and Lossen (97) have provided hydration numbers (16 and 21) for methyl and ethyl ammonium ions. The

hydration numbers reported by Hertz, *et al.* (57) are somewhat higher. Therefore, we believe that our concept is physically reasonable, though it needs substantiation by further research. These data allow a close correlation with our findings that the long-range structure of vicinal water in electrolyte solutions is sensitively dependent on the net structure changing properties of the salts added to the solution.

3. Temperature-dependent Anomalies in Thin Layers of Aqueous Electrolyte Solutions. In a previous paper the existence of thermal anomalies in structured water layers were substantiated in terms of a series of disjoining pressure extrema occurring at characteristic temperatures (Figure 2). Using alkali-metal chloride solutions instead of pure water, and partially hydroxylated fused silica plates, impressive results were obtained as shown in Figures 15-17.

The most significant common feature of all three solutions is the almost traditional existence of disjoining pressure extrema at about 30-32°C as found in layers of pure water (Figure 2). These results clearly demonstrate the

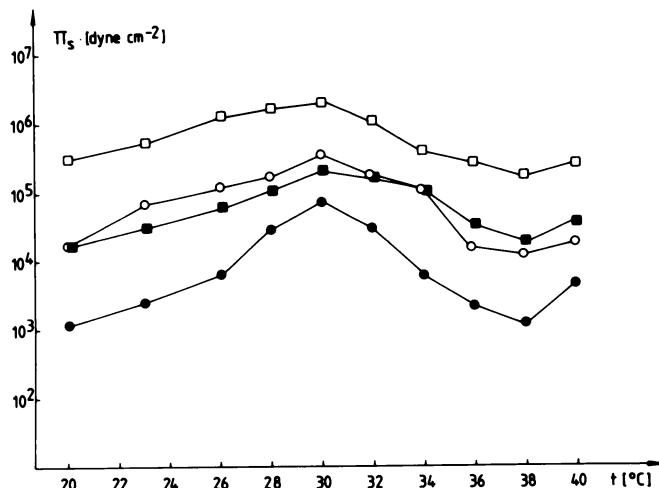


FIGURE 15. Temperature dependence of the disjoining pressure in thin layers of aqueous LiCl solutions between partially hydroxylated fused silica plates. $0 10^{-4} \text{ m}$; $\bullet 10^{-3} \text{ m}$; $[□] 10^{-2} \text{ m}$; $[■] 10^{-1} \text{ m}$. $h = 10^{-6} \text{ cm}$. (Uncorrected for the van der Waals attraction).

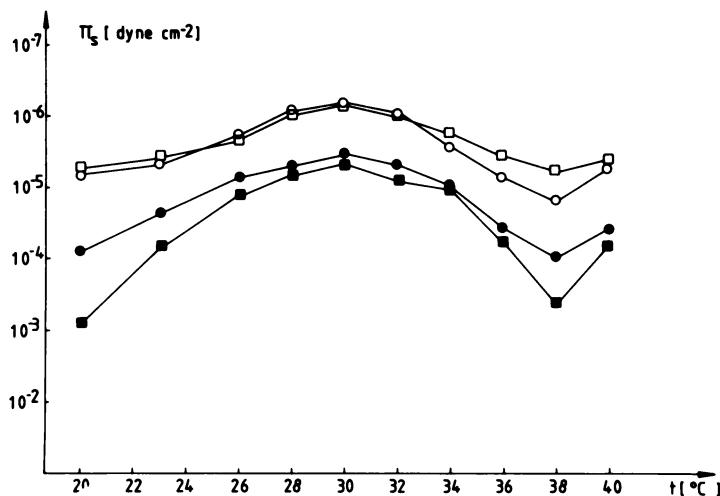


FIGURE 16. Temperature dependence on the disjoining pressure in thin layers of aqueous NaCl solutions between partially hydroxylated fused silica plates. $\circ 10^{-4}$ m; $\bullet 10^{-3}$ m; $\square 10^{-2}$ m; $\blacksquare 10^{-1}$ m. $h = 10^{-6}$ cm. (Uncorrected for the van der Waals attraction).

persistence of structural arrangements of water even in concentrated electrolyte solutions. A further noticeable effect is the occurrence of a second extremum which arises in LiCl solutions at about 34°C, and in KCl solutions at about 26°C. Since lithium ions are known to be water structure "enhancers" one would anticipate a structural transition of the water held by the hydration spheres somewhere above 32°C. Hence, the extremum at about 34°C might be related to such a transition. Potassium ions are structure breakers and their hydration envelopes, which have only a minor stability, might undergo a transition below 32°C. The generation of a pronounced maximum at about 26°C is consistent with this view.

The sodium ions are known to affect water structure to only a slight extent. It is reasonable to suppose that in this case the two maxima in question are closely spaced and are practically superimposed at about 32°C.

It should be noted that for LiCl and KCl solutions both extrema are clearly discernible only at sufficiently low electrolyte concentrations. In this context it is worth mentioning that Harned and Owen (52) presented a graph in

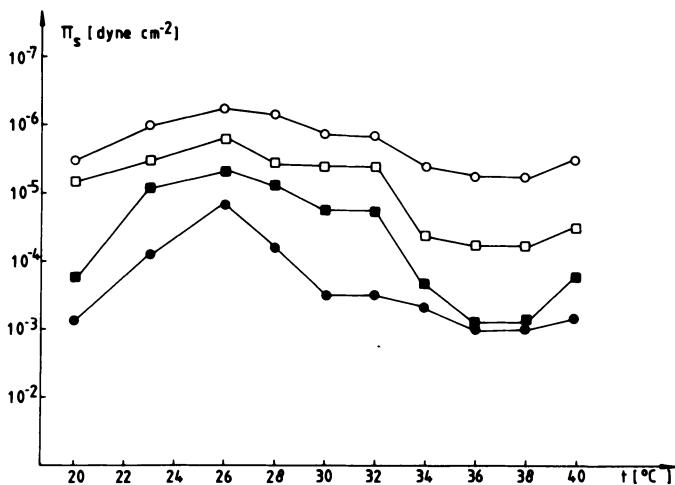


FIGURE 17. Temperature dependence of the disjoining pressure in thin layers of aqueous KCl solutions between partially hydroxylated fused silica plates. $\circ 10^{-4}$ m; $\bullet 10^{-3}$ m; $\square 10^{-2}$ m; $\blacksquare 10^{-1}$ m. $h = 10^{-6}$ cm. (Uncorrected for the van der Waals attraction).

which the activity coefficient of NaCl in bulk aqueous solution is plotted against temperature for various NaCl concentrations. Apparently abrupt changes are visible at about 30°C.

V. THE DISJOINING PRESSURE IN THIN LAYERS OF AQUEOUS UREA SOLUTIONS

The studies of electrolyte solutions in thin layers revealed intrinsic complications which derive from the electrostatic interaction between the ions and the charged solid surface. Hence, the disjoining pressure in thin layers of aqueous nonelectrolyte solutions should exhibit a less complex concentration profile. Figure 18 shows a study of aqueous urea solutions. Starting from the conditions for pure water the structuring of the surface zone gets smaller with increasing urea concentration and vanishes at about 10^{-3} m. At concentrations of about 1 m, or somewhat higher, a maximum seems to arise which might be due to a structural transition in the bulk solution when the hydration spheres

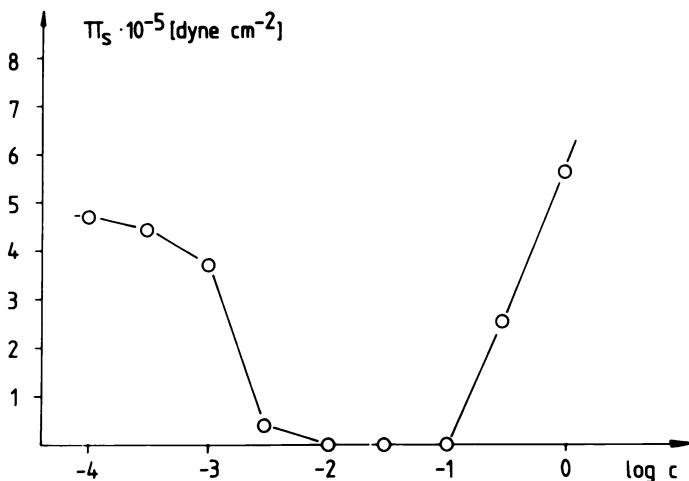


FIGURE 18. Structural disjoining pressure in thin layers of aqueous urea solutions between fully hydroxylated fused silica plates. $h = 10^{-6}$ cm, $t = 20^\circ\text{C}$. (Uncorrected for the van der Waals attraction).

overlap. Direct evidence for such a process is, however, still lacking. It is rather difficult to detect such a transition since urea is known to be only moderately hydrated; however, viscosity measurements might be useful in this regard.

According to Franks (48) urea behaves as a statistical structure breaker and, therefore, should have a marked destroying influence on surface-structured water. With regard to electrolyte layers, we have to state that the extremum, otherwise existent at about 10^{-2} m, is absent in the case where electrostatic interactions are of no importance.

VI. ION DISTRIBUTION BETWEEN BULK AQUEOUS SOLUTION PHASE AND LAYER

From what is known now it seems that surface-structured water represents a fluid phase showing individual properties such as an altered solvent power for ions, particularly for "structure makers".

Wiggins (116) carried out an excellent series of model experiments in which silica gel was equilibrated with mixtures of aqueous NaCl and KCl solutions. Analysis after equilibration was reached revealed that water in the pores of the gel, which was believed to be structured water, exhibited pronounced selectivity effects for potassium ions relative to sodium ions. Further support for the concept of potassium ions being accumulated in surface-structured water could be derived from measuring the temperature dependence of ion selectivity. At about the characteristic temperatures found for water near silica surfaces (Figure 2), Wiggins (117) detected extrema for the ion selectivity coefficient introduced in her paper.

The significant results of Wiggins have been confirmed by Hurtado and Drost-Hansen (this volume). This problem is of utmost interest in biology since ion distribution between extracellular and intracellular water is commonly found to be highly asymmetric. Most cell types have relatively high concentrations of potassium ions which, in the light of recent evidence, might be due to a large extent to the particular structure of intracellular water being subject to the influence of many intracellular surfaces. The present authors have attacked this fascinating problem by using the disjoining pressure method previously outlined. When using mixed electrolyte solutions we are faced with a multicomponent system whose description requires a more complex theoretical background. A conceivable situation is one where the layer consists of n components, each of which contributes to the structural disjoining pressure operative in the layer. Let us, with regard to the present case, deal with an aqueous electrolyte solution containing $(n-1)$ electrolyte components. By generalizing equation (16) the total disjoining pressure in this layer having a thickness, h , is given by

$$\Pi_t(h) = \Pi_{t,s}(h) + \Pi_{t,el}(h) + \Pi_{vw}(h) \quad (19)$$

i.e., the sum of the solvation, electric and van der Waals components.

The free excess energy for extruding the lamella (discussed in Section III) into the bulk phase might be defined by

$$\Delta(\Delta F)^E_t = -\Pi_t(h)\Delta h \quad (20)$$

Then

$$\Delta(\Delta F_t^E)_{t,s} = [\Pi_{t,e1}(h) + \Pi_{vw}(h) - \Pi_t(h)]_{\Delta h} \quad (21)$$

describes the steric portion of the free energy for extrusion which, on the other hand, can be written

$$\Delta(\Delta F_t^E)_{t,s} = \sum_{v=1}^n s_{nv} \Delta(\Delta F_{vm}^E)_h + \Delta(\Delta F_o^E)_h \quad (22)$$

where $\Delta(\Delta F_{vm}^E)_h$ is the partial differential molar free excess energy of the n^{th} component in the structured lamella. s_{nv} is the molar number of the v^{th} species present in this lamella. $\Delta(\Delta F_o^E)_h$ is a differential term independent of the electrolyte concentration and evidently associated with the influence of the solid wall on the boundary layer. For not too close plate distances one may put as a first crude approximation $s_{nv} = n_v$, where n_v is the molar number of the v^{th} component of the lamella if this would display bulk structure.

The quantity $\Delta(\Delta F_{vm}^E)_h$ can easily be derived from equation (22) by differentiation

$$\Delta(\Delta F_{vm}^E)_h = \frac{\partial \Delta(\Delta F_t^E)_{t,s} h}{\partial s_{nv}} \quad (23)$$

in which, by regarding equation (21) Π_{e1} is computed according to equation (11). Π_{vw} , in an approximate manner, can be derived from equation (15).

Figure 19 describes the concentration profile of the disjoining pressure of NaCl (a) and KCl (b) solutions, respectively, which in a more crude form is a characteristic noted in Figure 7. The outstanding feature of this plot is the minimum in the vicinity of 0.2 m which agrees with the ionic strength usually assigned to cell fluids (40). From this picture it follows that living tissue avoids large structuredness of water by establishing convenient electrolyte concentrations. For the sake of clarity, the experimental data are omitted from the diagram.

Moreover, two additional plots describe the splitting behavior of mixed electrolyte solutions. In case (c) the NaCl concentration of 0.1 m is kept constant and the concentration of KCl is varied in the range 5×10^{-2} up to 6×10^{-1} m; in case (d) the concentration of 0.1 m KCl is kept constant and the concentration of NaCl is varied from 5×10^{-2} up to 6×10^{-1} m. The electrostatic splitting pressure is negligible in this range and is, therefore, discarded.

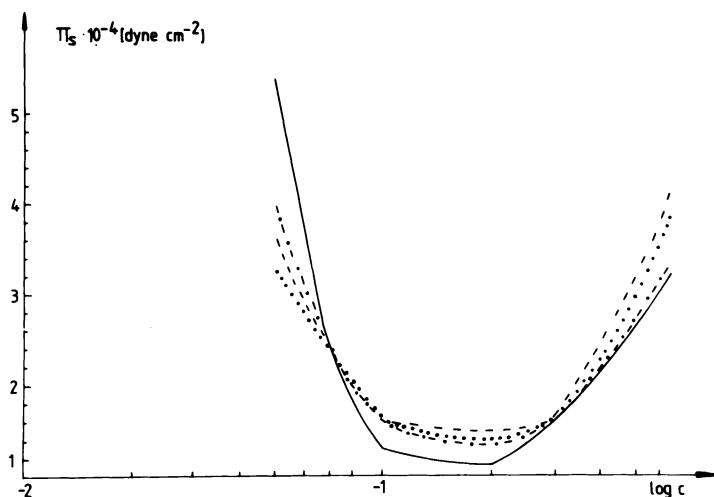


FIGURE 19. Structural disjoining pressure in thin layers of aqueous NaCl, KCl, and mixed NaCl/KCl solutions between partially hydroxylated fused silica plates. (—) NaCl; (---) KCl; (-.-.) KCl, 10^{-1} m kept constant, NaCl varied between 5×10^{-2} and 6×10^{-1} m; (....) NaCl, 10^{-1} m kept constant, KCl varied between 5×10^{-2} m and 6×10^{-1} m. $h = 10^{-6}$ cm, $t = 20^\circ\text{C}$. (Uncorrected for the van der Waals attraction).

It is interesting to note that KCl solutions produce the higher disjoining pressure at physiologically relevant concentrations. In order to analyze the graph in detail let us select a mixed solution with 0.1 m NaCl and 0.1 m KCl, having the same ionic strength as an 0.2 m KCl solution. When comparing both the disjoining pressure values they surprisingly turn out to be almost identical.

These striking results can only be explained by assuming that the generation of the splitting effect must be due exclusively to KCl enriched in the boundary layer with NaCl being expelled from this region. This is just the effect observed by Wiggins (116) in her extensive adsorption studies of mixed electrolyte solutions on silica powder, and confirmed by Hurtado and Drost-Hansen (this volume).

Estimations of the quantity $\Delta(\Delta F_{\text{vm}}^E)_h$ for $h = 10^{-6}$ cm, $\Delta h = 10^{-7}$ cm, and $c = 0.1$ m lead to values of the order -1 cal/mole for NaCl, -2.6 cal/mole for KCl and +1 cal/mole for water. The negative sign points to a decrease in the dis-

joining process with increasing electrolyte concentration. These estimates also indicate that the presence of KCl in the structured layer, at the ionic strength considered, might be energetically favored compared with NaCl. But attention must be drawn to the fact that, at lower concentrations, NaCl accumulates in the structured surface zone. This effect has, to our knowledge, not yet been reported by other authors and, therefore, requires confirmation.

VII. CONCLUDING REMARKS

As long as bulk water structure remains a matter of debate, the problem of vicinal water structure cannot likely be solved satisfactorily. The often used term "structured" is obviously a paraphrase for the phenomenon of alignment of distinct geometric arrangements of water molecules near interfaces. In the case of electrolyte solutions, direct evidence for the presence of clathrate-like hydration clusters near interfaces could be obtained. The extended hydration spheres of the ions might mostly be latently present in the bulk solution, but immediately near a solid wall they become, via adsorption processes, energetically stabilized so that disruptions are less probable. More distance from the interface the excess energies reach average values far below the mean thermal energy as was proven for pure water in thin layers (87).

Regarding this point one should recall that many authors in the field of water structure in cells have raised the question as to the proportion of intracellular water that is actually structured. We think this question can only be answered if a distinct lower limit for the total excess energy is fixed. Below this level, intracellular water might be considered to be "free". Therefore, it will be a matter of opinion regarding statements about the amount of "structuredness" of intracellular water. Our disjoining pressure apparatus is sensitive enough to detect total excess energies as low as about 1 cal/mole. Choosing this value as the lower limit we have the feeling that all the water within cells could be modified in its structure. Highly schematized models for water structure near polar or ionic and non-polar solid surfaces, respectively, were reported by Drost-Hansen (44). He believes that water molecules near polar surfaces are subject to dipole-dipole interactions which give rise to a long-range oriented layer. This zone is followed by a disordered one for which conclu-

sive experimental evidence is still lacking. Farther away from the surface, the structure of ordinary bulk phase is predominant. According to Drost-Hansen, water exists in the form of clathrate-like entities near hydrophobic surfaces. The misfit between the structure of the boundary layer and the bulk seems to be of minor importance, since the disordered zone is regarded to be only small.

Unfortunately, the disjoining pressure is a thermodynamic equilibrium quantity and allows no conclusions with respect to the specific structure of vicinal water; only thermodynamic excess functions are accessible to experiment.

The model studies in the present work have been conducted with hydrophilic surfaces which might simulate macromolecular surfaces in tissue fairly well. There is little question that cellular biopolymers analogous to inanimate silica surfaces are covered with a relatively thick hydration sheath, the formation of which seems to be favored if the hydrogen-bonding sites on the macromolecular surfaces fit the regular water lattice. Certainly we have to anticipate a feedback process in that water, in turn, impresses its structure on the conformation of polypeptide chains or other polymers. This view is held by Warner (114). Generally, the existence of vicinal water is deduced from the occurrence of thermal anomalies arising at the characteristic temperatures (see Section III). Some impressive examples are illustrated by Drost-Hansen (45).

That these anomalies are actually created by structural transition of vicinal water appear to be clearly established by the study of Peschel and Adlfinger (87) which has been firmly corroborated by the excellent work of Dreyer, *et al.* (41) who determined the diffusion coefficient of thiourea in water using capillaries and a pulsation method.

Since living cells are rich in interfaces it is evident that discrete effects at the characteristic temperatures should be concomitant in a great number of intracellular processes. Part of these effects are reviewed by Drost-Hansen (45). The body of evidence has grown so large that any comprehensive coverage lies beyond the scope of the present paper. But it is worth pointing out that many bacteria show a temperature dependence of their growth rate which strongly resembles the profile depicted in Figure 2 (87). Marked minima in growth are found where disjoining pressure maxima arise. Moreover, enzyme activities usually show a break in their activation energies at about the characteristic temperatures indicating a transition from one confor-

mation to another stabilized by vicinal water structure. (See also the article by Etzler and Drost-Hansen in this volume).

If surface active biopolymers which are known to produce vicinal water are accumulated at the liquid/gas interface, the surface tension of this solution should also be affected at the temperature in question and display anomalies. This phenomenon was actually detected by Heinzelmann, *et al.* (55) who measured the surface tension of a cell-free "leakage" medium of *Saccharomyces cerevisiae* (Figure 20). Similar findings for bovine serum albumin solutions, confined by a hexane phase were reported by Lobos and St.-Pierre (70).

The immense importance of intracellular structured water in the transfer of solutes between the extracellular and intracellular space has not been widely accepted since the concept of specific membrane pumps for solutes is widespread,

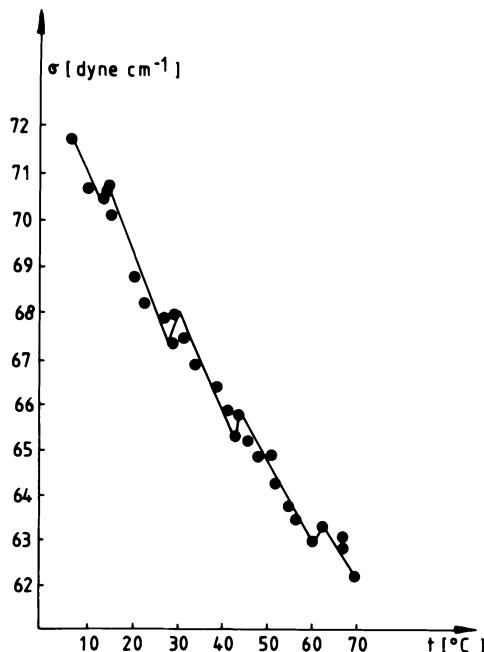


FIGURE 20. Surface tension of cell-free leakage medium of *Saccharomyces cerevisiae* vs. temperature. (Reproduced with permission from Heinzelmann, *et al.* [55]).

and hampers more detailed research on alternative explanations (66). The question arises as to whether solute transport via cell membranes actually represents an "active" transport as defined by current views based on the functional role of membrane pumps. In the light of recent evidence, the driving force for transport is the difference in the free energy of hydration of a solute outside and inside the cell. A measure for this difference according to our work is the disjoining pressure.

Some interesting views about this problem have been advanced by Wiggins (this volume). Her work referring to the intracellular pH (115) likewise deserves attention since her findings are compatible with the assumption of extended water structuring in cells which changes the dissociation constant of weak acids. It is natural to speculate on the dissociation constant of structured water which should be less than 10^{-14} .

There is surely urgent need for detailed research in the field of vicinal water utilizing comprehensible model systems as much as possible. This has been emphatically stated by Tait and Franks (108) who have excellently outlined a number of problems associated with the structure of water in biological systems.

NOTE ADDED IN PROOF

Much of the controversy in the field of vicinal water research is still centered on the question of whether there is a reliable method for determining the effective thickness of stagnant water layers at solid/liquid interfaces. This problem was reviewed in a recent paper by Lyklema (123) who, by an electrokinetic approach, argued that there are no convincing indications for the existence of immobilized water sheaths exceeding more than a few molecular layers. He particularly outlines that evidence presented by retarded viscous flow of aqueous solutions in thin capillaries might chiefly be ascribed to counter-electro-osmotic flow. An important argument against a great variety of experiments is that repulsion forces in boundary layers which are believed to stem from structuring processes have been obtained via the algebraic sum of electrostatic and van der Waals forces. This way of procedure, to be sure, should be rejected if a direct proof of solvation effects were possible.

Our experimental approach might be satisfactory since, apart from the structural disjoining process, even the electrostatic one could be independently determined and compared with theory. The central question left partially unanswered is the possible occurrence of gel formation on the surface of the silica plates used. We, therefore, performed a number of tests (124) with high-grade polished rutile plates which are commonly believed to show no swelling effects on their surfaces when being in contact with aqueous systems. The disjoining pressure results were hardly different from those obtained with silica surfaces which indicates that all our results might reflect genuine structuring effects in boundary layers.

Further support of our view, that the disjoining pressure extrema mostly found at an electrolyte concentration of about 10^{-2} m are produced by electrostatic effects, is given by a number of further tests (124) carried out with silica and rutile plates that were placed in aqueous electrolyte solutions having pH values which rendered the plate surfaces uncharged. Under these conditions the extrema at about 10^{-2} m vanished, but those which occur at about 0.5 m and are derived from another mechanism were maintained without notable change.

Clifford (125) in an article devoted to water in thin films discusses boundary water structure in detail. His point of view is that long-range structuring in vicinal water can become important when water molecules can form stronger hydrogen bonds to surface groups than among themselves and that the influence of a surface on water structure might extend up to about 100 Å.

Berendsen (126) assumes that hydrogen bonding sites on the surface of an adsorbent can stabilize water structure nearby if the sites do fit well into the regular water lattice.

Conway (127) in a particularly careful study of hydration phenomena at interfaces presents some objections to thick structured water layers. He believes that appreciable structure changes should occur only over the first 2 to 4 molecular layers. But this is just the conclusion we have drawn from our experiments when evaluating the average thickness of stagnant water layers in aqueous solutions near surfaces.

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DOES WATER PLAY A ROLE IN THE
STABILITY OF THE MYOFILAMENT LATTICE
AND OTHER FILAMENT ARRAYS?

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I. INTRODUCTION

Numerous biological systems appear to consist of ordered parallel arrays of long protein filaments: myofilament lattice, actin network in non-muscular motile cells, and tobacco mosaic virus gels, for instance. The equilibrium of these ordered arrays most likely involves similar types of forces. It is widely agreed that each protein filament possesses a net charge on its surface and is surrounded by a diffuse layer of counter-ions. Interfilament water is considered to be purely passive and to possess the properties of bulk water. Under these conditions, it is possible to calculate the electrostatic repulsive forces and the Van der Waals-London attractive forces exerted on each filament. A stable equilibrium of the filament array is assumed to result from the equality of these two kinds of forces. In 1976, however, Parsegian and Brenner (40) clearly showed that the balance between these two forces, calculated on the basis of the usual theory, cannot explain the properties of the gels of tobacco mosaic virus (TMV). We show here that the usual concepts are also unable to describe the equilibrium of the myofilament lattice and that it is necessary to introduce additional concepts to explain the stability of the sarcomere and, in a more general way, the stability of all filament arrays.

In 1968, Elliott (24) presented a complete theoretical study of the electrostatic and Van der Waals-London energies in hexagonally packed polyelectrolyte systems, and of the equilibrium of the myofilament lattice. His study was based on application of classical concepts. He showed that a stable equilibrium could be achieved under the action of the electrostatic and Van der Waals-London forces. However, he considered that the ionic strength of the interfilament aqueous solvent was only 0.015, a value which is significantly lower than the physiological ionic strength (usually taken to be about 0.15) (26). The calculations of Elliott (24) therefore, considerably over-estimate the repulsive forces and we present here calculations more related to the physiological data. We study in the present paper the case of the myofilament lattice in the absence of overlap between the thin actin filaments and the thick myosin filaments. We have, indeed, recently proposed a model of muscle contraction in which the cross-bridges attached to the thin filaments exert restoring forces on the latter (38). In order to eliminate the influence of these restoring forces, it is necessary to have no attached cross-bridges and therefore, no overlap between both types of filaments (for more details on the ultrastructure of the myofilament lattice, see, for instance, references 36, 37).

II. THE USUAL CONCEPTS ARE UNABLE TO PREDICT A STABLE EQUILIBRIUM

According to Elliott (24), the Van der Waals-London energy of a filament (E_w) and its electrostatic energy (E_e) can be written, on the basis of the usual concepts proposed by Deryaguin and Landau (18) and Verway and Overbeek (47) (DLVO theory):

$$E_w(c) = -v\lambda \frac{3\pi A}{20} \Psi_w(c) \quad (1)$$

$$E_e(c) = +v\lambda \frac{16}{\pi} \left(\frac{kT}{\epsilon}\right)^2 D \frac{R}{\ell} \gamma^2 \Psi_e(c) \quad (2)$$

where: c - center-to-center distance of the filaments; v - number of neighbors of each filament (3 for the actin filaments and 6 for the myosin filaments in a frog sartorius muscle); γ - length of the filament (1 μm for the actin filaments and 1.6 μm for the myosin filaments in a frog sartorius muscle); A - Hamaker constant; k - Boltzman constant; T - absolute temperature; ϵ - charge of the electron;

D - dielectric constant of the aqueous solvent; R - radius of the filament; $\lambda = \tanh(z/4)$, with $z = \epsilon \Psi_0/kT$, where Ψ_0 is the electric potential on the filament surface; and ℓ - Debye length defined by (24) as follows:

$$\ell = \left(\frac{8\pi\epsilon^2 N}{1000 D kT} \right)^{-1/2} I^{-1/2} \quad (3)$$

where N is the Avogadro number and I the ionic strength of the aqueous solvent. $\psi_w(c)$ and $\psi_e(c)$ are defined by (24):

$$\begin{aligned} \psi_w(c) &= \int_{\rho=c}^{\rho=R} \frac{r}{\rho} d\rho = C + R \int_{r=c}^{r=R} \frac{r}{r} dr = \theta + R, \quad \frac{\rho}{r^4} \cos^{-1} \left(\frac{c^2 + \rho^2 - R^2}{2 \rho c} \right) \\ &\quad \cos^{-1} \left(\frac{\rho^2 + r^2 - R^2}{2 \rho r} \right) dr d\rho \end{aligned} \quad (4)$$

$$\psi_e(c) = e^{-\frac{c}{\ell}} \int_0^{\frac{\pi}{2}} \cos \theta e^{-\frac{2R}{\ell} \cos \theta} d\theta \quad (5)$$

The Van der Waals-London force (F_w) and the electrostatic force (F_e) which act on a filament are given by (10):

$$F_w(c) = - \frac{\partial E_w}{\partial c}(c) = + v \lambda \frac{3\pi A}{20} \frac{\partial \psi_w}{\partial c} \quad (6)$$

$$F_e(c) = - \frac{\partial E_e}{\partial c}(c) = - v \lambda \frac{16}{\pi} \left(\frac{kT}{\epsilon} \right)^2 D \frac{R}{\ell} \gamma^2 \frac{\partial \psi_e}{\partial c}(c) \quad (7)$$

$$D \frac{R}{\ell} \lambda^2 \frac{\partial \psi_e}{\partial c}(c) \quad (7)$$

Let c^* be the center-to-center spacing corresponding to the physiological equilibrium of the filament lattice. At this particular spacing we necessarily have:

$$F_w(c^*) + F_e(c^*) = 0 \quad (8)$$

from which we immediately deduce (see relations 6,7):

$$v \lambda \frac{3\pi A}{20} = v \lambda \frac{16}{\pi} \left(\frac{kT}{\epsilon} \right)^2 D \frac{R}{\ell} \gamma^2 \frac{\frac{\partial \psi_e}{\partial c}(c^*)}{\frac{\partial \psi_w}{\partial c}(c^*)} \quad (9)$$

At the equilibrium center-to-center distance c^* we have thus:

$$\frac{E_w(c^*) + E_e(c^*)}{kT} = v\lambda \frac{16}{\pi} \frac{kT}{\epsilon^2} D \frac{R}{\lambda} \gamma^2$$

$$[\psi_e(c^*) - \psi_w(c^*)] \frac{\frac{\partial \psi_e}{\partial c}(c^*)}{\frac{\partial \psi_w}{\partial c}(c^*)} \quad (10)$$

The sarcomere volume is approximately constant in a frog sartorius muscle and we have $Lc^2 \sim 1.25 \cdot 10^6 \text{ nm}^3$ (L :sarcomere length). The absence of overlap is obtained at $L = 3.6 \mu\text{m}$ and the corresponding values of c^* are:

Actin filaments		$c^* = 19 \text{ nm}$
Myosin filaments		$c^* = 33 \text{ nm}$

$\psi_w(c^*)$, $\psi_e(c^*)$, $\partial \psi_w / \partial c(c^*)$ and $\partial \psi_e / \partial c(c^*)$ are calculated numerically from relations (4) and (5) (see Table I), by assuming the following values: $R = 3 \text{ nm}$ for the actin filaments; $R = 7 \text{ nm}$ for the myosin filaments; $I = 0.15$, $T = 298^\circ\text{K}$ (25°C); $D = 78.5$. We hence obtain:

$$\text{Actin filaments } \frac{E_w(c^*) + E_e(c^*)}{kT} = -4.2 \cdot 10^{-3} \gamma^2 \quad (11)$$

$$\text{Myosin filaments } \frac{E_w(c^*) + E_e(c^*)}{kT} = -1.1 \cdot 10^{-5} \gamma^2 \quad (12)$$

γ^2 being by definition less than unity (see above), the energy corresponding to the stable equilibrium of both the actin and the myosin filaments is therefore considerably lower than the thermal energy kT . We conclude that the usual DLVO theory applied here is insufficient to predict the equilibrium of the myofilament lattice. As pointed out by relation (8), the filaments are in principle in equilibrium at the distance c^* , but this equilibrium is easily displaced by the thermal motion. No stable equilibrium can thus be predicted on the basis of the DLVO theory and this result is clearly in contradiction with the experimental data, which show that the myofilament lattice is well ordered under all circumstances studied *in vivo*.¹ On the

¹If instead of the approach of Elliott (24) we make use of the approach of Brenner and McQuarrie (10) and Parsegian

other hand, relation (9) permits the calculation of the Hamaker constant, A, by introducing the numerical values of $\partial\psi_e/\partial c(c^*)$ and $\partial\psi_w/\partial c(c^*)$. We get the following values:

$$\begin{aligned} \text{Actin filaments: } A &= 1.5 \cdot 10^{-15} \gamma^2 \text{ erg} \\ &< 1.5 \cdot 10^{-15} \text{ erg} \end{aligned} \quad (13)$$

$$\begin{aligned} \text{Myosin filaments: } A &= 5.4 \cdot 10^{-19} \gamma^2 \text{ erg} \\ &< 5.4 \cdot 10^{-19} \text{ erg} \end{aligned} \quad (14)$$

These values are considerably lower than the physical values of A, which vary between 10^{-14} and 10^{-11} erg (6,10,24,27,47). This result is another argument showing that the application of the DLVO theory to the myofilament lattice leads to serious difficulties. It is obviously possible to refine the calculation of E_w and E_e , but these refinements should lead only to secondary modifications in the values of these energies. Thus, we can hardly expect that such refinements should be sufficient to get correct values of the potential wells [$E_w(c^*) + E_e(c^*)$], say 2 to 10 kT. We can even expect that these refinements should lead to lower values of the potential wells. Indeed, according to Mitchell, Ninham and Richmond (34) the introduction of the retarded Van der Waals interactions, for instance, leads to lower values of $E_w(c^*)$ when $c^* > 10$ nm, which is the case for the actin and myosin filaments.

III.DISCussion ON THE ROLE OF THE INTERFILAMENTARY WATER

In their paper, Parsegian and Brenner (40) also observed that the usual concepts are unable to predict the behavior of TMV gels and they concluded: "We do not believe that the long-range protein attraction is a strong determinant of order in either viral or cellular protein arrays". This conclusion is a possible one, but we think that the situation is probably much more complicated. We cannot dismiss the role of the electrostatic and Van der Waals-London forces without a detailed analysis. It is well known, indeed, that pH and

and Brenner (40), and if we assume a surface area of 4 nm^2 per ionizable group on the filaments (10), we approximately obtain the same values for the potential wells [$E_w(c^*) + E_e(c^*)$]/kT $\sim 10^{-4}$ to 10^{-6} . Our conclusion is hence independent of the mathematical treatment.

ionic strength play an important role in the filament spacing of the myofilaments as well as of the TMV rods (9,45,46). This strongly suggests that the electrostatic, and most likely, Van der Waals-London forces, play a role.

We think, however, that the usual theories present a serious weak point. In the case of the myofilament lattice and most of the other biological systems, water represents 70 to 80% of the mass. Usual theories do not account for the particular properties of this interfilamentary water. We consider that this water most likely does not play only the role of an inert solvent. The myofibrillar lattice is indeed a succession of water-solid interfaces (the solid surfaces being those of the filaments). Thus, we think that the interfilament water has properties similar to those of the vicinal water found near solid surfaces, and therefore, different from the properties of bulk water.

The presence of vicinal water (and, more generally, vicinal liquids) with modified properties near solid surfaces has been proposed many years ago by several authors. In 1913, Hardy (28) proposed that liquid layers on solids can have unexpected properties and Bangham and Razouk (5) deduced from their experiments that the polymolecular layers adsorbed on a solid do not simply have the properties of the bulk liquid and that many layers are necessary before true bulk-liquid properties are reached. Since those studies, and especially since the experiments of Deryaguin (16) and Deryaguin and Obuchov (19), much evidence has accumulated that solid surfaces can indeed alter the structure of an adjacent liquid layer (17,20,21,22,23,31,32,35,39,44; see also other chapters in this volume). Such influences occur up to thicknesses > 100 nm (1,23). This phenomenon is assumed to be caused by the solid surface inducing a molecular long-range orientation in the vicinal liquid. Vicinal water near solid surfaces has very specific properties, different from those of bulk water and we think that these specific properties play an important role in the surface effects. This is also the opinion of Pethica (42), who wrote:

"... To the extent that, in dilute electrolyte systems, the long-range nature of the surface effects due to the electrical double layers and Van der Waals' forces is commonly accepted, the issue is not simply one of the distance over which surface forces act in liquid layers, but rather as to whether or not the liquid itself can play a more direct role than that of providing a fluid

dielectric with convenient dispersion characteristics. In principle, the situation can be covered by using more refined two-force models. Such models will take account of saturation effects, ion volume, dipole terms, asymmetry of polarizability, etc., and hence they will include more directly the involvement of the solvent."

Some of the most interesting experimental findings which support the hypothesis of long-range ordering in the vicinal liquids have been obtained from studies of the effects of temperature variations on the properties of boundary layers. Many authors have indeed shown that temperature variations play a major role in the behavior of boundary layers near a solid surface (1,4,11,12,13,43,48; see also Peschel and Belouschek, this volume). Now, temperature variations play a minor role in the classical DLVO theory, where water plays a passive role, but a major role in any model explicitly involving long-range ordering in the vicinal liquid (42). The usual theories cannot, therefore, predict the behavior of the boundary layers and it is necessary to admit the existence of vicinal water, whose properties are strongly affected by temperature variations (20,21,22).

It is very tempting to apply results obtained by physical chemists to biological systems. We propose that the interfilament water which exists in the myofibrillar lattice, and in other filament arrays, is indeed vicinal water, with properties different from those of bulk water. Unfortunately, the presence of vicinal water in muscle and other tissues is not definitely proved, although many authors have provided evidence for the presence of oriented water molecules in muscle and other cells (7,8,14,15,25,29,30). The weak point of these studies is that they all make use of a unique technique (nuclear magnetic resonance), which is apparently insufficient to provide unequivocal proof for the presence of vicinal water. Other kinds of experiments have, however, shown that a noticeable fraction of the myofibrillar water is osmotically inactive and thus strongly bound to the myofilaments (3). Finally, we have seen above that the DLVO theory, which considers water as a passive solvent having the properties of bulk water, is unable to predict a stable equilibrium of the myofilament lattice. This theoretical result is, therefore, further indication that interfilament water plays an active role in the stability of the myofilament lattice and, most likely, in the other filament arrays found in biology.

The problem now arises as to the exact role of this interfilament water. In a previous paper (38) we have considered, as a simplifying hypothesis, that polymolecular layers of vicinal water are strongly bound to the filaments and that these layers exclude the K^+ counter-ions. The interfilamentary water located beyond these bound water layers is assumed to be able to dissolve the K^+ ions. The actual radius of the filaments is therefore greater than the measured radius and the diffuse layers of K^+ counter-ions are closer to each other. This possibility has also been evoked by Miller and Woodhead-Galloway (33) and is compatible with the opinion of Wiggins (49), who considers that "all ions might be expected to have diminished solubility in cell water". In a more recent paper, Wiggins (50) has experimentally found that water structure-making ions (H^+ , Li^+ , Na^+ , Mg^{++} , Ca^{++}) are to some extent excluded from the vicinal water present in the pores of a silica gel and that water structure-breaking ions (K^+ , Rb^+ , Cs^+ , Cl^-) are to some extent accumulated in vicinal water. Our hypothesis, which considers that the K^+ ions are excluded from the vicinal water layers surrounding the filaments seems therefore to be in contradiction with the findings of Wiggins (50). Nevertheless, this contradiction is only an apparent one (see Appendix).

With such a proposed structure, the problem of the stability of the myofilament lattice can be easily solved. In the case of an actin filament, for instance, surrounded by a strongly bound water layer excluding K^+ ions and having a thickness of 4 nm, the actual radius of the filament is $R' = R + 4 \text{ nm} = 7 \text{ nm}$. The interfilament water beyond the strongly bound water layer being assumed, as a first approximation, to have the dielectric constant of bulk water, we can apply the DLVO theory to the filament of radius R' and we get:

$$\frac{E_w(c^*) + E_e(c^*)}{kT} = 165 \gamma^2 \quad (15)$$

The electric potential, ψ_0 , on the surface of the bound water layers being most likely of the order of 50 to 100 mV, we have $\gamma^2 \sim 0.2$ to 0.6 and thus:

$$\frac{E_w(c^*) + E_e(c^*)}{kT} = -30 \text{ to } -100 \quad (16)$$

The equilibrium of the filament lattice becomes, therefore, very stable and cannot be displaced by thermal motion, which is compatible, of course, with the experimental observations.

TABLE I. Calculations Dealing with Electrical Potentials

	$\psi_e(c^*)$	$\psi_w(c^*)$	$\psi'_e(c^*)$	$\psi'_w(c^*)$	$\psi'_e(c^*)/\psi'_w(c^*)$	$\frac{16}{\pi} \frac{(kT)^2 D}{\epsilon} \frac{R}{\lambda}$	$\frac{16}{\pi} \frac{kT}{\epsilon^2} D \frac{R}{\lambda}$
	cm^{-1}	cm^{-1}	cm^{-2}		cm	$dyne$	cm^{-1}
Actin filaments	$\begin{cases} R = 3nm \\ c^* = 19nm \end{cases}$	$0.983 \cdot 10^{-8}$	$9.460 \cdot 10^2$	0.177	$2.722 \cdot 10^9$	$0.650 \cdot 10^{-10}$	$1.082 \cdot 10^{-5}$
Myosin filaments	$\begin{cases} R = 7nm \\ c^* = 33nm \end{cases}$	$0.200 \cdot 10^{-11}$	$2.060 \cdot 10^3$	$0.360 \cdot 10^{-4}$	$3.578 \cdot 10^9$	$1.006 \cdot 10^{-14}$	$2.524 \cdot 10^{-5}$
							$0.630 \cdot 10^9$

In these conditions, the values of the Hamaker constant A for the actin filaments are:

$$A = 8 \cdot 10^{-13} \gamma^2 \text{ erg} \sim 1.6 \cdot 10^{-13} \text{ to } 4.8 \cdot 10^{-13} \text{ erg} \quad (17)$$

which correspond to the physical order of magnitude (6,10, 24,27,47).

Another consideration appropriate to our topic will be considered next. It has been shown experimentally that the dielectric constant of surface zones is significantly less than the bulk dielectric constant (2,39,41). On such a basis it is reasonable to propose that the dielectric constant of the interfilament water is lower than the bulk dielectric constant. Under these conditions, the dissociation of KCl into K^+ and Cl^- ions would be less than in bulk water. The concentration of the K^+ counter-ions being decreased, the screening effect of these ions is also decreased. As a result, $\psi_e(c^*)$ and $\partial\psi_e/\partial c(c^*)$ are considerably increased, and the potential well $[E_w(c^*) + E_e(c^*)]$ becomes much higher than predicted by the usual DLVO theory. It is evidently difficult to propose an example of calculation in this case, since the dielectric constant, D, and the relation between D and I (ionic strength) are unknown. However, as an illustrating example, let us assume the following values:

$$D = 30 \qquad \qquad \qquad I = 0.005 \quad (18)$$

$\psi_w(c^*)$ and $\partial\psi_w/\partial c(c^*)$ remaining unchanged (see Table I), we can calculate numerically the new values of the other parameters and we get, for the actin filaments:

$$\frac{E_w(c^*) + E_e(c^*)}{kT} = -12 \gamma^2 = -2.4 \text{ to } -7.2 \quad (19)$$

$$A = 1.4 \cdot 10^{-11} \gamma^2 \text{ erg} = 2.8 \cdot 10^{-12} \text{ to } 8.4 \cdot 10^{-12} \text{ erg} \quad (20)$$

The equilibrium of the filament is, therefore, very stable and the values of A lie in the physical range (6,10,24,27, 47).

IV. CONCLUSION

It has been clearly shown that the DLVO theory is unable to predict the equilibrium of the myofilament lattice and

this result also applies to other filament arrays found in biology. In our opinion, the only possible way to solve this problem is to consider that the interfilamentary water plays an active role because it has properties different from those of bulk water, even though the properties of this vicinal water remain to be described in detail. The concept of strongly bound water layers surrounding the filaments and excluding the counter-ions is certainly an over-simplification. Nevertheless, at the present state of our understanding the concept is quite useful (38). It is also possible to propose that the dielectric constant of the interfilament water is less than the bulk dielectric constant, and that the dissociation of KCl into K^+ and Cl^- ions is less than in bulk water, which leads to a reduced screening effect of the K^+ ions. These two hypotheses are by no means the only possibilities, and we may foresee that the near future will provide further information on the exact role of the vicinal water in biology.

V. SUMMARY

We have examined the electrostatic and Van der Waals-London energies of actin and myosin filaments, on the basis of the classical theories. The *in vivo* spacing between these filaments corresponds to energies of 10^{-3} to 10^{-5} kT. The myofilament lattice cannot, therefore, be in stable equilibrium and this result also applies to the other filament arrays found in biology. We propose that a stable equilibrium can be achieved only if the interfilament water plays an active role.

NOTE ADDED IN PROOF

Based on discussions with our colleagues in the field of muscle research, we would like to consider briefly two additional aspects of the stability of the myofilament lattice. Obviously, we cannot dismiss the possibility that the Z disks and the M lines, to which the actin and the myosin filaments are respectively anchored, may contribute to the stability of both types of filaments. However, the Z disks are located at one end of the actin filaments and the M lines in the middle of the myosin filaments; hence, these structures most likely cannot prevent rotational motions of the filaments about their points of fixation. In any case,

we have clearly shown that the Hamaker constant, A, is far too low, independent of the presence of the Z and M structures and our reasoning remains valid. On the other hand, Spencer (51) has experimentally shown that a stable equilibrium of actin filaments oriented in glass capillaries can be obtained and that the center-to-center distance of these filaments is close to the distance observed in intact muscle. No Z disk being present in this case, it seems evident that the Z disks, and most likely the M lines, play a minor role in the stability of the actin and myosin filaments.

In the main text, we have proposed two possible hypotheses to solve the problem of the stability of the myofilament lattice. These two hypotheses are by no means the only possibilities. Dragomir (52), for instance, proposes that the Kirkwood-Shumaker forces play a role in the stability of resting muscle. These types of forces would be a consequence of the existence of charged fluctuations due to the mobile protons at the surface of proteins. Such charge fluctuations would be able to generate attractive forces between the myofilaments, if water with a high degree of molecular arrangement lies at their surface. The introduction of this type of force leads, therefore, to a deeper potential well at the physiological spacing, c^* , and is another interesting way, also intimately related to the presence of vicinal water, to solve the problem of the stability of the myofilament lattice and perhaps of all the other filament arrays found in biological systems.

APPENDIX

Let us call (α) the bound water layers excluding the K^+ ions and (β) the water layers beyond the (α) layers, in which the K^+ ions are dissolved. On the other hand, let V_α be the volume of the (α) layers, V_β the volume of the (β) layers and C_β the concentration of the K^+ ions in the (β) layers. The apparent concentration, C_i^* , of the K^+ ions in the interfilamentary water, assumed to dissolve uniformly these ions, is given by:

$$C_i^* = \frac{V_\beta}{V_\alpha + V_\beta} C_\beta \quad (21)$$

Let us now consider a skinned muscle fiber, from which the membrane has been removed, bathing in an aqueous solvent containing a concentration, C_e , of K^+ . By using the notation of Wiggins (50), the partition coefficient, λ_0 , between the

intrafibrillar water and the external water is defined here by:

$$\lambda_o = \frac{C_i^*}{C_e} = \frac{V_\beta}{V_\alpha + V_\beta} \frac{C_\beta}{C_e} \quad (22)$$

If the (β) layers accumulate the K^+ ions, we have $C_\beta > C_e$ and λ_o can be greater than unity, as experimentally found by Wiggins (50). Our hypothesis, which considers that the (α) layers exclude the K^+ ions, is therefore not in contradiction with the experimental results of Wiggins (50), if we add the further hypothesis that the (β) layers accumulate the K^+ ions. Note that the presence of two phases (α, β) in the intramuscular water is in agreement with the experimental findings of Hazlewood, *et al.* (30).

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METABOLIC CONTROL OF THE PROPERTIES
OF INTRACELLULAR WATER AS A UNIVERSAL
DRIVING FORCE FOR ACTIVE TRANSPORT¹

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I. INTRODUCTION

Over the last few years an attempt has been made to develop an hypothesis of energy conservation as adenosinetriphosphate (ATP), and its use for active transport, which is simple, universal and yet sufficiently versatile to be able to account for the wide diversity of transport processes. The hypothesis ascribes to water a central role in many cellular processes, and requires that both its structure and its solvent properties be under some degree of metabolic control (1,2,3).

Active transport is defined initially as an energy-dependent movement of an ion (or other solute) against a real or apparent electrochemical potential gradient. For example, most cells loaded with Na^+ will, in the presence of energy, expel Na^+ ions against a concentration gradient until a steady state is reached. In the case of muscle, for example, the apparent concentration of intracellular Na^+ is about $25 \text{ Mole} \cdot \text{m}^{-3}$, and that of extracellular Na^+ about $145 \text{ Mole} \cdot \text{m}^{-3}$, while the potential difference across the membrane is -90 mV (inside negative relative to outside). In the absence of energy, on the other hand, muscle cells

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continue to take up Na^+ until the intracellular concentration is higher than the extracellular concentration, and the membrane potential is quite low, but still negative inside relative to outside. Similarly, many cells in the presence of energy accumulate amino acids and sugars to higher-than-extracellular concentrations and, therefore, against apparent concentration gradients. If cells are depleted of energy the concentrations on either side of the membrane become approximately equal. Those are examples of energy-dependent transport against apparent electrochemical or chemical-potential gradients. We cannot assert with any confidence that they are *real* gradients until we know more about the distribution and activities of the solutes and water among the many intracellular compartments. In order to arrive at a figure for concentration of a solute, the total amount of solute is divided by the total amount of water and the result expressed as Mole m^{-3} . That is legitimate for the extracellular solution which is homogeneous and in which all the Na^+ , for example, is indeed dissolved in all the water. It may not be legitimate, however, for the intracellular solution where water and Na^+ may not necessarily be distributed proportionally among the various intracellular sites. The real concentration gradient across the plasma membrane depends upon the amount of Na^+ in solution in the cytoplasm alone, divided by the amount of water in the cytoplasm: those are quantities which are not readily accessible experimentally. The second difficulty, that of deciding upon the activity of an intracellular solute, is even more severe. The activity can be regarded as the effective concentration of a solute, and is equal to the concentration only if the solute behaves entirely independently of every other solute species, and of water. It is therefore not safe to assume that activities and concentrations are the same inside the cell, or even that they bear the same mutual relationship that pertains outside the cell, because cells contain high concentrations of proteins with which either the solute or water (or both) might interact strongly. For example, excess amino acids accumulated in the presence of energy might be bound to proteins, or other sites; thus, their *effective* concentration may not be greater than in the absence of energy.

Energy-dependent transport against real electrochemical potential gradients is, however, observed in isolated epithelia where the movement is from one simple electrolyte solution through a bank of cells to another simple electrolyte solution. In that case, activities of the solutes in the two solutions can be measured and the reality of the

gradient is unequivocal. For example, the cells of an isolated toad bladder in the presence of an energy source, move Na^+ ions from a low activity in the mucosal solution to a higher activity in the serosal solution, against a transepithelial potential difference (5).

II. EVOLUTION OF ACTIVE TRANSPORT

Active transport must have evolved from very simple beginnings to its present level of complexity. Now it is uncommon to find a single ion or molecule, which, in the presence of energy, is distributed across the plasma membrane of any cell in the simple equilibrium fashion that is observed across permeable artificial membranes. But when the source of energy is removed, all solutes move across the plasma membrane until finally their distributions are not significantly different from those in the simpler systems (this, of course, may take a long time). Moreover, certain epithelia can actively transport many solutes (e.g., Na^+ , K^+ , Cl^- , amino acids and sugars) from mucosal to serosal solution, while others actively transport solutes (e.g., K^+ and Cl^-) from serosal to mucosal solution.

The primitive cell would not likely have already developed those specialized functions. But very early it would be faced with the problem of extracting nutrients from low concentrations in its bathing solution. An ability to concentrate nutrients might have been a biological advantage before the acquisition of many strictly metabolic processes. Thus, it is perhaps not too fanciful to suggest that the primitive cell might have happened upon a very simple chemical mechanism of accumulation, rather than one that today would be described as "biochemical".

The widely accepted view of active transport is that solutes are prevented from achieving an equilibrium distribution across single cell membranes and are transported by epithelial cells by specific membrane-bound carriers, each of which uses metabolically derived energy to change its affinity for its passenger on one side of the membrane or the other. Each carrier or pump can therefore pick up its specific ion or molecule from a low concentration on one side of a membrane and release it into a higher concentration on the other side. This view requires the parallel or sequential development of a profusion of independent mechanisms of energy use for active transport. It seems to me more probable

that the specialized functions of today's sophisticated cells evolved by means of elaborate variations upon the accidentally acquired concentrative ability of a single primitive cell. Like nucleation, the first step in an evolutionary process is the least probable and most difficult. Subsequent development by means of a long succession of minor modifications is relatively easy. It has seemed profitable, therefore, to try to view all active transport processes in contemporary cells as manifestations of Nature's known dexterity in achieving diversity from simplicity. By seeking a single attribute common to active transport to all solutes by all cells, one should be able to identify the original simple chemical mechanism of selectivity which, today, should be evident as a unifying principle.

III. A UNIFYING PRINCIPLE

A search of the literature has revealed that all cells (6,7,8) maintain low free concentrations of small cations (Na^+ , Ca^{2+} , Mg^{2+} , and H_3O^+) and that if any cell shows energy-dependent concentrative uptake of a solute, it is always of a large cation (K^+ , Rb^+ , or Cs^+), an anion (Cl^- , I^- , many organic anions), an amino acid or a sugar. The simplest possible explanation of such uniformity of cellular selectivity is that the cells contain a different solvent -- one that is a better solvent than extracellular water for the species they extract from aqueous solution, and a poorer solvent than extracellular water for the species that they left behind. But the intracellular solvent has always been water, so that in order to make use of that potentially valuable mechanism of selectivity, cells would somehow have to be able to change the properties of their contained water in such a way that it behaved as if it were a different solvent.

IV. WATER AT SURFACES

Water is a highly-associated liquid with an unequalled capacity for forming three-dimensional networks of molecules in which each water molecule is joined to up to 4 neighbors by hydrogen bonds (9,10). The clusters or networks of molecules are extremely short-lived and collapse and reform on a time scale of about 10^{-11} sec. The structure or hydrogen-bondedness of liquid water can be modified in a number of ways which include interaction with solutes (11) and proximity to surfaces (12). There is considerable controversy

about the number of water molecules which are affected by proximity to a surface. Two extreme positions tend to be taken: either that the surface perturbs water molecules for only 2 or 3 molecular diameters after which water resumes its normal bulk phase properties (13); or that a surface can exert an influence upon the structure of water up to 0.1μ into the liquid phase (14). The present unpopularity of the concept of long-range effects of surfaces upon water's structure may stem partly from the history of anomalous water (15,16). Initially the strange properties of water condensed from the vapor phase into small capillaries were ascribed to long-range effects of the quartz surface upon water structure. When, however, it became clear that measurements were, in fact, being made upon water contaminated with impurities (17,18), the change in its properties was no longer so surprising. There is now, therefore, a tendency to regard all apparent long-range effects as being due to the presence of impurities and, given the experimental difficulties of producing clean surfaces, such a possibility cannot often be ruled out. Moreover, many experimental techniques cannot distinguish between a drastic effect upon a few water molecules and a more modest effect upon a large number of molecules. Where there is no need to invoke long-range effects, experimental results are usually interpreted in terms of short-range effects, thus avoiding both mathematical and conceptual complexity. Finally, although there is a large amount of evidence which is consistent with long-range effects, a mechanism by which surfaces could exert such effects is not obvious, so that again it seems safer to interpret results in terms of high-energy, short-range effects which are well understood.

In the following development a mechanism by which the inner surfaces of a membrane might initiate medium-to-long-range changes in the structure and solvent properties of intracellular water will be considered, and then the possible consequences of such changes upon the distribution of solutes across cell membranes will be discussed.

Quantum mechanical calculations (19) have shown that hydrogen-bonding between water molecules is cooperative (i.e., that in favorably oriented chains or rings of water molecules the hydrogen bonds are stronger than are single hydrogen bonds). A surface carrying suitably directed and spaced hydrogen bond donors and acceptors, by participating in hydrogen bonding with immediately adjacent water molecules could therefore strongly reinforce the intermolecular hydrogen bonding within those nearest bonded regions of water.

Further, it seems probable that the stability of any given region or cluster of water molecules would be influenced by the stability of neighboring regions. If hydrogen-bonding is truly cooperative, then whole bonded regions must collapse and reform as entities, with freshly aligned hydrogen bonds (10). Collapse of a whole network would release molecules with greatly increased translational energy, and therefore, greatly increased probability of initiating the collapse of a neighboring cluster by bombardment. Increased stability of one cluster should, therefore, increase the stability of its neighbors. Thus, if on the inner surface of the membrane of a primitive cell, there were patches of strongly water-structure-promoting hydrogen bonding areas, the increased stability of those bonded regions of water might be propagated some distance into the cell through the operation of the stabilizing influence of a few long-lived clusters upon a whole population of clusters.

An extension of the concept of cooperativity of hydrogen bonding suggests a possible role for cytoplasmic proteins in stabilizing the increased structure initiated at the inner surface of the membrane. If collapse of an entire bonded region or cluster follows the breaking of one of its hydrogen bonds, then the probability of its collapse must increase with the number of its hydrogen bonds, so that the frequency with which clusters collapse increases with cluster size.

Formation of a cluster, on the other hand, depends upon the probability of formation of the first hydrogen bond, and to quote Frank (10): "*on the momentary locations and motions of the molecules in the volume element in question*". The frequency of formation of clusters is, therefore, probably less dependent upon cluster size, so that, given a distribution of cluster sizes in bulk liquid water (2) the smaller clusters should have longer lifetimes than the large clusters. If that were so, then one would expect that water vicinal to surfaces, and particularly water contained within small volumes bounded by surfaces, should be characterized by hydrogen bonds of longer average lifetimes than those in bulk water. Lack of room, when the water domain size was kept small, would prevent formation of the larger bonded regions with their inherent relative instability.

Clifford and Lecchini (21) have shown that the effect of domain size upon the properties of water is greater than the effect of a single surface. Clifford (22) suggested that the reason for this might be that bulk phase structures have no room to form and that the structures which do form are less

stable and, therefore, more readily dominated by surface interactions.

Drost-Hansen (23) pointed out that Clifford's suggested mechanism was entirely consistent with his many observations that the properties of vicinal water (e.g., the temperatures at which thermal anomalies are observed, the degree of change in heat capacity, etc.) are largely independent of the chemical nature of the surface, and of the nature and concentrations of solutes (unless they are too high). An increased stability of the structures permitted in small volumes might be expected to show greater independence of the chemical nature of the surface, since specific water-surface interactions would be less effective in perturbing intrinsic water structures. It is suggested, therefore, that the high concentrations of intracellular proteins and other macromolecules, by keeping the water domain size small, prevent formation of large bonded regions of relative instability. Such a consideration would increase the average lifetimes of hydrogen bonds in intracellular water, and also amplify and help to propagate the stabilizing effect of the specific structure-promoting inner surface of the membrane. If the structure of water inside a primitive cell were modified in this way due to interactions with the inner membrane surface, one might expect that like other vicinal water it should have increased viscosity (24,25), lowered melting point (27) and lower mobility (28). Of principal interest to the problem of cellular selectivity, however, is the possibility of a change in its properties as a solvent. A few years ago (29) we used a small-pored silica gel, in which all the water was dispersed in small volumes bounded by surfaces, to see whether such conditions would, in fact, affect the affinity of water for different solutes. We equilibrated the gel with electrolyte solutions at low pH so that ionization of the surface OH groups was negligibly small, and having corrected for the degree of exclusion due to ion size, we assumed that the partition coefficients (λ) for Rb^+ and Cl^- were equal, and derived individual ionic partition coefficients for the other ions. In Figure 1, $\log \gamma$ is plotted against ΔE^* (30), the contribution of the ion to the activation energy for viscous flow, which is related to the water structure-making and breaking properties of ions. Small cations which have a large surface charge density and therefore, interact strongly with a few water molecules of primary hydration, impose upon water a greater structure than its intrinsic structure, and increase the activation energy for viscous flow. Larger cations and anions have less need for primary hydration and, as they do not fit into the cavity

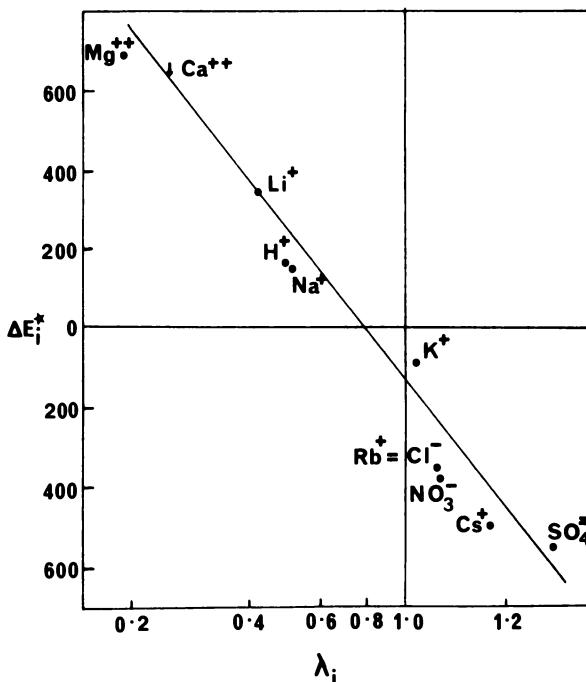


FIGURE 1. Derived individual ionic partition coefficients, λ , between water contained in the small pores of a silica gel and a bulk aqueous phase outside the gel shown as a function of ΔE^* , the individual ionic contribution to the total activation energy for viscous flow.

ties normally existing in liquid water, they cause the structure to collapse around them and decrease the activation energy for viscous flow.

Ions for which $\lambda < 1$ are relatively excluded from the water of the gel. Ions for which $\lambda > 1$ are relatively accumulated. The small cations Na^+ , H^+ , Li^+ , Ca^{2+} and Mg^{2+} all have positive values of ΔE^* and $\lambda < 1$. The larger cations and anions, K^+ , Rb^+ , Cs^+ , NO_3^- , Cl^- and SO_4^{2-} all have negative values of ΔE^* and $\lambda > 1$. The water inside the silica gel is indeed behaving as if it is a different solvent from the water in the bulk phase outside the gel. Most interesting, of course, is the observation that an inorganic gel shares the uniformity of selectivity displayed by cells. Both cells and gel water exclude small cations; both cells and gel water accumulate large cations and anions. If, therefore, the primitive cell contained water with the properties

of the water in the silica gel, it would already be better at accumulating those species which cells do accumulate and excluding those species which cells do exclude, than would a cell containing water with bulk phase properties. Further indications that ion distribution in cells and gel are at least partly determined by the properties of vicinal water are shown in Figures 2 and 3 (31) where the relative distributions of Na^+ and K^+ in the silica gel and in rat kidney cortex slices show typical Drost-Hansen thermal anomalies. In the kidney slices some of the anomalous temperature dependence might have been due to a phase change of lipids, but in the silica gel there can be no other phase change in the temperature range studied. These findings have been corroborated by Hurtado and Drost-Hansen (see chapter in this volume).

V. MECHANISM OF SOLUTE SELECTIVITY

If, in vicinal water, the average lifetime of intermolecular hydrogen bonds is increased, water is less readily available to hydrate ions. Those ions with the greatest need for primary hydration are, therefore, accumulated into the bulk aqueous phase where hydration is easier. Two possible mechanisms for accumulation of other solutes suggest themselves. First, if the unit of water as a solvent is a cluster, rather than a single water molecule, vicinal water containing a large number of small clusters should mix with more units of any solute species than the same volume of bulk water containing a smaller number of larger clusters. Vicinal water should, therefore, accumulate all solutes to higher molar (or molal) concentrations. Opposing that accumulating tendency of vicinal water would be the specific need of a particular solute for primary hydration. The range of selectivity found in Figure 1 is consistent with such a mechanism. The degree of accumulation of an ion in the pore water decreases as its activation energy for viscous flow increases; i.e., as it interacts more strongly in the primary hydration shell. The second possible mechanism of accumulation follows from the Principle of Le Chatelier: vicinal water with a higher degree of hydrogen-bonded structure should accumulate water structure-breaking solutes from bulk water of less hydrogen-bonded structure.

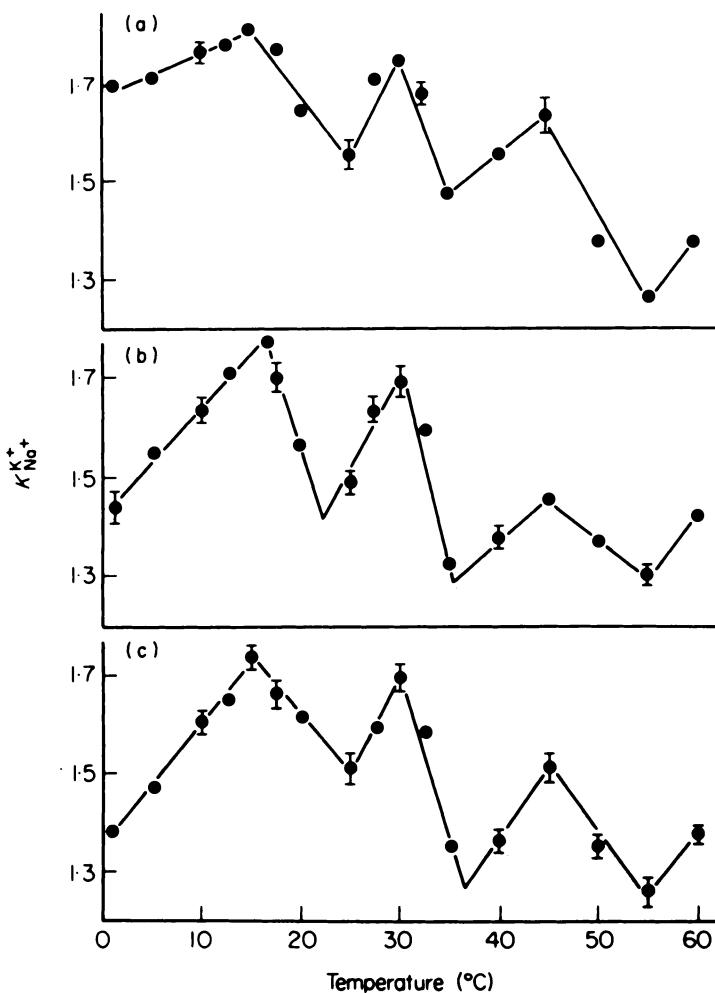


FIGURE 2. Variations of the selectivity coefficient, $K^{K^+}_{Na^+}$ (defined as $K^{K^+}_{Na^+} = [K^+]_i [Na^+]_e / [K^+]_e [Na^+]_i$) with temperature in Davison silica gel code 950. The accompanying anions are (a) SO_4^{2-} ; (b) I^- ; (c) Cl^- . Each point is the mean of selectivity coefficients calculated from six or eight equilibrations. Vertical lines indicate standard deviations. The figure is reproduced with the permission of Clin. Exp. Pharmacol. and Physiol.

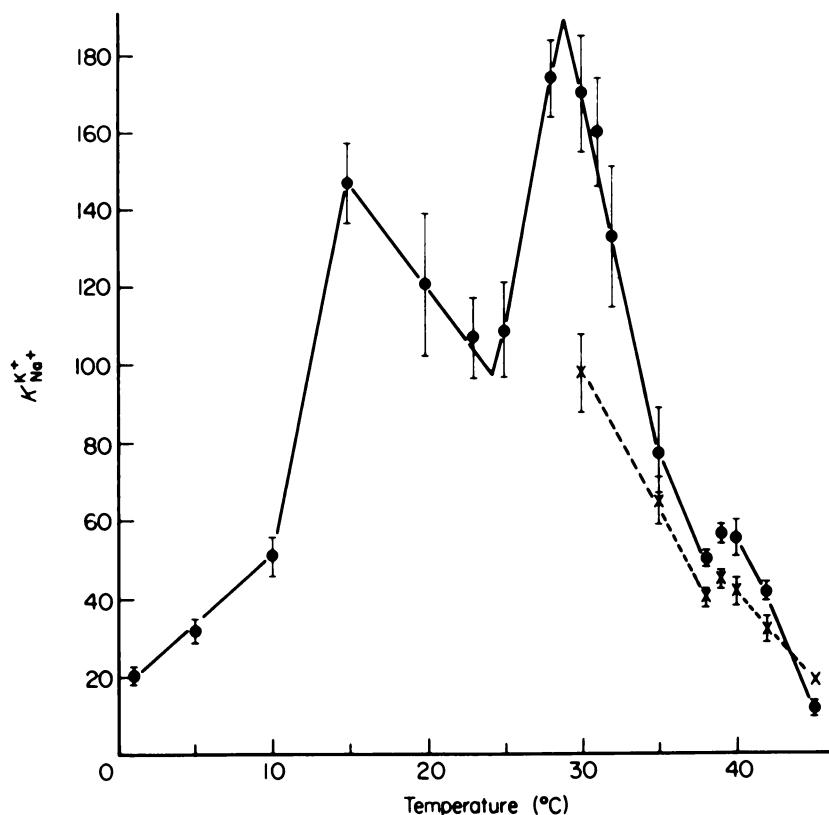


FIGURE 3. Variation of the selectivity coefficient, K^{K+}_{Na+} (as defined in the legend of Figure 2) with temperature in rat kidney cortex slices. ● phosphate medium; ✕ trimetamol medium. Each point is the mean of six to eight determinations. The vertical lines represent standard deviations. The figure is reproduced with the permission of *Clin. Exp. Pharmacol. and Physiol.*

VI. THE SIMPLE CENTRAL THEME

It is suggested, therefore, that the germ of a simple universal mechanism of active transport might be found in a change in the solvent properties of intracellular water. The plausibility of this mechanism rests on the uniformity of cellular selectivity, the qualitative resemblance between ion distribution in a silica gel and across cell membranes, and the suggested possible mechanisms by which cell mem-

branes might induce long-range order in intracellular water such that it might have appropriately changed affinities for different solutes.

VII. DEVELOPMENT OF THE HYPOTHESIS

While a static equilibrium mechanism of selectivity would be an advantage to a primitive cell, enabling it to concentrate nutrients from low concentrations in its bathing solution and maintain substrates at effective concentrations, it would not be adequate to serve the specialized functions of more sophisticated cells.

The next step in the development of the hypothesis, therefore, was an attempt to decide upon the fewest additional postulates that would be necessary in order that active transport of all solutes by all cells might be driven by a mechanism which depended ultimately upon a change in the properties of intracellular water.

The first and most obvious deficiency of the equilibrium model is that cells in fact require a continual expenditure of energy in order to generate and maintain solute gradients (32). In an equilibrium system such as the silica gel, on the other hand, the free energy needed to partition solutes is permanently built into the surface-water interactions. That requirement would be met if the water structure-promoting inner membrane surfaces were created by an energy-consuming process, collapsed spontaneously, and were reformed by a further expenditure of energy. Since in animal and plant cells the plasma-membrane-bound transport adenosinetriphosphatases (ATPases) are undoubtedly directly involved in whatever processes drive active transport (32), the next logical addition was to suppose that the energy to form the structure-promoting surfaces must arise from hydrolysis of ATP by the ATPases of such cells. In mitochondria and bacteria the same function is performed either directly by respiration, or by hydrolysis of ATP by the Mg^{2+} -dependent proton translocating ATPase (33, 34). It then seemed probable that any conformational change of a membrane might involve changes in permeability of that membrane. Surprisingly, these few additions have proved almost adequate to endow the mechanism with the versatility demanded by the extremely varied requirements of different cells, without destroying its essential chemical simplicity and universality.

The scheme in Figure 4 summarizes the present state of development of the hypothesis. There are two conformational states of membrane, which have been called *ordered* and *disordered* because of the degree of organization of a zone of intracellular water that is associated with each of them. In the absence of energy a membrane is permanently in the *disordered* state; its inner surface has no special water structure-promoting properties, and intracellular water in this case has properties not widely different from those of extracellular water; however, by virtue of its containment within small volumes bounded by surfaces, one would expect it to be similar to other vicinal water. On hydrolysis of ATP, the ATPase-containing portion of the membrane undergoes a conformational change to the *ordered* state and, after a short time interval, collapses spontaneously as a result of thermal fluctuations, back to the *disordered* state. Thus, in the presence of energy the ATPase-associated membrane (or the parts of the mitochondrial membrane containing the cytochrome chain) oscillate between two conformations. There are two properties of the *ordered* state which are different from the *disordered* state. First, the inner surface of the ATPase has hydrogen-bonding donor and acceptor groups oriented in a way that enables them to participate without strain in intrinsic water structure. Bonded regions or clusters of water molecules immediately adjacent to that part of the membrane are greatly stabilized and a degree of stability is thus transmitted through an associated zone of intra-

TWO CONFORMATIONAL STATES OF A MEMBRANE

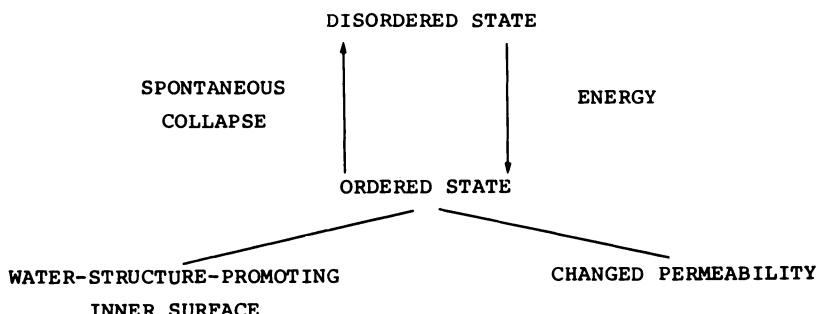


FIGURE 4. Scheme representing the universal use of metabolic energy in generating a change in the conformational state of a membrane. For explanation see text.

cellular water. The stabilizing influence of the water structure-promoting surface is amplified and its propagation aided by the presence of closely spaced protein surfaces, which keep the water domain size small and thus prevent formation of large clusters which are inherently less stable. That affected zone of water has increased structure, increased viscosity, and profoundly changed affinities for all solutes: it almost totally excludes small cations, and accumulates large cations, most anions, amino acids, sugars, etc. to varying degrees. Secondly, there are changes in the permeability of the part of the membrane associated with the ATPase (or the cytochrome chain). Some changes are not specific, but others are highly specific. For example, the ($\text{Na}^+ + \text{K}^+$)-ATPase generates a highly specific Na^+ -ionophoric channel; the Ca^{2+} -stimulated ATPase of the plasma membrane or the sarcoplasmic reticulum generates a Ca^{2+} -ionophore; the proton ATPase of plant, bacterial and mitochondrial membranes generates a proton ionophore. For those small cations it is as if part of the membrane opens and closes in phase with the ordering and disordering of the zone of intracellular water. Previously it was thought that if the mechanism were to function at all, it would be necessary both that all intracellular water were involved in the changes in structure and solvent properties, and also that on one membrane all ATPases operated in phase. It has since been realized that neither of those strictures, each of which strained the credibility of the hypothesis, is, in fact, necessary. It is true, however, that the efficiency of the mechanism increases with the relative amount of cell water involved, and also with the degree of synchronization of the ATPases.

The scheme in Figure 4 emphasizes the fundamental simplicity of the hypothesis. All cells drive active transport by using metabolically derived energy to generate a single type of conformational change in a membrane with associated changes of just two of its properties. In spite of that basic simplicity and universality, however, there is now limitless scope for specificity of behavior from cell to cell and from solute to solute, since the factors which determine the steady-state concentration of any solute in any cell are: (a) its extracellular concentration; (b) its affinities for the zone of metabolically controlled water inside the membrane in its two states; (c) the permeabilities of the active segment of the membrane in its two states; (d) the density of ATPases on the membrane; (e) the constant passive permeability of the inactive membrane segments; and (f) the overall requirements of electroneutrality and osmotic equilibrium.

Briefly, the properties of the highly *ordered* water determine whether a particular solute is accumulated or excluded by all cells, but the degree of selectivity by a given cell is largely dictated by the relative permeabilities of the active segment of membrane in its two states for that particular solute, and on the leak permeability of the rest of the membrane.

VIII. STEADY-STATE DISTRIBUTION OF Na^+ AND K^+

The operation of the pump can best be illustrated by describing the distribution of Na^+ and K^+ across the membrane of a nerve or a muscle cell. Figure 5 shows a strip of membrane separating the inside from the outside of a cell during a *disordered* period. Through it run aqueous channels just large enough to allow free passage of the hydrated K^+ . Since the *ordered* structures have collapsed, water inside the membrane is not very different from water outside the membrane, and there is a strong driving force for influx of Na^+ , for which intracellular and extracellular water have similar

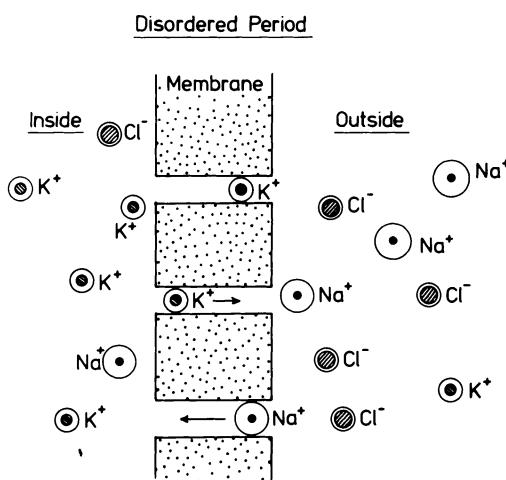


FIGURE 5. Diagrammatic representation of a strip of membrane separating the inside from the outside of a cell during a disordered period. Most aqueous channels are just large enough to allow passage of a hydrated K^+ ion, but not of a hydrated Na^+ ion.

affinities down its concentration gradient. However, very little movement of Na^+ can in fact take place because, although the Na^+ ion is smaller than the K^+ ion in its hydrated form, with its large tightly held sheath of water molecules, it is too big to pass through most of the pores. There is just a slight leakage of Na^+ inward through the very few pores (such as the one shown at the bottom of Figure 5) which are large enough. Figure 6 represents the conditions which exist during an *ordered* period. In this representation two adjacent ($\text{Na}^+ + \text{K}^+$)-ATPases have changed simultaneously to the *ordered* conformation. Two changes have taken place: first, the water in a zone just inside the membrane has greatly reinforced hydrogen-bonded structure, and is not readily available to provide primary hydration for the ions. The difficulty ions experience in achieving primary hydration is represented in a highly simplistic way by drawing them without hydration shells. Secondly, associated with each ($\text{Na}^+ + \text{K}^+$)-ATPase there has appeared a Na^+ -specific ionophoric channel, which just fits a dehydrated Na^+ ion, and perhaps provides it with a protective tunnel of

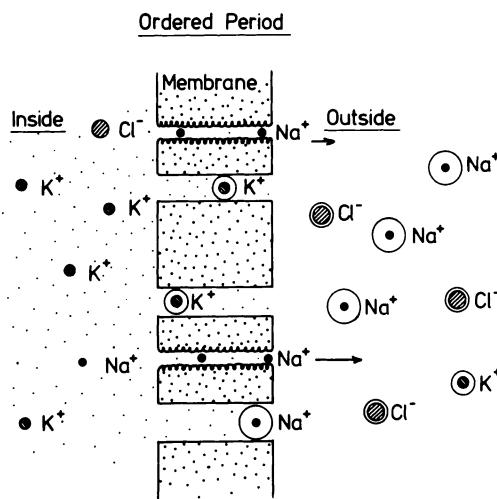


FIGURE 6. Diagrammatical representation of a strip of membrane separating the inside from the outside of a cell during an *ordered* period when two adjacent ($\text{Na}^+ + \text{K}^+$)-ATPases have undergone simultaneous conformational changes. Water inside the membrane is highly ordered and poorly accessible to hydrate ions, which are therefore depicted without hydration shells. Association with each ($\text{Na}^+ + \text{K}^+$)-ATPase has opened a Na^+ specific ionophoric channel.

O atoms to replace its water of hydration. Na^+ can move rapidly in either direction through those pores, but now the driving force for its net movement has changed sign, because water inside the membrane has a structure which is incompatible with the stable existence of a Na^+ ion with its absolute requirement for primary hydration. Thus, the driving force is for net efflux of Na^+ into the extracellular solution where hydration is easier. The overall effect for Na^+ is that it is held at a very low intracellular concentration, far from equilibrium, because when the driving force is for net influx the membrane is practically impermeable to Na^+ ; when the driving force is for net efflux the permeability of the membrane is both specific and high. K^+ can still move in and out readily through the pores that it used during the *disordered* period. Since it is a large cation and a water structure-breaker it finds the structured water a favorable environment, and tends to be accumulated both because of its water structure-breaking powers, and because of the change in the colligative properties of vicinal water containing a large number of small clusters. If there is no change in K^+ permeability associated with the *ordered* state, then K^+ is in equilibrium with the average properties of intracellular water. If, however, there is a change in permeability, K^+ will also reach a steady-state, not an equilibrium state. Since K^+ is the only cation from the extracellular solution that can go freely into the cell to maintain electroneutrality it is accumulated to relatively high concentrations, and because of its high permeability its distribution largely determines the membrane potential.

If, in Figure 6, the two ATPases operate out of phase, and if each has associated with it in the ordered state a rather small localized region of ordered water, movements of ions other than those which were discussed above can take place. For example, if only the lower of the ATPases (Figure 6) is in the ordered state there are driving forces for Na^+ in that region to move not only out of the extracellular water, but also sideways into a zone of unstructured intracellular water and inward to the bulk of unstructured intracellular water. Since there are no diffusional barriers to such intracellular fluxes, the probability that a Na^+ will leave the cell through its ionophoric channel is greatly decreased. It must be realized, however, that random movements of ions inside the cell during an ordered period do not detract from the finality of loss of those Na^+ ions which do pass through the pump channel. When the ordered state collapses the local zone of structured water reverts to bulk phase-like properties, and is restocked with Na^+ ions dif-

fusing in from other parts of the cell interior. With further hydrolysis of ATP by the same ATPase, some Na^+ ions again are irretrievably lost to the cell, even if there is much random movement of ions within the cell. If ATPases operate independently and each generates the minimal thickness of modified water capable of moving Na^+ outward during the ordered period, then these pumps will use as much energy as conventional carrier pumps. As, however, the thickness of the zone of structured water increases, the driving force for ions near the membrane to move backward into the cell interior during the ordered period decreases, and the probability that Na^+ ions will choose the path with its very large driving force through the ionophoric channel increases. Thus, the rate of Na^+ efflux increases with thickness of the zone of ordered intracellular water. The energy requirement for transport also decreases as the zone of affected water increases in thickness and thus increases the average chemical potential of intracellular Na^+ . The average amount of energy required to move each Na^+ ion is least when all intracellular water is ordered, and most if only the minimal membrane-associated water is ordered. Efficiency must obviously be compromised in the case of a large cell; thus, it is extremely improbable that a high degree of water structuring could be propagated from the axonal membrane across 1 mm of squid axon, or that ATPases could be synchronized down a long segment of axon.

IX. Ca^{2+} IN MUSCLE AND MITOCHONDRIA

The versatility of the mechanism is further illustrated by the behavior of Ca^{2+} ions in muscle fibers and in mitochondria. Muscle cells contain concentrations of free Ca^{2+} as low as 10^{-4} Mole $\cdot \text{m}^{-3}$ (35) although the equilibrium concentration calculated from the membrane potential and the extracellular concentration should be about 10^3 Mole $\cdot \text{m}^{-3}$. Such extreme exclusion of Ca^{2+} is similar in operation to the extreme exclusion of Na^+ .

During the *disordered* period the sarcolemma and the sarcoplasmic reticulum are impermeable to Ca^{2+} ; in the *ordered* period both Ca^{2+} -stimulated ATPases generate a highly specific Ca^{2+} ionophore through which Ca^{2+} ions rapidly escape the incompatible highly structured water for the bulk phase-like water of the extracellular space or sarcoplasmic reticulum. Mitochondria, however, accumulate Ca^{2+} . There need be no difference in the kind of water generated inside a mitochondrion, or in its lack of affinity for Ca^{2+} . The

difference lies in the permeability of the membrane in its two states. In the absence of energy (i.e., in the disordered state) the inner mitochondrial membrane is permeable to Ca^{2+} (36). Therefore, in a steady-state in the presence of energy, Ca^{2+} can move readily across the membrane during the disordered period when water inside the mitochondrion is a favorable environment for it. Ca^{2+} is therefore not totally excluded from matrix water as it is from muscle water.

Nevertheless, relative to K^+ , Ca^{2+} is excluded by matrix water. In the presence of energy, mitochondria generate a potential gradient, inside negative, in response to which cations can be taken up in exchange for H_3O^+ ions. Massari and Pozzan (37) showed that the accumulation ratio of Ca^{2+} in the presence of energy was much lower than that of K^+ in the presence of valinomycin under comparable conditions. So the generalization holds even for this apparent exception. The properties of the highly ordered water determine that Ca^{2+} ions are excluded to some degree from all cells, but the degree of exclusion depends upon the relative permeability of the membrane in its two states.

X. TRANSEPITHELIAL TRANSPORT

Perhaps the most stringent of any hypothesis of active transport is its ability to account for the many different modes of active reabsorption and secretion that epithelial cells can perform. Figures 7 and 8 show schematically that the mechanism, as it has been outlined so far, is capable of transcellular transport without any additional assumptions.

As an aid to explaining the mechanism of net active transport, the most favorable set of conditions has been illustrated in Figure 7, such conditions being complete impermeability of the serosal membrane during the *disordered* period and ordering of all intracellular water during the *ordered* period. It should become clear that the requirements for net transport are not as extreme; these are that the serosal membrane has increased permeability during the *ordered* period, and that an appreciable zone of intracellular water associated with each ATPase is *ordered*.

For simplicity of presentation only Na^+ ions have been shown, with equal arbitrary concentrations in the mucosal and serosal solutions and a low steady-state concentration inside the epithelial cell. The $(\text{Na}^+ + \text{K}^+)$ -ATPases are located pre-

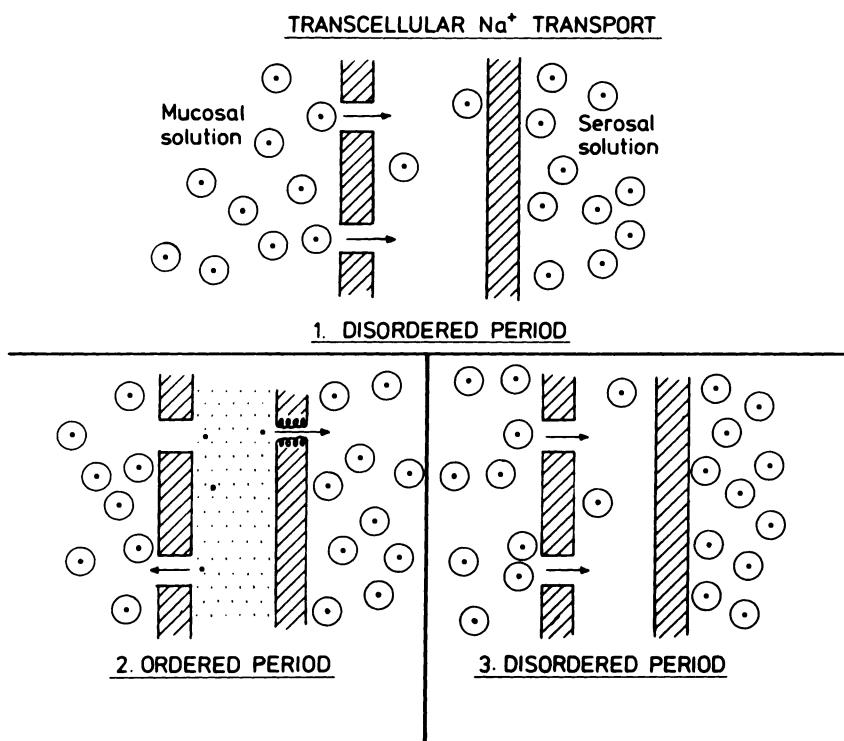


FIGURE 7. Scheme illustrating the ability of the $(\text{Na}^+ + \text{K}^+)$ -ATPase on the serosal membrane to achieve active Na^+ reabsorption against a real electrochemical potential gradient.

dominantly or exclusively on the serosal membranes of epithelial cells (38). The first frame illustrates the conditions during a *disordered period*.

Water inside the cell has properties that are relatively slightly different from those of water in both extracellular solutions, so that there is a strong driving force for influx of Na^+ from both solutions down its electrochemical potential gradient. The serosal membrane is represented as being totally impermeable to Na^+ , which can enter the cell only through the permeable mucosal membrane. Therefore, at the beginning of the *ordered period* in terms of the arbitrary numbers of ions shown in Figure 7, the cell has acquired two extra Na^+ ions from the mucosal solution. Intracellular water is now highly ordered and incompatible with the stable

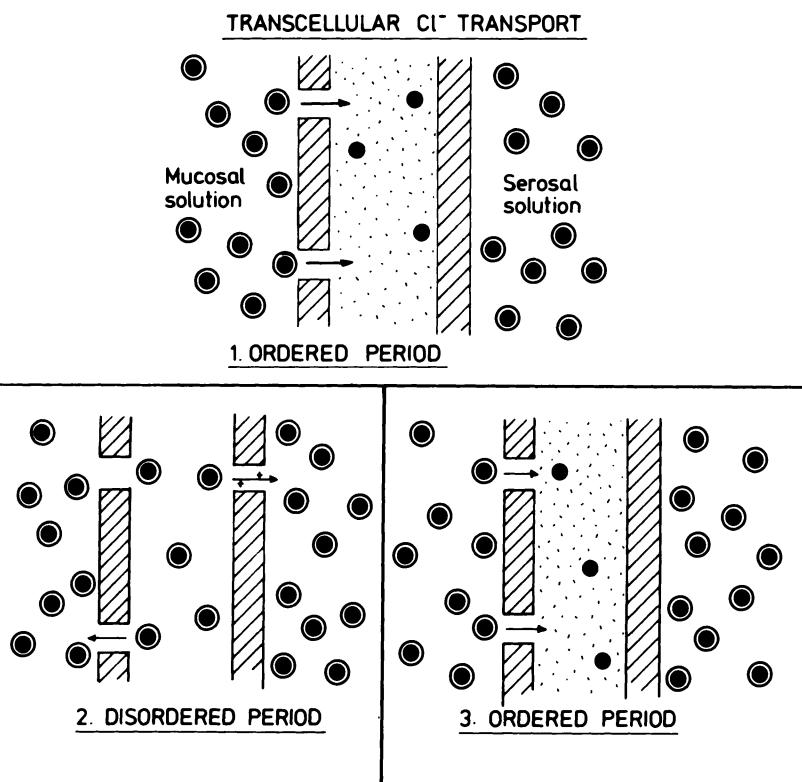


FIGURE 8. Scheme illustrating the ability of the $(\text{Na}^+ + \text{K}^+)$ -ATPase on the serosal membrane to achieve active Cl^- reabsorption against a real electrochemical potential gradient.

existence of a Na^+ ion. Consequently, there is a driving force for the net efflux of Na^+ , which can either go back through the unchanged mucosal membrane, or out through the newly-opened ionophoric channel generated by the $(\text{Na}^+ + \text{K}^+)$ -ATPase on the serosal membrane. When the *ordered* state collapses spontaneously in the third frame, the mucosal solution has lost one Na^+ ion to the serosal solution and the cell has regained its original quota of 2. Thus, in each cycle of the order-disorder transitions generated by each $(\text{Na}^+ + \text{K}^+)$ -ATPase, there is net transport of Na^+ from the mucosal to the serosal solution until the concentration of Na^+ is so high in the serosal solution that net efflux during an ordered period can take place only into the mucosal solution. Transport is inhibited by ouabain in the serosal solution. Macroscopic electroneutrality is maintained by movements of K^+ and

of Cl^- in proportions and in directions depending upon the permeability of the mucosal membrane, and the relative permeabilities of the serosal membrane in its two states.

Figure 8 illustrates how the same mechanism could drive active Cl^- reabsorption. Again, extreme conditions have been depicted and this time only Cl^- ions are included. The features that differ from those of active Na^+ reabsorption are: (a) the driving force for the net Cl^- influx comes into operation at the beginning of the *ordered* period when intracellular water has a high affinity for the water structure-breaking Cl^- ion; (b) the driving force for net Cl^- efflux operates during the *disordered* period, when previously accumulated Cl^- ions tend to leave the cell down their electrochemical potential gradient; (c) the serosal membrane is more permeable during the *disordered* than the *ordered* period. Under these conditions, for each cycle of the order-disorder transitions generated by each $(\text{Na}^+ + \text{K}^+)$ -ATPase on the serosal membrane there is net transport of Cl^- from mucosal to serosal solution until the electrochemical potential of the Cl^- ion in the serosal solution is equal to that inside the serosal membrane at the beginning of the *disordered* period. Electroneutrality is maintained by movement of other ions, and transport is inhibited by ouabain in the serosal solution. Such Cl^- reabsorption takes place in the thick ascending limb of the loop of Henle (39). If the changes in serosal membrane permeability were opposite to those shown in Figure 8 (i.e., if the permeability to Cl^- were greater during the *ordered* than during the *disordered* period) there would be net active transport of Cl^- from serosal to mucosal solution, as is observed, for example, in the salt-secreting rectal gland of the dogfish (40). Again, active Cl^- secretion would be inhibited by ouabain in the serosal solution.

Figure 9 shows how active stereospecific amino acid reabsorption can be driven by activity of the $(\text{Na}^+ + \text{K}^+)$ -ATPase on the serosal membrane. Since amino acids make no great demands for primary hydration they are accumulated to higher-than-extracellular concentrations, and conditions for reabsorption are the same as those for Cl^- (i.e., influx during the *ordered* period, efflux during the *disordered* period, and an increased permeability of the serosal membrane during the *disordered* period). The only difference is that there is a high affinity stereospecific binding site for the amino acid, accessible from either side of the mucosal membrane. The amino acid binds during the *disordered* period and dissociates from its binding sites when the intracellular water becomes highly ordered and has increased affinity for the amino acid.

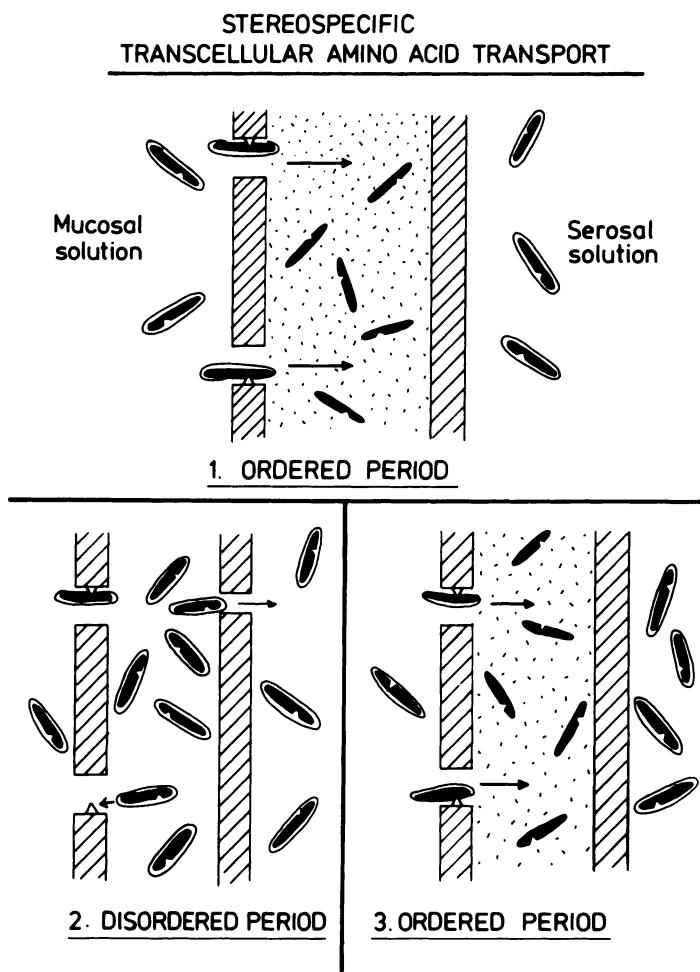


FIGURE 9. Scheme showing the ability of the $(\text{Na}^+ + \text{K}^+)$ -ATPase on the serosal membrane to achieve stereospecific amino acid reabsorption against a real chemical potential gradient.

If the zone of *ordered* water does not reach as far as the mucosal membrane, the dissociation of the amino acid from its binding site still takes place as intracellular amino acids diffuse down their chemical potential gradients into the zone of *ordered* water adjacent to the serosal membrane, and the intracellular concentration adjacent to the mucosal membrane is decreased. In either case, the amino acid dissociates as a result of increased affinity for intracellular water, but not as a result of a changed intrinsic affinity

for its binding site. With the collapse of the *ordered* state, the amino acid either recombines with its binding site or diffuses out through the newly-opened permeable channel in the serosal membrane. Again, transport is inhibited by ouabain in the serosal solution. The biological advantage of the high affinity stereospecific binding site is an increase in the rate of transport since the amino acid can be scavenged from low concentrations in the mucosal solution, and be poised ready on the membrane when the driving force for accumulation comes into operation.

In various epithelia there are different combinations of active reabsorption and active secretion of ions. The examples in Figures 7, 8 and 9 show how a single mechanism is, in principle, capable of any combination of active reabsorption or secretion of any number of ions or non-electrolytes, or indeed of water itself. The common driving force is the generation of *ordered* water by an ATPase on the serosal membrane; the extent and direction of movement of a particular solute is then dictated by a constant permeability of the mucosal membrane and a permeability of the serosal membrane which is different in the two states, together with the requirements of electroneutrality.

XI. TRANSLOCATORS OR PERMEASES

In addition to bilaterally accessible stereospecific binding sites which merely increase the rate of transport or of uptake, there are specific permeases or translocators which increase the level of accumulation of their particular substrate. Figure 10 which specifically describes the activity of the OH⁻-phosphate exchange translocator on the inner mitochondrial membrane illustrates the proposed mechanism. The principles involved are quite general and apply to stereospecific uptake of any solutes by "non-polar" cells (3).

The membrane carries a highly specific phosphate binding site which in the *disordered* state is accessible only from the outside. With the onset of the *ordered* state and the associated conformational change of the membrane, the bound phosphate comes into contact with intramatrix water, and dissociates because of its increased affinity for that highly *ordered* water. The small OH⁻ ion which is one of the few strongly hydrated anions, and therefore has decreased the affinity for the *ordered* water, binds to the now-vacant site

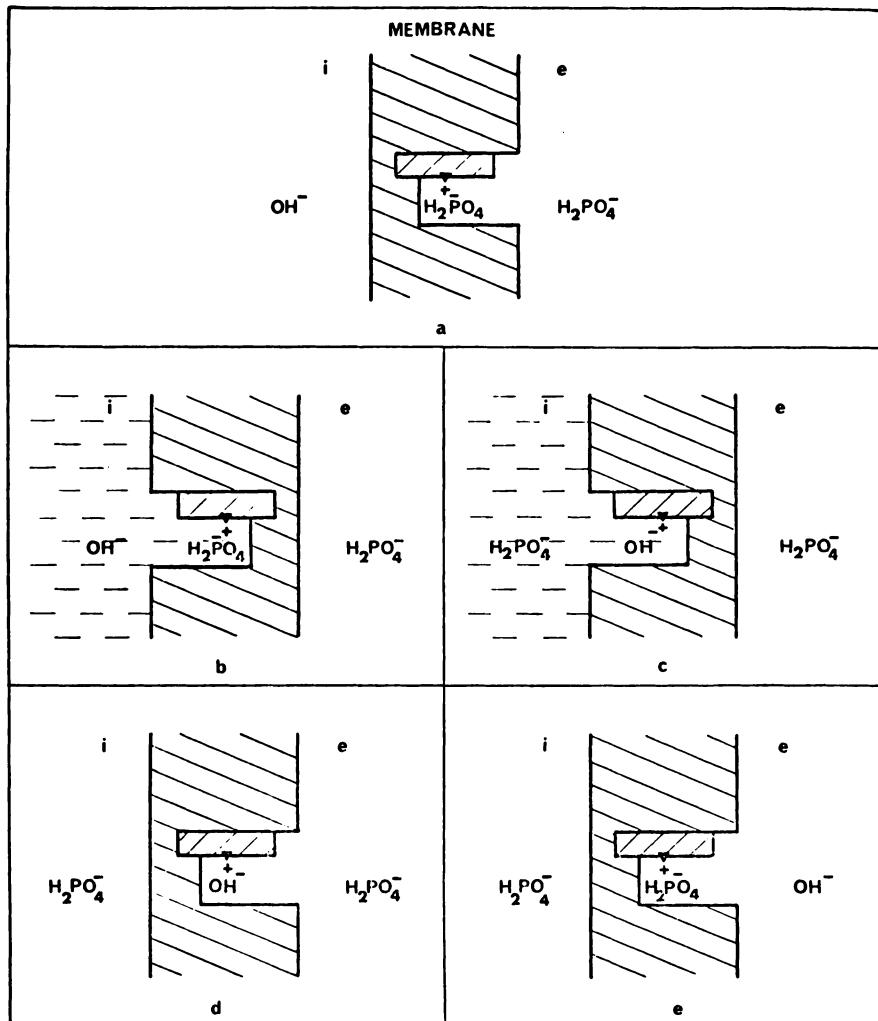


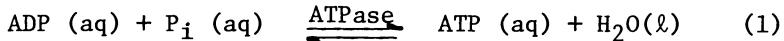
FIGURE 10. Diagrammatic representation of the action of the $\text{OH}^- - \text{H}_2\text{PO}_4^-$ exchange translocator across the inner mitochondrial membrane. i, internal solution; e, external solution. See text for explanation. The figure is reproduced with permission from *Medical Hypotheses*.

(Figures 10b and c). With the collapse of the ordered structures the binding site is again accessible only from the outside (Figure 10d) and since it has a higher intrinsic affinity for H_2PO_4^- than for OH^- , it exchanges its OH^- , which can readily find its primary hydration in the outside solution, for H_2PO_4^- . Thus, in one cycle an internal OH^- has exchanged for an external H_2PO_4^- . Energy has been used not to change the intrinsic affinity of the binding site for its anion,

but to change water-anion affinity, and also the orientation of the binding site.

XII. EQUILIBRIA INVOLVING HYDRATED SPECIES

Upon generation of the *ordered* state all equilibria involving hydrated species in the zone of ordered water should be displaced. That general phenomenon has already been illustrated by the examples of dissociation of bound substrates from membranes on coming into contact with ordered water. Of particular interest is the equilibrium between ATP and ADP. George, *et al.* (41) have pointed out that the equilibrium should be written:



where (aq) indicates that the species is in aqueous solution and therefore, hydrated and the symbol (ℓ) indicates that water is in the liquid state. The reaction as written to the right has a positive standard free energy *in vitro* of about 40 kjoule/mole (42), so that with the usual concentrations of the three species, the spontaneous direction of the reaction is from right to left, with hydrolysis of ATP. George, *et al.* (41) showed that the rather small free energy change of the overall reaction is largely determined by the much greater free energies of hydration of ADP, P_i and ATP. In other words, the position of the equilibrium and, therefore, the spontaneous direction of the reaction depends principally upon the affinities of the participating species for water. If, therefore, those affinities are all changed by generation of the *ordered* state, the magnitude of ΔG^0 must change, and it is possible that its sign may change so that synthesis of ATP becomes a spontaneous process. It has been suggested, therefore (3), that if in mitochondria and bacteria the conformational change involving generation of *ordered* water is initiated by the transfer of electrons down the respiratory chain, then the ATPase is free to catalyze the ADP/ATP reaction which now goes spontaneously in the direction of synthesis of ATP.

XIII. ISOLATED MEMBRANES

If cytoplasmic proteins played no part in the generation and maintenance of ion gradients one would expect that isolated membranes devoid of cytoplasm would be as effective as

the cells from which they were derived. Generally, that is not the case. Neither red cell ghosts nor isolated squid axonal membranes have been shown to be capable of performing as extensive an exchange of Na^+ for K^+ as do the intact cells, although under suitable conditions ghosts do effect a slight active exchange (43). Both membranes, however, show an increased ouabain-inhibitable Na^+ efflux when they contain ATP. It has been suggested (2) that the behavior of such isolated membranes can be explained in terms of a dissociation of the two changes which accompany activity of the $(\text{Na}^+ + \text{K}^+)$ -ATPase. That is, in the absence of cytoplasmic proteins, when the water domain size is large, membranes are incapable of ordering enough water to generate steep Na^+/K^+ gradients, but that the opening of the Na^+ ionophoric channel associated with the ATPase increases the permeability to Na^+ and therefore, its unidirectional efflux. Such an interpretation is consistent with the existence of a ouabain-inhibitable Na^+-Na^+ exchange in the absence of K^+ . Isolated membrane vesicles from bacteria, on the other hand, can effect high degrees of accumulation or of exclusion. The water content of such vesicles, however, is only about $2.2 \mu\text{l}/\text{mg}$ membrane protein (44), and the water domain size is still small enough (approximately 10 nM diameter) for the isolated membrane to have a profound effect upon all its contained water in the absence of cytoplasmic proteins.

XIV. RECONSTITUTED ATPases

Purified ATPases and parts of the cytochrome chain (45, 46) have been reconstituted in artificial liposomes and shown to be capable of pumping ions. Goldin and co-workers (47,48) showed that whereas the ouabain-inhibitable $(\text{Na}^+ + \text{K}^+)$ -ATPase from brain catalyzed an active exchange of Na^+ for K^+ , that from the canine renal medulla actively transported Cl^- with Na^+ . The transport of both K^+ and of Cl^- was shown to be active, and not merely a passive response to potentials generated by Na^+ transport. In the discussion on transepithelial transport, the existence of a ouabain-inhibitable active Cl^- reabsorption in the thick ascending limb of the loop of Henle was mentioned as a possible example of the ability of a serosal membrane-bound $(\text{Na}^+ + \text{K}^+)$ -ATPase to drive active Cl^- transport. If the principles applied to intact epithelia are applied to reconstituted ATPase vesicles, it can be shown that only slight modifications in the ATPase complex would be necessary for active co-transport of Cl^- to predominate over the more usual forced Na^+/K^+ exchange.

Figure 11 represents a single inside-out ($\text{Na}^+ + \text{K}^+$)-ATPase from renal medulla reconstituted in a lipid membrane. In the absence of energy (Figure 11a) the ATPase incorporates a single channel large enough to admit a Cl^- ion, and is perhaps positively charged so that it excludes the K^+ ion which is approximately the same size. For simplicity, the behavior of just the two Na^+ ions and the two Cl^- ions shown on the outside of the vesicle will be considered. It is assumed

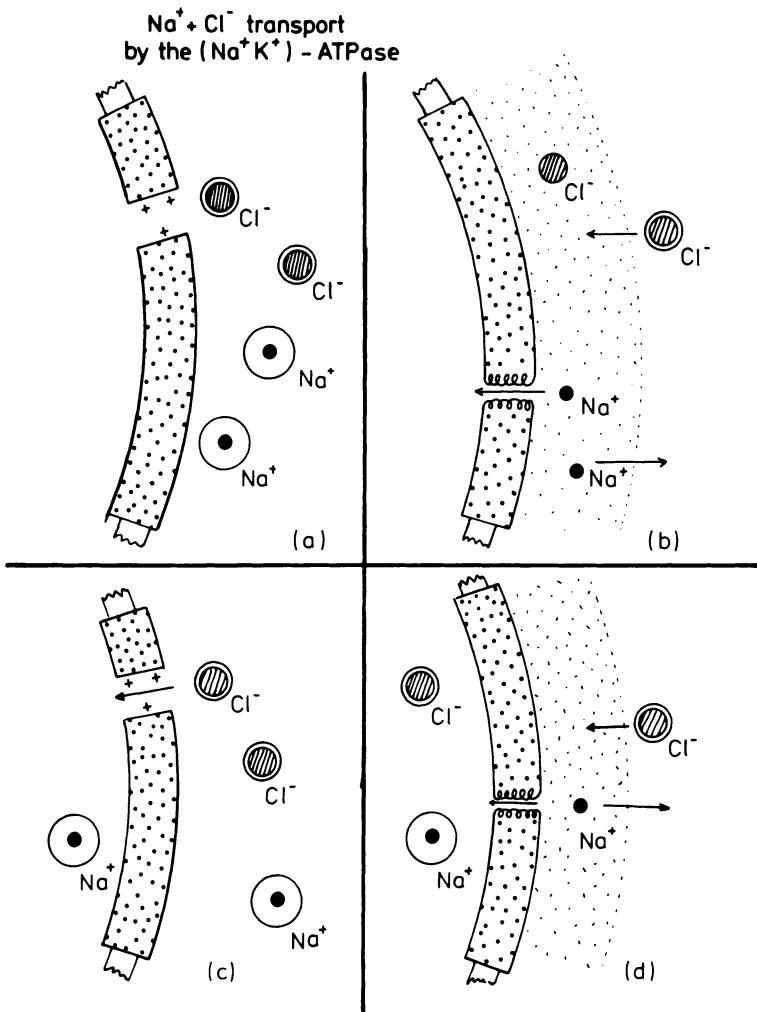


FIGURE 11. Scheme showing a mechanism by which the $(\text{Na}^+ + \text{K}^+)$ -ATPase isolated from renal medulla and reconstituted in an artificial liposome could actively co-transport Na^+ and Cl^- .

that the concentrations of NaCl inside and outside the vesicles are initially the same. On hydrolysis of ATP the ATPase undergoes a conformational change which has the two familiar effects. First, the outer surface (which in an intact cell would be the inner surface) becomes strongly water structure-promoting so that a zone of water vicinal to the ATPase has greatly increased hydrogen-bondedness, decreased availability for solvation and, as a result, changed affinities for all solutes (Figure 11b). Na^+ , which has the strongest requirement for primary hydration, has least affinity for the ordered water and is forced out of the vicinal zone. Most Na^+ ions (e.g., the lower one in Figure 11b) diffuse out into the bulk aqueous phase where hydration is easier. A few, however (e.g., the upper one in Figure 11b) pass into the vesicle through the newly-opened specific Na^+ ionophoric channel. During the same ordered period (Figure 11b) Cl^- ions are attracted into the ordered water for which they have greater affinity than they have for bulk phase water. Since the Cl^- channel is now closed, Cl^- ions from the bulk aqueous phase only can accumulate in the vicinal zone. When the *ordered* state collapses as a result of thermal fluctuations, water reverts to more bulk phase-like properties, and the membrane regains its normal permeability with a Cl^- channel, but no Na^+ channel (Figure 11c). Na^+ which entered the vesicle during the *ordered* period is now trapped inside the impermeable membrane, but Na^+ which was forced outward from the poorly hydrating vicinal zone can now return. Cl^- ions which have accumulated in the vicinal zone now diffuse down a concentration gradient, either into the bulk aqueous phase or through the specific channel which is open again, into the interior of the vesicle. Finally, (Figure 11d) the *ordered* state is regenerated by a further hydrolysis of ATP, and the Cl^- is now trapped inside an impermeable membrane. Thus, in one cycle of the *order-disorder* transitions both Na^+ and Cl^- have been taken up into the vesicles against concentration gradients. Figure 12 illustrates how the same principles would apply to an artificial lipid vesicle incorporating brain ($\text{Na}^+ + \text{K}^+$)-ATPase which forces an active exchange of internal K^+ for external Na^+ . Figure 12a shows the state of the ATPase in the *disordered* period when it is impermeable to both cations, and water in the vicinal zone has bulk properties. On hydrolysis of ATP the *ordered* state is generated, water vicinal to the outside of the ATPase becomes a hostile environment for Na^+ , but a relatively favorable environment for the larger water structure-breaking K^+ ion.

Na^+ ions are forced out into the bulk aqueous phase, or through the now open specific ionophoric channel. K^+ is

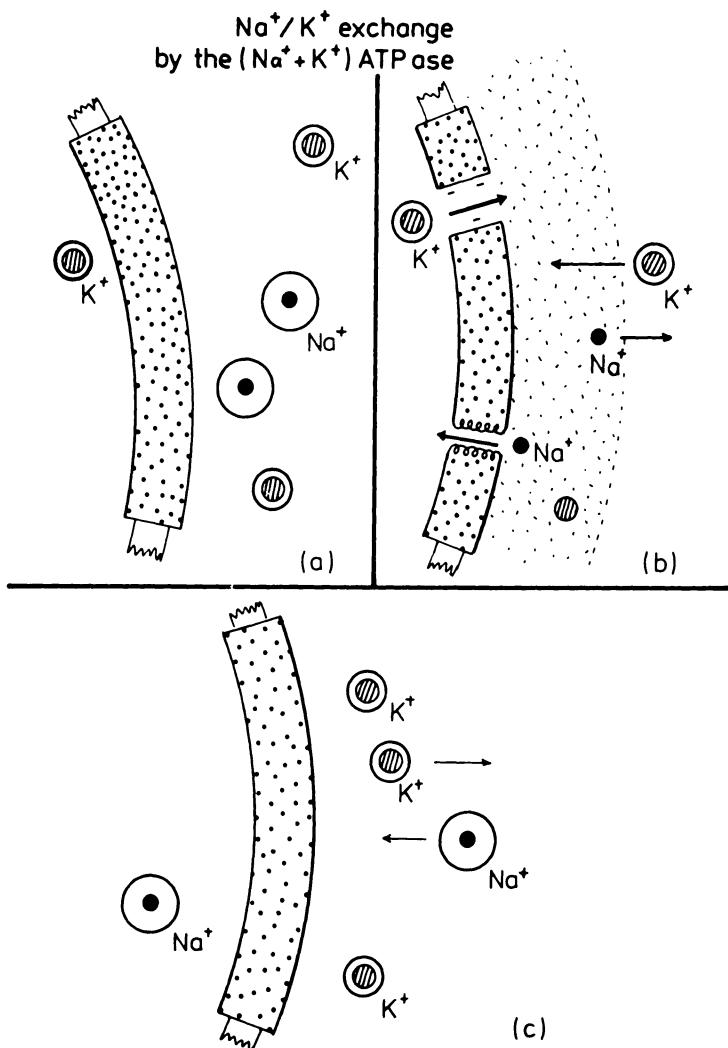


FIGURE 12. Scheme showing how the $(\text{Na}^+ + \text{K}^+)$ -ATPase isolated from the brain tissue and reconstituted in an artificial liposome could actively force a Na^+/K^+ exchange.

attracted into the vicinal zone from both the bulk phase and from the interior of the vesicle which it can leave through a specific channel which has now opened for it. On collapse of the *ordered* state (Figure 12c) the membrane becomes impermeable to both cations, vicinal water reverts to its bulk phase structure and an internal K^+ has exchanged for an external Na^+ . Although for the purpose of illustration the

ATPase has been made absolutely impermeable to an ion during one phase of its cycle of activities, a relative decrease in permeability would allow active transport to take place. The only difference between the two ATPases, from renal medulla and from brain, need be that the channel which allows movement of K^+ or Cl^- is positively charged in the kidney ATPase and negatively charged in the brain ATPase, and that in both cases, generation of the ordered state makes the channel more negative.

One would expect that in such inside-out vesicles the degree of ordering of the zone of vicinal water would be less than that of the equivalent zone inside an intact cell where cytoplasmic proteins, by keeping the domain size small, should amplify the effect of the structure-promoting surface of the ATPase. In fact, the selectivity achieved by reconstituted systems is less than that achieved by intact cells, in spite of the fact that the passive leak in such vesicles is quite low. Hilden and Hoken (49), however, reported impressively high ratios of 7 and 1/6 for Na^+ and K^+ , respectively, in vesicles incorporating purified $(Na^+ + K^+)$ -ATPase from the rectal gland of *Squalus acanthias*.

XV. SPECIFIC TESTS OF THE HYPOTHESIS

The accumulated experimental data in the literature are so vast that most of the experiments that one might think of as specific tests of the hypothesis have already been done. For example, it has been shown that ouabain inhibits active transport of Cl^- (50), amino acids (51), sugars (52), and uptake of I^- in cells of the thyroid gland (53). Although those results are all consistent with a direct involvement of the $(Na^+ + K^+)$ -ATPase, in driving the transport of species other than just Na^+ and K^+ , they are generally interpreted in terms of the more popular Na^+ gradient theory (54) according to which the $(Na^+ + K^+)$ -ATPase operates indirectly, by generating a Na^+ gradient, the energy of which then drives the transport of other species.

Kimmich (55), however, using chick epithelial cells, showed that inhibition of galactose uptake by ouabain took place before there could be appreciable dissipation of the Na^+ gradient. Preliminary results by Mr. C. P. Main in our laboratory have shown that in mouse mastocytoma cells ouabain inhibits uptake of α -aminoisobutyric acid before there has been dissipation of the Na^+ gradient at all, and that uptake is unimpaired when the Na^+ gradient is abolished by

replacing some extracellular Na^+ with choline.

The most crucial test of the hypothesis, of course, involves measurement of properties of intracellular water first when the ATPases are functioning, and then with the ATPases inhibited, in order to see whether their activity does, in fact, affect properties of intracellular water. Most investigations of the properties of intracellular water have been made using the techniques of nuclear magnetic resonance (NMR) or electron spin resonance (ESR). While both of these techniques are non-destructive in the sense that the spectral measurement itself does not change the properties of the water it is measuring, the gross departure from physiological conditions which seems inevitable in order for a significant intracellular signal to be obtained, detracts from their value as probes of the effect of metabolism upon water structure. Tissue is dissected from an animal after death, blotted dry, and packed into glass tubes for the spectral measurements. We have found by mimicking such conditions of preparation, that within a few minutes muscle lactate levels are high and ATP levels reduced; in tissues such as kidney and brain with high ATP turnover rates, ATP is undetectable after a few minutes. It therefore seems probable that at the time when the spectral measurement is made, the ATPases, which are very sensitive to pH and, of course, require ATP, are not functioning optimally. Since big changes are found in spin-spin and spin-lattice relaxation times for water in concentrated protein solutions or in dead muscle (56) compared with bulk water, any change in those quantities produced by metabolic process would appear as a small difference between two large quantities and would be particularly hard to detect if the tissue were already physiologically impaired. Two observations suggest that under the usual conditions of NMR measurements a metabolic component is indeed lost. Chang, Hazlewood and Woessner (57) showed that the spin-lattice relaxation time of muscle protons changed most rapidly in the first 2 hours after the death of the animal. Sachs and Latorre (58) found that the microviscosity of water in barnacle muscle fibers was higher in fresh than in starved barnacles. We have, therefore, used the technique of polarization of fluorescence to test directly the effect of the $(\text{Na}^+ + \text{K}^+)$ -ATPase upon the microviscosity of intracellular water. Our general experimental approach was to carry out paired experiments. In the first, the intensity and polarization of the fluorescence of intracellular fluorescein was measured in a cell suspension incubated under physiological conditions. In the second, the same measurements were made under identical conditions with the single exception that ouabain was added just before the measurement

was made. Briefly, those experiments showed that the $(\text{Na}^+ + \text{K}^+)$ -ATPase had two effects in all the cells investigated (human peripheral lymphocytes, mouse mastocytoma cells, and rat and guinea pig kidney cortex cells). Its activity increased both the polarization and the intensity of the fluorescence of intracellular fluorescein. (See Noted Added in Proof for further details).

The experiments have been interpreted as showing an increase in the microviscosity of intracellular water and a displacement of the fluorescein binding equilibrium induced by activity of the $(\text{Na}^+ + \text{K}^+)$ -ATPase. Assuming a microviscosity of 1 cp in the presence of ouabain, the highest average intracellular microviscosity obtained was 18 cp for guinea pig kidney cortex cells under physiological conditions. A simple calculation has shown that if the zone of water affected by the ATPase were such that the fluorescein ion did not rotate at all during the lifetime of its fluorescence the thickness of that zone would be approximately 0.5 μ . Thus, the result is consistent with a highly cohesive narrow zone of water of minimal thickness 0.5 μ , or with a wider zone of water with less drastically changed properties.

The observation that the fluorescein binding equilibrium is displaced in the direction of release of the water structure-breaking fluorescein ion from its binding sites is direct evidence for a change in the affinity of some intracellular water for solutes.

Another test of the hypothesis concerns the volume of kidney cells. When rat kidney cortex slices were incubated in the presence of graded concentrations of ouabain, not only was there an exchange of intracellular K^+ for extracellular Na^+ , but there was an interesting change in the steady-state intracellular Cl^- concentration, and in the cell volume. At low concentrations of ouabain, both intracellular Cl^- concentration and cell water content decreased, reached minimal values together, and then increased at higher concentrations. The changes were small, but significant. Since the exchange of Na^+ for K^+ was practically one-for-one, the change in volume was dictated by the change in intracellular Cl^- . The minimum in the Cl^- concentration was consistent with two opposing effects caused by ouabain. First, as the $(\text{Na}^+ + \text{K}^+)$ -ATPase was progressively inhibited, the number of functioning ATPases decreased, and therefore, the degree of accumulation of the water structure-breaking Cl^- decreased; secondly, however, as the concentration of intra-

cellular K^+ decreased the membrane potential became more positive, decreasing the degree of exclusion of all anions. In other experiments it was shown that urea, at low concentrations, was accumulated by rat kidney cortex slices with cellular swelling, and that both accumulation of urea and swelling were prevented by ouabain. As the concentration of urea increased, intracellular K^+ exchanged for Na^+ , and there was a decrease followed by an increase in intracellular Cl^- concentration and cell water content. At the same time, the accumulation ratio of urea itself decreased. The similarity between the effects of increasing concentrations of urea and of ouabain suggest that as the concentration of urea increased, it interfered with the water structures generated by ATPase activity, causing a general decrease in selectivity. In the silica gel it had also been found that urea decreased the selectivity of the gel water to K^+ relative to Na^+ (29).

If the $(Na^+ + K^+)$ -ATPase were simply a membrane-bound carrier exchanging intracellular K^+ for extracellular Na^+ and opposing the spontaneous leak of both ions through the membrane, the rate of exchange of intracellular Na^+ for extracellular K^+ should be fast when the ATPase was functioning, and slow when it was inhibited. However, the rate of exchange through leak pathways of intracellular K^+ for extracellular Na^+ should be slow, and independent of ATPase activity.

The present hypothesis predicts, however, that Na^+/K^+ exchange should be fast in either direction when the $(Na^+ + K^+)$ -ATPase is functioning and providing Na^+ with a highly permeable channel, and slow in either direction when that channel is closed by inhibition of the $(Na^+ + K^+)$ -ATPase. This prediction was confirmed in rat kidney cortex slices: when loaded with either Na^+ or K^+ exchange with the other cation was completed within 10 minutes in the absence of ouabain, and over a period of about 1.5 hours in the presence of ouabain (see Note Added in Proof). Similar experiments with red cell ghosts are proving more complex. Since, unlike kidney cortex cells, ghosts have a low and approximately equal permeability to Na^+ and K^+ in the absence of energy, acceleration of Na/K exchange cannot be induced, whatever the direction of the driving force, merely by providing a permeable channel for Na^+ whose movement is still limited by the relatively low permeability to K^+ . If, however, K^+ permeability was increased by the presence of valinomycin, ATP-containing ghosts showed rapid net movement of Na^+ either inward or outward, depending upon the conditions of the experiments (i.e., the functioning ATPase pro-

vides a Na^+ -specific channel, and if the conditions of the experiments provided a driving force for Na^+ movement inward, and allowed K^+ to move freely, intracellular K^+ exchanged rapidly with extracellular Na^+ , but only in the presence of ATP). If ghosts did not contain ATP rapid movements of K^+ and Cl^- took place, but there was no movement of Na^+ at all.

Another prediction that has been made for ghosts (2) already has some experimental support. When ghosts are made by the technique of Schwoch and Passow (59) they contain, depending upon the precise conditions of haemolysis and reconstitution, from 11 to 20 g water/g dry weight. Ghosts of that size can effect very slight active exchange of Na^+ for K^+ under the most favorable conditions. Preliminary experiments in our laboratory have shown that their ability to move Na^+ outward against a concentration gradient increased as their volume decreased. The rationale of those experiments is that the pump should be more efficient at generating highly ordered water structures if the water domain size adjacent to the pump is kept low, so that the stabilized clusters are protected from the inherent instability of large clusters.

XVI. CONCLUDING REMARKS

The hypothesis that has been described is both fundamentally simple and yet sufficiently versatile to account for the wide diversity of transport processes that different cells perform. It differs in many important respects from both the widely accepted carrier-mediated transport theory, and the less widely accepted structured water theory in its different manifestations. It shares with the pump theory the concepts that most ions are in free solution inside cells, that pumps do exist, and that most species are held in a steady-state far from equilibrium by means of processes which occur at membranes. There are, however, only three or four kinds of pumps in this hypothesis and they all use energy, not to change the affinity of a membrane-bound carrier for its passenger, but to change the affinity of a zone of intracellular water for all solutes, and at the same time change the permeability of the membrane to many solutes.

The hypothesis shares with the structured water theories the concept of changed properties of intracellular water, but suggests that all cell water need not be involved, and that as suggested by the work of Garlid (60, and the present

volume) and Hinke (61), there may be two different kinds of water with different solvent properties in a single cell. The present hypothesis would put the profoundly changed water in a zone adjacent to the inner membrane surface, and have it firmly under the control of the ATPases. The more bulk phase-like water should be found in the interior of the cell (or mitochondrion).

NOTE ADDED IN PROOF

Dr. E. D. Lewis of our laboratory has recently completed a computer simulation of the "water-powered pump" (paper in preparation) and has shown that conditions under which such a pump should work efficiently do exist. The outstanding problem in the qualitative description of the pump had been that to a Na^+ ion, for example, which suddenly found itself in a zone of highly hydrogen-bonded water generated by the $(\text{Na}^+ + \text{K}^+)$ -ATPase, the membrane, in spite of its specific ionophoric channel, still presented a formidable barrier to diffusion; whereas, an interface between two aqueous phases presented none. It was difficult to assess, therefore, the probability that some Na^+ ions would, in fact, escape to the extracellular solution rather than all diffuse backward into the interior of the cell. The solution of that problem was shown to lie in the increased viscosity and therefore decreased rate of diffusion in the ordered zone. If diffusion were the same as in normal water, there would be time for all Na^+ ions in the ordered zone to diffuse into the interior of the cell before the ordered state collapsed (a time interval of the order of msec). Therefore, the pump would not work at all. If, however, diffusion was sufficiently slow so that a Na^+ ion near the ATPase surface could move only a short distance before the ordered state collapsed, the probability that it would diffuse out through the ionophoric channel was high. Efficiency of the pump, therefore, increases with decreasing diffusion coefficient of all ions, decreasing duration of the ordered state, and increasing thickness of the ordered zone. Efficiency is also increased by a property of the $(\text{Na}^+ + \text{K}^+)$ -ATPase which was not included in the computer model. ATPase activity increases with intracellular Na^+ concentration. A Na^+ ion must bind to the inner surface of the complex (62) before the ordered state can be generated. In other words, the ionophoric channel is "opened" by a Na^+ ion inside the cell. That Na^+ ion, at least, has a high probability of escaping into the extracellular solution.

Recent evidence has reinforced the idea that there might be a single basic mechanism which cells adapt for the performance of many different kinds of work. Tada, Yamamoto and Tonomura (63) recently drew attention to the many similarities that exist in the thermodynamics and the partial reactions of the $(\text{Na}^+ + \text{K}^+)$ -ATPase, the Ca^{2+} -stimulated ATPase of sarcoplasmic reticulum, and the ATPase of the actomyosin system. Properties that many energy-transducing mechanisms appear to have in common are (1) that an early event in the series of processes that culminate in the performance of work, is a conformational change of a protein complex (64); which is accompanied by (2) an increase in the fluorescence intensity of 1-anilino-naphthalene-8-sulphonate (ANS) (65), an amphipathic probe, the fluorescence intensity of which is greatly enhanced at a water-hydrocarbon interface; (3) an increase in β -pleated sheet configuration (66) and (4) by a large increase in entropy (63).

These observations are all consistent with an energy-linked partial unfolding of the protein complex, with generation of a hydrocarbon-water interface. Properties of such interfaces have not been investigated in great detail, because their extent is always severely limited in any equilibrium system by the large decrease in entropy accompanying their formation. Thus, alkanes are only slightly soluble in water, and must be studied in extremely dilute solution; lipids form micells, which minimize the hydrocarbon-water interface, and proteins are folded in such a way that most nonpolar side chains are buried in their hydrophobic interior, out of contact with water. Nevertheless, there is no doubt that hydrocarbons have a profound ordering effect upon water, and that the effect is transmitted over considerable distances. For example, solutions of "essentially hydrophobic" compounds, such as alcohols, show solute-solute interactions at surprisingly low concentrations (67). Owicki and Scheraga (68) recently found in an *ab initio* calculation of energies in an aqueous methane solution that each methane molecule ordered 23 water molecules. A most dramatic example of the existence of a long-range force extending into an aqueous solution from a hydrocarbon surface is the observation of Gingell and Todd (69) that negatively charged red blood cells took up an equilibrium position below a negatively charged hydrocarbon-water interface. They calculated that a weak attractive force must be operative at least 240 nm from the surface.

The possibility is now being considered, therefore, that the common feature of mechanisms by which cells perform work at a molecular level might be an energy-linked conformational

change which generates a hydrocarbon-water interface and extensive long-range hydrophobic hydration. The inherent instability of such an interface allows it only a transient existence before it is obliterated, and the protein complex reverts to its more stable conformation. Water in a zone vicinal to the ATPase then oscillates between a state in which it constitutes a region of hydrophobic hydration, and a state in which it has properties resembling those in a bulk aqueous phase. Rapidly-induced oscillations in chemical potential of all solutes and functional groups within the altered aqueous zone then drive the association-dissociation cycles needed to provide the energy for muscle contraction, active transport or oxidative phosphorylation. Notice that each ATPase requires, in addition to this common feature, a specific attribute which determines the particular kind of work that it performs. Thus, the ($\text{Na}^+ + \text{K}^+$)-ATPase, the Ca^{2+} -ATPase and the actomyosin ATPase might all establish the same transient gradients in chemical potential of all solutes within the region of hydrophobic hydration, but the ($\text{Na}^+ + \text{K}^+$)-ATPase pumps Na^+ , and the Ca^{2+} -ATPase pumps Ca^{2+} , because of their highly specific permeability changes, and the myosin ATPase causes the filaments to slide because of the relative positions of the actin binding sites and the ATPase.

If an ATPase does indeed operate by inducing zones of hydrophobic hydration, it should be possible to detect changes in average properties of water in a concentrated suspension of an ATPase coincident with its activation. Two isolated ATPases which are particularly suitable for testing predictions of the model are the Ca^{2+} -ATPase of sarcoplasmic reticulum and the actomyosin ATPase. Fragmented sarcoplasmic reticulum (SRV) isolated by differential centrifugation, forms vesicles so oriented that the surface that faced the cytoplasm *in vivo*, faces outward. The putative zones of hydrophobic hydration, therefore, are external to the vesicle, and experimentally accessible. The ATPase is Mg^{2+} -dependent, and is activated by Ca^{2+} . When actomyosin is extracted from homogenized muscle together with the regulatory proteins troponin and tropomyosin, its ATPase activity is also activated by Ca^{2+} in the presence of Mg and ATP. Again the hypothetical zones of hydrophobic hydration are accessible since actomyosin is a membrane-free system. The most stringent tests of the model are those experiments designed to test changes in properties of water that are essential for efficient performance of work. For example, the computer simulation showed that an ATPase could not pump ions unless high viscosity in the zone of hydrophobic hydration slowed diffu-

sion through the zone. Accordingly, changes in the microviscosity of water in a concentrated suspension of SRV have been followed by measuring the polarization (p) of fluorescence of fluorescein. An increase in p reflects an increase in the rotational relaxation time of the fluorescein ion, and therefore in the microviscosity of the water in which it is dissolved. Previously we had shown that p decreased with inhibition, and increased with stimulation, of the $(\text{Na}^+ + \text{K}^+)$ -ATPase in intact cells in which the water should be ordered at the inside surface of the membrane (70,71). Figure 13 shows that in a concentrated suspension of SRV activation of the ATPase with ATP in the presence of Mg^{2+} and Ca^{2+} resulted in an immediate increase in p/p_0 (where p_0 is the value of p before activation), followed by a steady decline back to unity as ATP was consumed. The magnitude of the increment increased in the presence of poly (ethylene oxide), (PEO)-6000 (a stabilizer of water structure) and decreased in the presence of urea, which disrupts intrinsic water structure, at concentrations much lower than those required for disruption of membranes or denaturation of proteins. Each experiment on suspensions of SRV has been repeated by Dr. V. A. Knight of our laboratory, on suspensions of actomyosin, which gave a similar result to that shown in Figure 13; the only important difference was that p/p_0 continued to rise, reaching a maximum just before ATP was exhausted, a time when the actomyosin ATPase has been shown to have maximal activity. Again PEO increased and urea decreased the increment (72) (paper submitted for publication).

A second essential requirement of the model is that activation of an ATPase should increase activities of all small cations, both in the zones of hydrophobic hydration, and, to the extent that cations diffuse out of those zones before their collapse, in the bulk aqueous phase. Therefore, a sodium-selective electrode or a pH electrode sensing the bulk aqueous phase of a suspension of isolated ATPase, should record a transient increase in activity when the ATPase is activated; and that should be true in spite of the fact that the SRV ATPase pumps only Ca^{2+} (neither Na^+ nor H^+) and the actomyosin ATPase pumps no ions at all. Figure 14 shows the result of a typical experiment. As with the fluorescence experiments, an effect should be detectable only in a highly concentrated suspension where a significant fraction of the total water would be ordered at any one time. Upon activation of the ATPase with ATP in the presence of Ca^{2+} and Mg^{2+} , the activity of Na^+ increased immediately to a maximum, and then returned to its initial level as ATP was consumed. The effect was rather small. That is consistent with diffusion

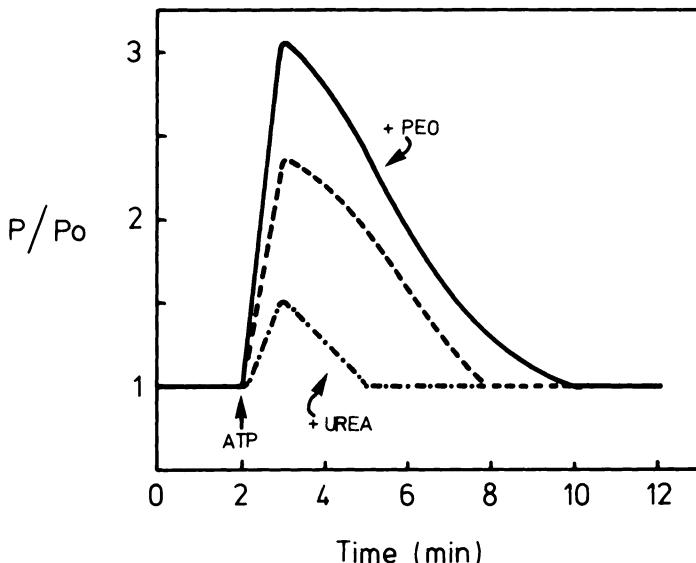


FIGURE 13. The effect of activation of the ATPase upon the polarization of fluorescence of fluorescein in suspensions of SRV. Conditions were: protein, 50 mg cc^{-1} ; MgCl_2 , 50 mol m^{-3} ; CaCl_2 , 5 mol m^{-3} ; fluorescein, $7.5 \times 10^{-6} \text{ mol dm}^{-3}$; ATP (50 mol m^{-3}) was added at the time indicated by the arrow. PEO (3.3 mol m^{-3}) or urea (50 mol m^{-3}) were added before ATP. All solutions were made up in tris(hydroxymethyl)-aminomethane (tris) maleate (100 mol m^{-3}).

of some Na^+ ions out of the hydrophobic zones, and their return, as ATP was consumed, back into previously ordered zones. The effect upon the proton is more dramatic. Protons produced by hydrolysis of ATP would originate in an ordered zone. Unlike other small cations, their diffusion would not be significantly slowed by the increased viscosity, since they move through water by protonic conduction from one water molecule to another; increased hydrogen-bondedness might even increase their rate of diffusion. The combination, therefore, of high activity in the ordered zone, and rapid conduction through it, could result in the large increase in activity of the proton sensed by a pH electrode in the bulk aqueous phase (Figure 14). That high level of activity should not be sustained, however, because as ATPase activity declined a larger volume of water would become accessible to

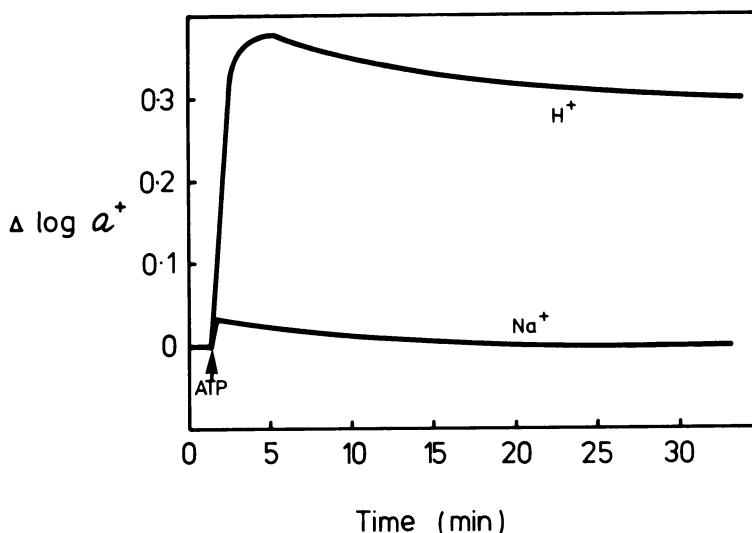


FIGURE 14. The effect of activation of the ATPase upon the activities of Na^+ and H^+ , in suspensions of SRV. Conditions were the same as in Figure 13, except that NaCl (50 mol m^{-3}) was added to the suspension instead of fluorescein, and activation was with tris ATP.

protons, and their concentration in the bulk phase would fall, as did the activity in Figure 14. When the same amount of ATP was hydrolyzed by a dilute suspension of vesicles, the activity of the protons rose steadily and became constant without an overshoot. Concentrated suspensions of actomyosin gave a similar result, but the maximum in a_{Na^+} was not reached until ATPase activity was at its highest, just before ATP was exhausted. In both systems the increments in activity were increased by PEO and decreased by urea (paper in preparation).

Outgrowths of ordered water accompanying ATPase activity should also change the intensity of light scattered by suspensions of ATPases. Figure 15 shows the changes in light scatter observed at 90° when the SRV-ATPase was activated by a small amount of Ca^{2+} in the presence of excess MgATP . With each small addition of Ca^{2+} , scatter increased, flattened, and then returned to a steady level higher than before addition of Ca^{2+} ; the extent of the return decreased with sequential additions until the signal rose with the final addition and remained constant. That result can be

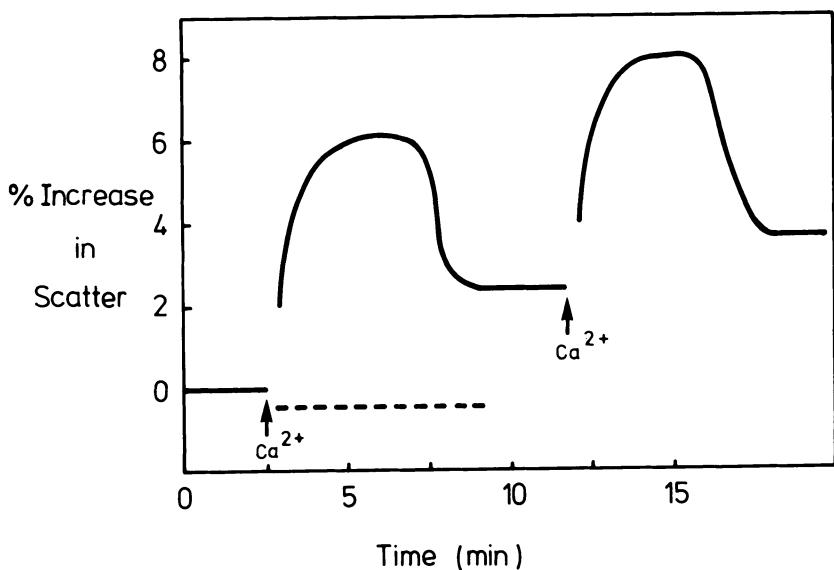


FIGURE 15. Changes in light scatter observed at 900 when the ATPase of SRV was sequentially stimulated by small additions of Ca^{2+} . Conditions were: protein, 1 mg cc⁻¹; MgCl_2 , 5 mol m⁻³; ATP, 5 mol m⁻³; CaCl_2 (0.1 mol m⁻³) was added at the time indicated by each arrow; the wavelength was 600 nm.

explained in the following way: activation of the ATPase with Ca^{2+} increased scatter, partly because of ATPase activity *per se*, and partly because of swelling or precipitation of phosphate following active uptake of Ca^{2+} . When a critical amount of Ca^{2+} has been sequestered, further decrease in external Ca^{2+} decreased activity of the ATPase; the level to which the signal returned was determined by the total osmotic change that had taken place, and the level of residual ATPase activity set by the new steady-state concentration of external Ca^{2+} . It was possible to separate effects of Ca^{2+} accumulation from effects of ATPase activity *per se* by performing the same experiments on vesicles which had been shown to have become so leaky to Ca^{2+} that they were incapable of retaining Ca^{2+} that was pumped in. With such vesicles a single addition of Ca^{2+} resulted in maximal rise of the scattered signal, because one addition was enough for the ATPase to become and remain maximally stimulated. That increment in scattered signal was increased by PEO and decreased by urea. A detailed analysis of the results suggested

that both particle size and relative refractive index increased with ATPase activity. When the actomyosin ATPase was activated by Ca^{2+} there was an immediate rise in scatter which continued to increase until ATP was exhausted (papers submitted for publication).

Many other water-related properties of the suspensions of SRV and actomyosin are in the process of being measured; it is well known that the amount of "bound water" in a system varies widely with the method of measurement. It is hoped that estimates of the degree of structural alteration of water and the number of water molecules associated with hydrophobic hydration of a single ATPase will finally emerge when more techniques are used to follow changes in water-related properties accompanying activation of an ATPase.

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IONIC SELECTIVITIES OF VICINAL WATER
IN THE PORES OF A SILICA GEL

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One of the most fundamental features of biological systems is the unequal distribution of potassium ions and sodium ions inside and outside the cell.

Wiggins (1,2,3) - among others (4,5,6) - has proposed that changes in the structure of water proximate to an interface may play a role in the selective enhancement of potassium ions over sodium ions; we shall refer to such structurally modified interfacial water as "vicinal water". To test this proposal Wiggins carried out studies of both a model system (a silica gel) and a cellular system (rat kidney cortex). For the silica gel measurements were made to determine the distribution of sodium and potassium ions between the solution in the capillary pores of a silica gel and the bulk solution as a function of temperature. She observed that the concentration of potassium ions was selectively enhanced in the pores of the silica gel over the concentration of sodium ions. Furthermore, this selectivity was found to possess a complex temperature-dependence, showing maxima at about 15, 30 and 45°C - independent of the nature of the accompanying anion: sulfate, iodide or chloride. Similarly,

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Wiggins measured the distribution of potassium and sodium ions in rat kidney cortex slices and again found the selectivity of the system to be highly temperature-dependent, with sharp maxima at about 15 and 29°C. The similarities of the data obtained for the silica gel model system to the data for the cellular system suggested that intracellular water structuring plays a significant role in determining the ion distribution. (See Wiggins [3, this volume]; see also [7]). Because of the importance of Wiggins' findings, it was of considerable interest to test her findings. Hence, we present in this paper the results of our own study on the potassium-sodium ion distribution in a silica gel. In addition, we report on similar experiments to determine the potassium-magnesium distribution and the potassium-calcium distribution (the latter in the presence and absence of ouabain).

Experimental

Experiments were performed with a silica gel with an average pore diameter of about 140 Å (Davison silica gel, Code 62; pore volume: 1.15 cm³/gm; surface area 340 sq. m/gm). The gel was cleaned by careful washing in a vertical column using 1.5 normal nitric acid. The acid wash was followed by flushing with distilled water until the effluent had a pH between 2 and 3. Samples of the effluent water were analyzed by atomic adsorption to insure the complete removal from the gel of the ions to be studied: sodium, potassium, magnesium and calcium. Finally, the gel was dried at 110°C for at least 24 hours. Salt solutions were prepared from either analytical trade reagents, or ultra-pure reagents and used without further purification. The following set of solutions were prepared: sodium-potassium chloride ($7.5 \cdot 10^{-4}$ M NaCl + $7.5 \cdot 10^{-4}$ M KCl); magnesium-potassium chloride ($7.5 \cdot 10^{-4}$ M MgCl₂ + $7.5 \cdot 10^{-4}$ M KCl) and calcium-potassium chloride ($7.5 \cdot 10^{-4}$ M CaCl₂ + $7.5 \cdot 10^{-4}$ M KCl). Finally, an equimolar solution of CaCl₂ and KCl was prepared both with and without $1 \cdot 10^{-3}$ M ouabain. Equilibration was achieved in a polythermostat (Temperature Gradient Incubator, Model 675, Scientific Industries, Inc., Bohemia, Long Island, New York). The polythermostat provides 30 different constant temperatures simultaneously, thus permitting the study of the ion selectivity of the gel as a function of temperature at closely spaced intervals. The gradient bar of the polythermostat is mounted in such a fashion as to provide a rocking motion around an axis parallel to the length-axis of the gradient bar. In this manner, the samples were continuously agitated

(oscillation frequency approximately 60 per minute). Runs were made between 8° and 60°C. The composition of the supernatant liquid was determined at different pre-selected mixing times to ascertain the time required for complete equilibration between the liquid in the pores and the bulk. Analyses were performed on a Perkin-Elmer Atomic Adsorption Spectrophotometer (Model 403).

Data Treatments

The results are expressed in terms of a selectivity coefficient, K, (Wiggins, 1) defined as the ratio of the apparent partition coefficients where the apparent partition coefficient (λ), is the ratio of the concentration of the salt in the pore water of the gel to its concentration in the bulk phase at equilibrium. In other words,

$$K = \frac{\lambda_k}{\lambda_{Na}}$$

where

$$\lambda_k = \frac{[K^+]_i}{[K^+]_o}$$

and

$$\lambda_{Na} = \frac{[Na^+]_i}{[Na^+]_o}$$

As indicated by Wiggins, the selectivity coefficient, K, is less affected by systematic errors (due to difficulties in analytical techniques) as such errors will tend to cancel out when forming the ratio of the apparent partition coefficients.

Results

The selectivity coefficient (K) for the partitioning of the potassium and sodium ions is shown in Figure 1. As reported by Wiggins, the values of K are all larger than 1, and exhibit distinct peaks at almost precisely the same temperatures as reported by Wiggins, namely, 14, 30 and 45°C.

Figure 2 shows the selectivity coefficient (K) for an equimolar mixture of potassium chloride and magnesium chloride.

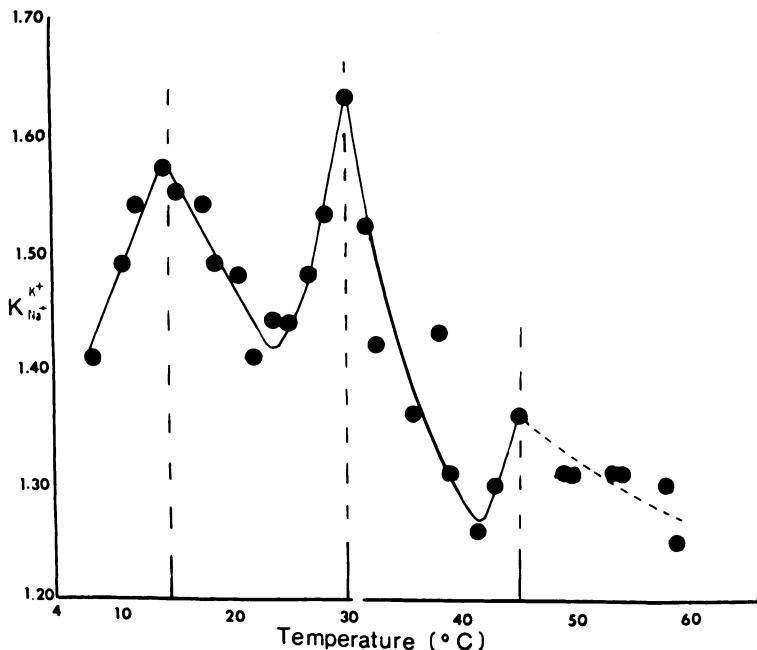


FIGURE 1. Selectivity coefficient, K , for potassium/sodium ions between the pores of a silica gel and bulk solution as a function of temperature.

Again, maxima are observed in the distribution coefficient (K) for equimolar solutions of calcium chloride and potassium chloride (over a limited range of temperatures). In this graph are shown the data obtained for equimolar potassium and calcium ions in the presence and absence of ouabain (10^{-3} molar).

It is seen that both sets of data exhibit a sharp maximum near 15°C . The presence of ouabain does not appear to influence the distribution of the two ions in the silica gel.

Discussion

The results obtained in this study have confirmed, essentially quantitatively, the data reported by Wiggins (1973). Thus, potassium ion concentration is selectively enhanced in the pores of the silica gel over the sodium ion concentration and the distinct temperature dependence, namely, near 15 , 30 and 45° , has been reproduced. Note that in the

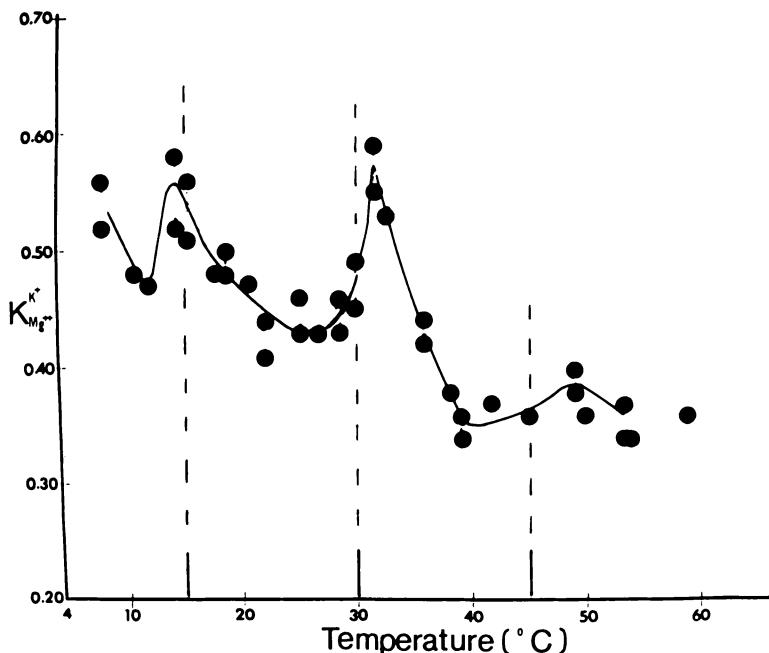


FIGURE 2. Selectivity coefficient, K , for potassium/magnesium ions between the pores of a silica gel and bulk solution as a function of temperature.

present study the silica gel had an average pore diameter of about 140 Å compared to the pore diameter of 25 Å for the silica gel used by Wiggins (Davison gel, Code 950). In spite of the differences in pore diameters and in electrolyte concentration, the observed distribution coefficients are essentially identical.

Table I shows a comparison of the distribution coefficients obtained by Wiggins and by the present authors.

The agreement shown in Table I is satisfactory. Furthermore, a t-test shows the values for K at the maximum near 15°C compared to the K values near the minimum (25°C) to be statistically significant, at least on the 95% confidence level, and in the case of the 30° anomaly, on the 99% confidence level. It is seen from Figure 2 that the distribution of potassium and magnesium also is highly temperature dependent, again showing sharp maxima at the temperatures of vicinal water structure transitions. For the potassium-magnesium data, a t-test also confirmed that the relative

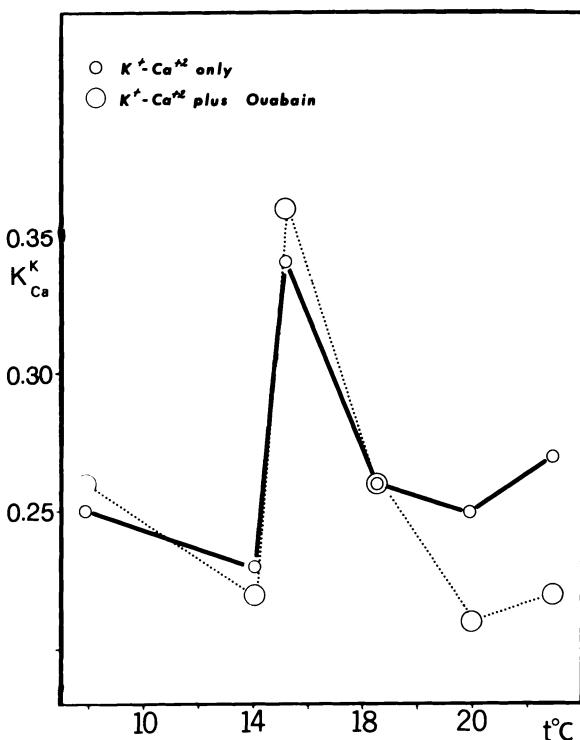


FIGURE 3. Selectivity coefficient, K , for potassium/calcium ions between the pores of a silica gel and bulk solution as a function of temperature. Small circles: K^{+}/Ca^{2+} only. Large circles: in the presence of 10^{-3} molar ouabain.

maxima and minima were statistically significant, at least at the 95% confidence level. Finally, in the case of the partitioning of potassium and calcium, reliable data were obtained only near the first of the vicinal water transition temperatures. However, these data do confirm the existence of a pronounced temperature dependence, again giving rise to a peak in K near 15°C . Furthermore, as would be expected, if only solvent structure is involved in establishing the observed ion partitioning in the model system, the presence of ouabain does not influence the ion distribution.

The agreement between the results obtained by Wiggins and those reported in the present study is of considerable interest. Note that the pore diameters of the two gels used differed by almost a factor of six (25 vs. 140 Å) and hence

TABLE I

<i>T</i> ($^{\circ}$ C)	<i>T</i> _{average} ($^{\circ}$ C)	<i>K</i> _{Na⁺} ^K	Percent Difference	Source
14 $^{\circ}$	14.50	1.58 (Max.)	8	H & D-H
15 $^{\circ}$		1.72 (Max.)		W
24 $^{\circ}$	24.50	1.41 (Min.)	7	H & D-H
25 $^{\circ}$		1.52 (Min.)		W
30 $^{\circ}$	30 $^{\circ}$	1.65 (Max.)	3	H & D-H
30 $^{\circ}$		1.70 (Max.)		W
37 $^{\circ}$	39.00	1.26 (Min.)	0	W & D-H
41 $^{\circ}$		1.26 (Min.)		H

Symbols:

- Max.: Maximum in K
 Min.: Minimum in K
 W: Wiggins
 H & D-H: Present study

the cross-sectional areas differed by a factor of nearly 36. Furthermore, the concentrations of electrolytes differed by a factor of 100. Nonetheless, our results agree almost quantitatively; we take this agreement to indicate that vicinal water in general extends beyond the larger of the pore radii employed (i.e., 70 Å or 0.0070 μm), consistent with the notion that vicinal water may extend over distances of 0.01 to 0.1 μm (8,9,10). The independence of the electrolyte concentration is also in agreement with the "paradoxical effect"; i.e., the observation that, to a first approximation, vicinal water structures are independent of the chemical nature of the confining solid and independent of the nature and concentration of the solutes present (at least up to about 0.5 molar)(11,12).

The observed ion distributions strongly suggest the notion that vicinal water affects ionic solute activities. In a sense, vicinal water represents a solvent phase with properties which are distinctly different from those of a bulk aqueous phase. As discussed elsewhere (12) vicinal water may be induced by proximity to both ionic, polar or

non-polar solid surfaces. It would appear that this observation, together with the observed temperature dependencies, cannot readily - or at all - be explained in terms of Ling's "polarized multilayers" (13,14).

Summary

The remarkable results reported by Wiggins for the potassium/sodium ion selectivity of pore water in silica gel have been reproduced, essentially quantitatively. In addition, the results obtained with potassium/magnesium and potassium/calcium also support the interpretation of the ion-partitioning phenomenon as due to the effects of vicinal water structure.

NOTE ADDED IN PROOF

A detailed description of the Temperature Gradient Incubator (Polythermostat) and some illustrative applications of this device are given in a forthcoming proceedings volume (15).

With regard to the "paradoxical effect", attention is called to recent studies from Prof. Peschel's laboratory, in which extrema in disjoining pressure are observed as a function of electrolyte concentration (16,17). These results suggest that as regards the solutes the notion of the "paradoxical effect" may need to be revised.

Finally, ultrasonic absorption and velocity measurements (18,19) and dielectric loss measurements (at 5 Gigahertz) (20) on polystyrene sphere suspensions have added further information regarding the properties and extent of vicinal water. The preliminary dielectric data agree with our previous suggestion (21) that electrical double layers cannot account for the observed temperature dependence of the vicinal water transitions (20) (nor do they appear to affect the vicinal water structures).

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A ROLE FOR WATER
IN BIOLOGICAL RATE PROCESSES¹

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I. INTRODUCTION

The properties of water adjacent to most solid interfaces differ from the corresponding bulk properties. Hence it must be concluded that the structure of the interfacial water differs from the structure of bulk water. Such "surface modified" water is referred to as vicinal water.

A considerable amount of literature exists in which various aspects of interfacial water are described. The reader is referred to brief reviews of this literature, presented in various chapters of this volume. In this Introduction, reference is made only to two sets of experiments which particularly illustrate the type of anomalous properties which vicinal water possesses. These two sets of experiments were carried out in the laboratories of two of the contributors to this volume.

Peschel and Adlfinger (1,2) measured the disjoining pressure as well as the viscosity of water between two highly polished quartz plates. They observed maxima in the disjoining pressure near 15, 30 and 45°C for both the viscosity

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and the disjoining pressure. The plates were separated by distances varying between 0.01 and 0.09 μm . In all probability, the maxima observed reflect structural transitions of the vicinal water between the plates. The disjoining pressure is of particular interest as thermodynamic properties of vicinal water may be deduced from such measurements.

Clifford (3) has reviewed some aspects of disjoining pressure. Following Padday (4), Clifford splits the disjoining pressure into several additive components:

$$\Pi = \Pi_{\text{SL}} + \Pi_{\text{LL}} + \Pi_{\text{EL}} + \Pi_i \quad (1)$$

In this equation, Π is the observed (total) disjoining pressure; and Π_{LL} and Π_{EL} are the disjoining pressures due to London dispersion forces (SL referring to solid/liquid interactions, while LL refers to liquid/liquid interactions); Π_{EL} is the disjoining pressure due to electrical interactions - primarily due to repulsive double layer forces, and Π_i is the disjoining pressure due to the effect of the solid surface upon the liquid structure. As all the components of the disjoining pressure, except Π_i , must be assumed to be monotone functions of temperature, Drost-Hansen (5) concluded that the maxima observed by Peschel and Adlfinger result from structural changes in the liquid near the solid interfaces. Unfortunately, at present, Π_i cannot be calculated theoretically. Thus, it is not possible to compare the observations of Peschel and Adlfinger to any theoretical model at this time. In addition to the maxima at 15, 30 and 45°C, Peschel and Adlfinger observed also that the structural effects imposed by the quartz surface appear to be manifested over distances of the order of 0.1 μ (1000 Å) although it is not necessarily implied that all the water, over this distance, is modified. The value of about 0.1 μ probably represents an upper limit for the distance over which structural modifications, imposed by a solid surface, may be observed. Figures 1 and 2 show the results by Peschel and Adlfinger.

The second important study to be discussed briefly is that by Wiggins (6) who observed maxima in the selectivity coefficients of potassium and sodium ions in the pores of a silica gel at 15, 30 and 45°C. The selectivity coefficient of potassium relative to sodium is calculated from the individual partition coefficients. Thus:

$$\lambda_{\text{Na}} = \frac{[\text{Na}^+]_o}{[\text{Na}^+]_i} \quad (2)$$

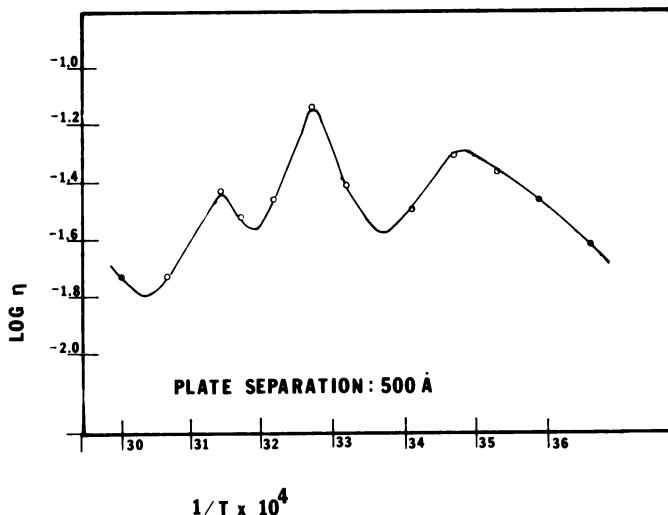


FIGURE 1. Viscosity of water between quartz plates as a function of reciprocal absolute temperature. The data shown are for a 500 Å plate separation. Data from Peschel and Adlfinger (1).

Here, λ_{Na} is the partition coefficient for sodium ions; $[\text{Na}^+]_i$ is the concentration of sodium ions inside the pores, while $[\text{Na}^+]_o$ is the concentration of sodium ions in the bulk phase. The partition coefficient for potassium ions is calculated similarly. The selectivity coefficient for potassium relative to sodium, $K_{\text{Na}^+}^{\text{K}^+}$, is then defined as:

$$K_{\text{Na}^+}^{\text{K}^+} = \lambda_{\text{K}}/\lambda_{\text{Na}} \quad (3)$$

In addition to the maxima in the $K_{\text{Na}^+}^{\text{K}^+}$ at 15, 30 and 45°, Wiggins found that the value for $K_{\text{Na}^+}^{\text{K}^+}$ was always larger than 1 at all temperatures, and independent of the type of anion present (sulfate, iodide or chloride). Recall, K^+ is selected over Na^+ in biological systems. As described in another chapter in this volume, the findings by Wiggins have recently been confirmed in the Laboratory for Water Research by Hurtado and Drost-Hansen (7). Wiggins' data are shown in Figure 3.

The results of these experiments by Peschel and Adlfinger and by Wiggins are consistent with the concept, introduced earlier by one of the present authors (W. Drost-Hansen, 8,9) of the existence of vicinal water. Thus, vicinal water appears to be ordered differently from bulk water due to its proximity to an interface and exhibits structural transitions

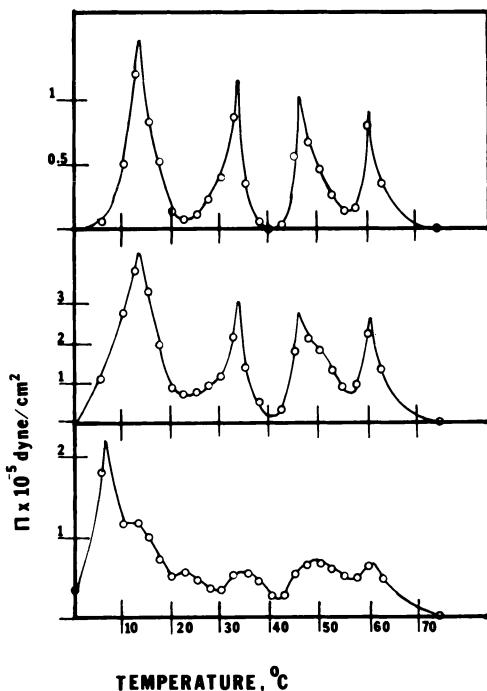


FIGURE 2. The disjoining pressure of water between quartz plates as a function of temperature for various plate separations. The plate separations from top to bottom are 500, 300 and 100. Note the maxima near 15, 30 and 45°C. Data from Peschel and Adlfinger (2).

near 15, 30 and 45° (and likely also near 60°). Additionally, this ordering appears to take place over distances of the order of 0.01 μ up to about 0.1 μ from any surface.

Anomalies in the temperature responses of a variety of physiological parameters for many organisms have frequently been reported at the same temperatures at which vicinal water undergoes transitions (see particularly Drost-Hansen, 9,10, 11; and Nishyama, 12,13). Thus, it is not unreasonable to conclude that at least some of the water in cells is "vicinally structured", considering the vast number of intra-cellular interfaces present (macromolecules, membranes, organelles, etc.). Wiggins, indeed, observed similar results when measuring the potassium-sodium distribution in rat renal cortex instead of a silica gel (see Figure 4).

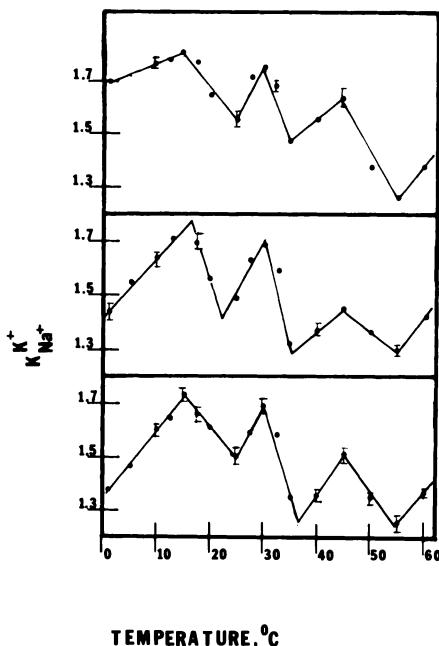


FIGURE 3. The selectivity coefficient ($K_{\text{Na}^+}^{K^+}$) for pore water in silica gel as a function of temperature for solutions initially equimolar in Na^+ and K^+ . The anions present are (from top to bottom): SO_4^{2-} , I^- and Cl^- . Note the maxima near 15, 30 and 45°C. Data from Wiggins (6).

Finally, we note that Braun and Drost-Hansen (14) observed that water adjacent to a variety of non-biological surfaces, as well as the "surface" of biologically important macromolecules, appears to possess a specific heat of approximately 1.25 calories/gram at 25°C, and Clegg (this volume) has observed a similar result in intact living cells of *Artemia*. It is of interest also that anomalously high values for specific heat of aqueous systems have recently been reported by Angell and co-workers (15) from studies of highly supercooled solutions.

In the following sections of this chapter we will discuss some notions regarding vicinal water in cellular systems, both qualitatively and quantitatively. The present authors' interest is in the likely role of vicinal water 1) in determining the rates of various metabolic events as well as 2) qualitative changes in metabolism apparently induced by changes in vicinal water structure. Specifically,

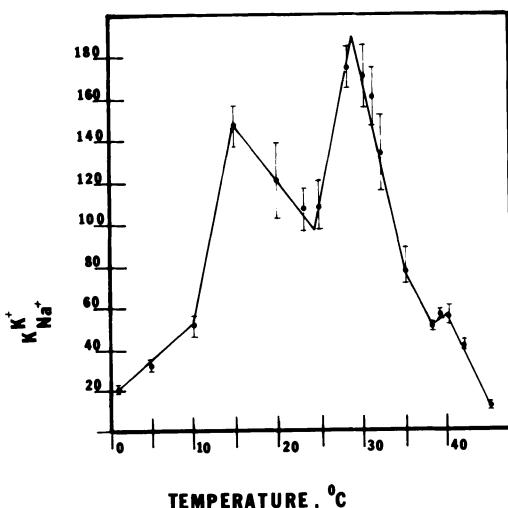


FIGURE 4. The selectivity coefficient ($K_{Na^+}^{K^+}$) for water in rat renal cortex as a function of temperature. Data from Wiggins (6).

the present authors propose the hypothesis that metabolic anomalies, near the vicinal water transition temperatures, arise from water-induced protein conformational or configurational transitions. Macromolecules other than proteins may also be affected by vicinal water structures.

II. EXPERIMENTAL STUDIES

Two systems have been studied recently in the Laboratory for Water Research; namely, germinating turnip seeds, and the growth of a green thermophilic alga (*Cyanidium caldarium*). In both of these systems, it appears that growth (or metabolism) is affected by vicinal water structure as manifested by abrupt changes in behavior near the vicinal water transition temperatures.

In the case of the germinating turnip seeds, abrupt changes in the energy of activation of the germination process occurs near 15 and 30°. Figures 5, 6 and 7 show some of the experimental results obtained (16). Figure 7 is particularly interesting: the maxima to either side of 30°C seem to indicate different metabolic regimes. Above 30°, the young plants, while growing quickly, do not develop their charac-

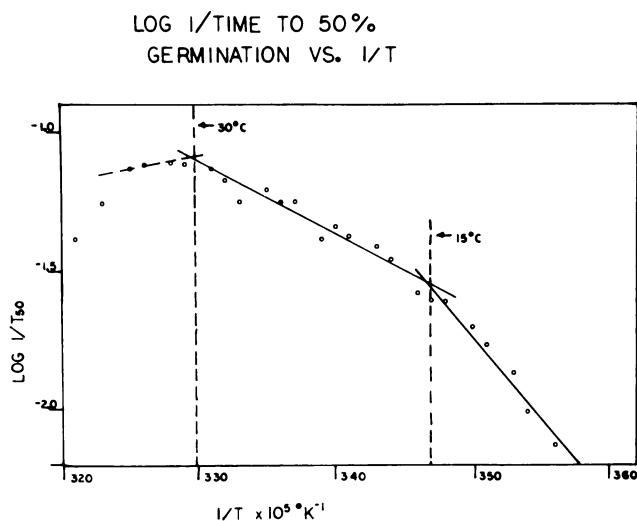


FIGURE 5. Log (reciprocal of time in hours to 50% germination) for turnip seeds as a function of reciprocal absolute temperature.

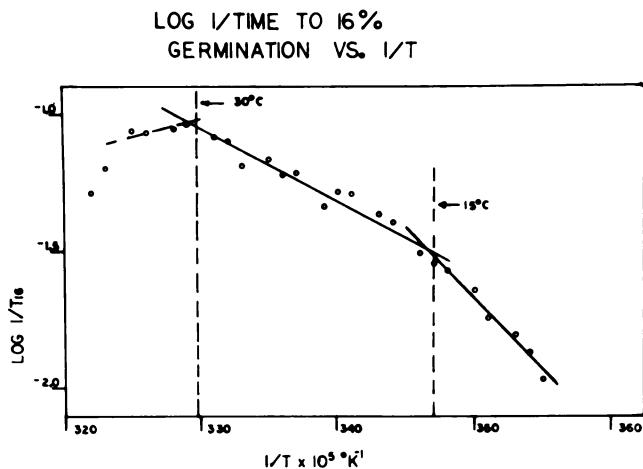


FIGURE 6. Log (reciprocal of time in hours to 16% germination) for turnip seeds as a function of reciprocal of absolute temperature.

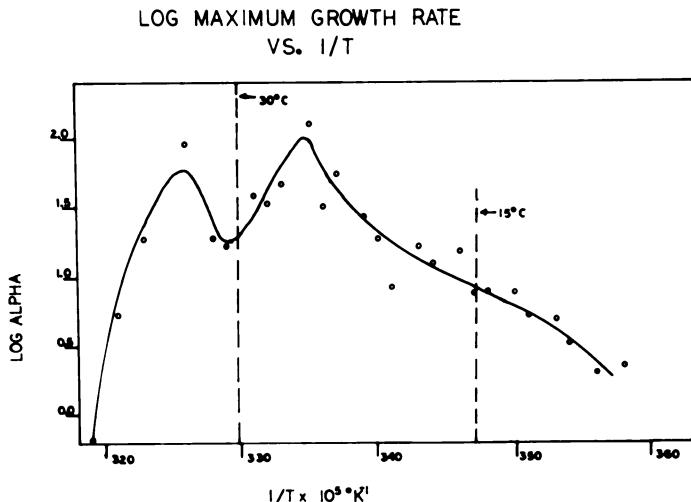


FIGURE 7. *Log (maximum germination rate) for turnip seeds as a function of reciprocal absolute temperature.*

teristic green color, indicating the absence (or minimal contents) of chlorophyll. Also, at the minimum near 30°, the roots emerge but the stems do not. Below 28° normal seedlings were produced.

Cyanidium caldarium also exhibits abrupt changes in behavior near the vicinal water transition temperatures. Figure 8 shows the growth of *C. caldarium* as a function of temperature; note the minima near 31 and 43°. In these experiments, the only nitrogen source was ammonium ion. It appears from the data of Rigano and Violante (17) that the relatively sharp growth maximum between 20 and 30° disappears when ammonium is replaced by nitrate as the nitrogen source. Testing is underway in this laboratory to examine this effect; see Figure 9. If the observation should prove correct, it would appear that at least some metabolic pathways are influenced - and possibly "regulated" - by the vicinal water structures.

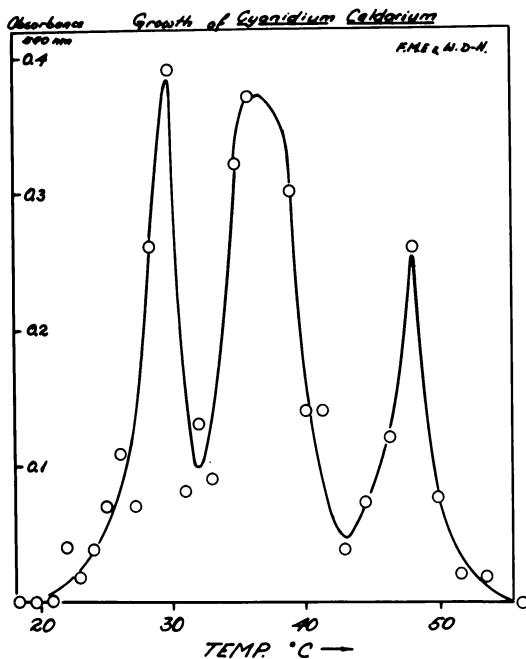


FIGURE 8. Amount of growth (measured as absorbance at 540 nm) of Cyanidium caldarium after 3 weeks, as a function of temperature.

III. DISCUSSION

A. Overview

In the previous sections we have indicated briefly some possible qualitative aspects of the role of vicinal water in cellular systems. In this section an attempt will be made to place these concepts into a unifying framework. Some historical information, not previously introduced, will be discussed first.

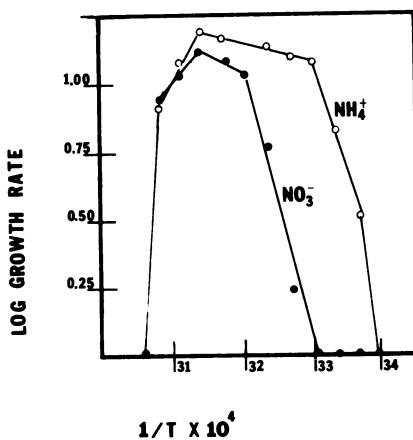


FIGURE 9. Arrhenius graph of the growth rate for *Cyanidium caldarium*. The solid circles indicate growth data obtained when NO_3^- was used for the nitrogen source. The open circles indicate data for growth in which NH_4^+ was used for the nitrogen source. Data from Rigano and Violante (17).

B. Development and Conclusions Regarding Rate Processes in Biological Systems

The study of rate processes in biological systems is almost as old as the quantitative study of rate processes in ordinary chemical systems. In fact, Arrhenius (18), in the late 19th and early part of the 20th century, published a number of papers on this subject; he felt that rates of reactions in biological systems could generally be expected to follow his rate expression:

$$k = Ae^{-E_a/RT} \quad (4)$$

where k is a rate constant, A a pre-exponential factor, E_a the activation energy and RT has its usual meaning. At high temperatures, however, the apparent E_a fell, supposedly due to denaturation of some enzyme. This view is "essentially" correct, but is indeed complicated by the role of vicinal water structures.

At about the same time, Blackman (19,20) and Pütter (21) independently proposed that a given metabolic process may be governed by a "controlling reaction" - the "master reaction".

This supposition was based on the apparent linearity, over rather small temperature intervals, observed in some Arrhenius graphs. This concept came to be known as the *Blackman-Pütter Principle*. Somewhat later, Crozier (22-31) modified and extended the work of Blackman and Pütter. Crozier suggested that at certain "critical temperatures" the so-called "rate limiting reaction" changed. This idea, however, was not well received, particularly as no fundamental reason for the abrupt changes existed. It now appears that Crozier's "critical temperatures" frequently (though certainly not invariably) correspond to the vicinal water transition temperatures.

More recently, Johnson and Eyring (32) proposed a well known equation for the rates of many biochemical processes. As is seen below, the equation does not yield a straight line in a typical "Arrhenius-type plot", but rather a broad maximum representing a more or less truncated peak. The equation proposed by Johnson and Eyring is:

$$G = \frac{cT e^{-\Delta H^\ddagger / RT}}{1 + e^{-\Delta H / RT} e^{\Delta S}} \quad (5)$$

where G is the rate of a given physiological process, ΔH^\ddagger is the enthalpy of activation, ΔH and ΔS are the changes in enthalpy and entropy respectively for the denaturation process. The denaturation is assumed to be straightforward ($N \rightleftharpoons D$). The constant, c , contains a number of terms, including ΔS^\ddagger , the entropy of activation.

Figure 10 shows an application of the Johnson-Eyring (32) equation. In this case, the phenomenon studied was the luminescence of *Vibrio phosphorescens*. Note the difficulty of fitting the equation to the data. The greatest deviations from the fitted curve arise below 15° and above 30°C. Figure 11 shows the same data as in Figure 10 fitted to straight line segments.

The present authors do not take issue with the derivation of the Johnson-Eyring equation as far as its correctness is concerned in terms of the original, simplified underlying theoretical assumptions. However, we propose that it is necessary to modify the Johnson-Eyring Approach to allow for the changes in vicinal water structures at the thermal transition temperatures (33). Thus, when structural transitions occur in vicinal water these may well impose conformational and/or configurational constraints upon the proteins. These

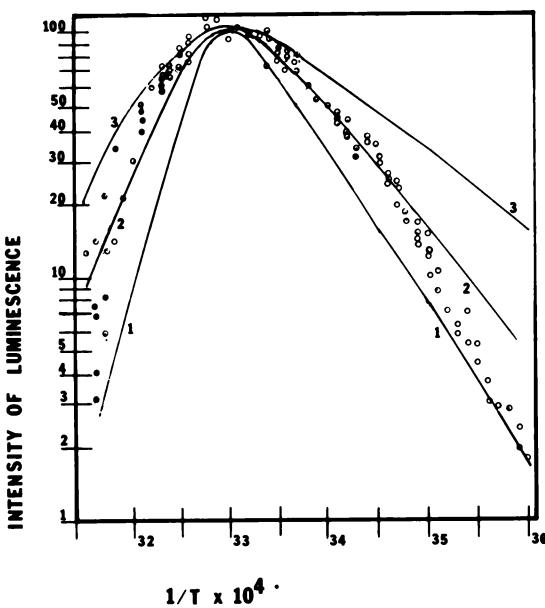


FIGURE 10. The intensity of luminescence for Vibrio phosphorescens as a function of reciprocal absolute temperature. The curved lines represent the Johnson-Eyring expression (Equation 5).

In Curve 1: $\Delta H^\neq = 30,000$
 $\Delta H = 100,000$
 $\Delta S = 328.29$
 $c = 0.475 e^{49.60}$

Curve 2: $\Delta H^\neq = 22,400$
 $\Delta H = 70,000$
 $\Delta S = 229.60$
 $c = 0.49 e^{36.96}$

Curve 3: $\Delta H^\neq = 15,000$
 $\Delta H = 50,000$
 $\Delta S = 163.58$
 $c = 149 e^{24.75}$

The reader should note that the curves generated from Equation (5) do not fit the data well below 14°C.

Data from Johnson and Eyring (32) redrawn by the present authors.

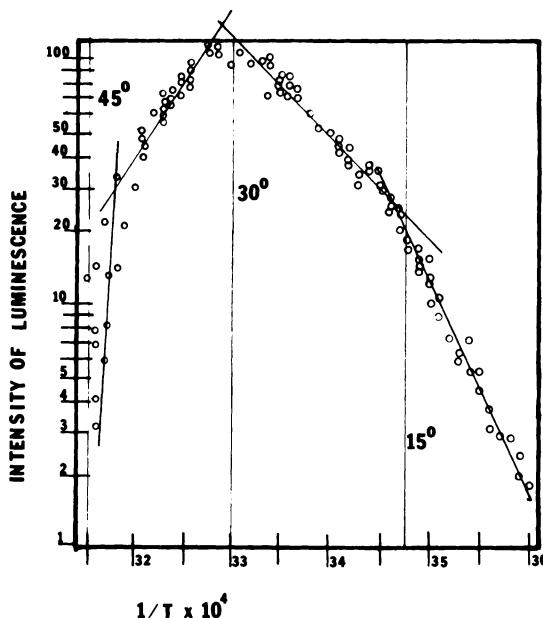


FIGURE 11. Another representation of the data from Figure 10. Straight-line segments have been fitted to the points.

changes will most likely affect both the ΔH^\neq and ΔS^\neq terms. Enzymes are effective in changing rates of reactions by altering activation energies (and not by affecting the thermodynamic functions, ΔH and ΔS , for the enzyme reactions). However, vicinal water structure transitions may well alter the ground state energy of the proteins, thus affecting the ΔG value for the denaturation equilibrium (see, for instance, 34 and 35). If the denominator of the Johnson-Eyring equation remains constant with temperature, one may suspect that an entropy-enthalpy compensation is operating - as discussed by Lumry and Rajender (36). Therefore, we conclude that structural transitions of vicinal water may affect both the kinetic and thermodynamic functions in the Johnson-Eyring equation. This means, in other words, that the thermodynamic functions would not remain constant with temperature, but be sensitive to the structural transitions of vicinal water at the transition temperatures. Hence, the present authors favor plotting such data in the manner of Crozier allowing for individual straight-line segments as opposed to smooth curves over the entire temperature interval studied. Recall also that the data for the germinating turnip seeds clearly show breaks in the energy of activation at the

vicinal water transition temperatures of 15 and 30°C.

Individual enzyme-catalyzed reactions in a metabolic sequence can be expected to have similar activation energies. If this is the case, then it is difficult to justify the existence of rate limiting processes. If "rate limiting" processes did not exist, then the Arrhenius plots would not be sharply curved or show "critical temperatures". This is not the case. Clegg (37) has provided some ideas which allow for the existence of rate limiting steps to be consistent with the complexity of cellular metabolism.

In all current theories of biological rate processes (of the type discussed in this paper) the operation of a "rate limiting enzyme" is implied. It is noted that this applies equally well to the older approach advocated by Crozier as well as to the more recent ideas of Johnson and Eyring. However, it would appear intuitively that while different (consecutive) reactions may have somewhat differing energies of activation, these differences could neither (a) lead to changes in rates as *abrupt* as those frequently reported, nor (b) account for the *regularity* of the occurrence of the transition temperatures observed. Clegg (37) proposed a model that might account for the occurrence of these events. Thus, he postulated that macromolecules have a greater affinity for the vicinal water phase than for the bulk part of the cytoplasm and suggested that "soluble enzymes" will, on the average, tend to occur in (loose) aggregates adjacent to interfaces such as a membrane/cytosol interface. On the other hand, many small molecules (of molecular weight less than about 500 to 1000 Daltons) may dissolve more readily in the more nearly bulk-like aqueous phase of the cell. Hence, the "bulk phase" serves as the transport media for small molecules within the cells. Clegg envisions that macromolecules involved in a given metabolic pathway (or sub-pathway) may be loosely associated in the vicinally structured phase. Therefore, it would be possible for initial substrates of a pathway to react with the enzyme "complex"; the subsequent end-product would be released into the bulk phase to be transported to other areas of the cell. This type of enzyme "complex" could then behave kinetically as a "single enzyme species", giving rise to Arrhenius graphs similar to those we observed for the germination of turnip seeds, and those reported by Crozier (22-31). The reader should note, however, that single enzymes in solution also frequently appear to exhibit breaks at or near the vicinal transition temperatures (Figure 12). This aspect is discussed in greater detail in later sections of this paper.

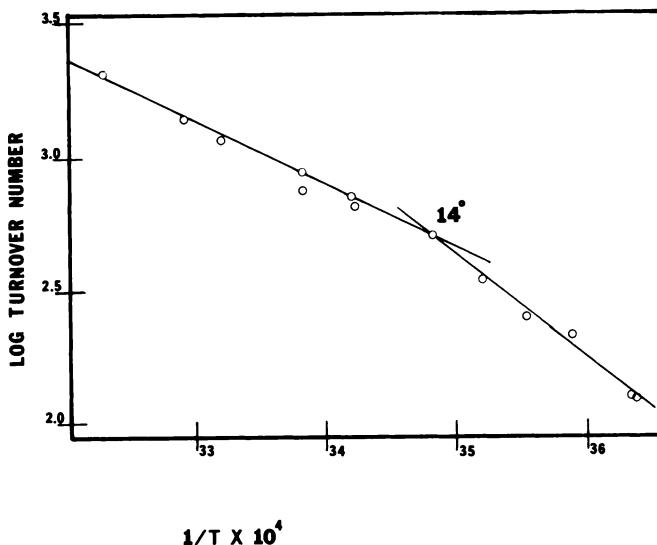


FIGURE 12. Turnover number as a function of reciprocal absolute temperature for D-amino acid oxidase D-alanine system. Data from Massey, *et al.* (38).

Some breaks in Arrhenius graphs for biological systems do not occur at the temperatures of the vicinal water transitions. This behavior may be accounted for by at least two different, independent arguments. If data are not obtained at sufficiently closely spaced temperature intervals, errors may readily result in determining the "critical temperatures". However, where good data are available, in the sense of carefully observed, closely spaced measurements, it appears that temperatures other than those of the vicinal water transition temperatures are still often represented. In these cases, it seems likely that lipid transitions are involved. As an example, ATPase in sarcoplasmic reticulum membranes shows a break in the Arrhenius graph near 19°C and EPR measurements by Inesi, *et al.* (39) indicate a break in the Arrhenius plot at the same temperature as observed for an order-disorder transition of the lipids. Similarly, other lipid systems show anomalies in apparent energies of activation (40). These experiments have several implications, not the least of which is that critical temperatures may appear in cellular systems at temperatures other than those of the vicinal water transition temperatures.

In addition to the anomalous responses induced by lipids, other drastic changes are frequently observed in the energy of activation at other temperatures. This is particularly the case at higher temperatures where denaturation most likely occurs. Furthermore, it is expected that the vicinal water transitions are manifested only in those systems where the energetics are favorable. It has been pointed out (41,42) that different vicinal water structures most likely differ energetically only slightly from one another. Thus, processes involving large changes in energies are likely to be relatively insensitive to the subtle details of the vicinal water structures. The energy of transition between one vicinal water state and another is considered to be at most on the order of one kcal/mole H₂O and likely far less. However, cooperative effects of many interfacial water molecules may induce energy changes on macromolecules which are notably greater than the small figure of about one kcal/mole or less. In any case, "ordinary" denaturations should be more energetic than the vicinal water-induced transitions.

In short, the present authors propose that vicinal water may induce relatively minor structural transitions upon macromolecules (notably proteins) in cellular systems by imposing configurational and/or conformational changes; in addition, however, lipid transitions may also be involved in some systems. Thus, Arrhenius plots for physiological systems may be expected to be complex, involving vicinal water anomalies, lipid transitions and ordinary denaturations.

C. Multiple Growth Optima

The notion that vicinal water transitions may alter the preferred metabolic sequences in organisms originated from the work of Oppenheimer and Drost-Hansen (43). This and subsequent studies (44) provided evidence for multiple growth optima, and it was suggested that such effects might be due to the use of different metabolic pathways. This idea has remained relatively dormant until the work of the present authors was initiated. We stress that multiple growth optima have been observed previously by several other authors. Frequently, these authors have not commented on possible mechanisms to account for this phenomenon - least of all in terms of invoking vicinal water structures. Mitchell and Houlahan (45) observed that a mutant of *Neurospora crassa* required riboflavin for growth above 30°, while it was not required for growth below this temperature (see Figure 13). Multiple growth optima were observed for

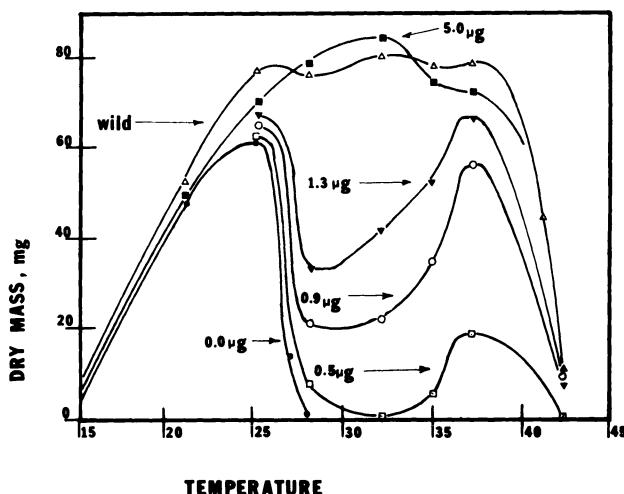


FIGURE 13. Growth of wild type and mutant strains of *Neurospora crassa* as a function of temperature for various amounts of riboflavin. Data from Mitchell and Houlahan (45).

Streptococcus faecalis by Davy, et al. (46). Other examples have been discussed elsewhere (9,10,44).

A general mechanism for the role of vicinal water in "regulating" metabolic pathways can readily be suggested from the "vicinal water network model" of Clegg (37) and those of the present authors. Recall that vicinal water apparently can influence or control the structure of proteins or enzymes in solution, and that "soluble" enzymes might be held in loosely bound complexes near interfaces within the cell. When the cell is not near one of the transition temperatures for vicinal water, cellular metabolism is orderly; enzymes are in complexes near various intracellular interfaces and bulk water "channels" may exist for the transport of small molecules within the cell. When the temperature of the cell approaches vicinal water transition temperatures the enzyme complexes dissociate and the bulk water "channels" become less well defined. Hence, metabolism slows down: a minimum would thus be observed. If, however, large amounts of substrate nutrient are provided to the cell a minimum may not be observed as the enzymes now become rate limiting rather than the substrates (as would be the case on minimal media). Upon passing through a temperature transition zone new enzyme complexes are expected to reform and metabolism, once again,

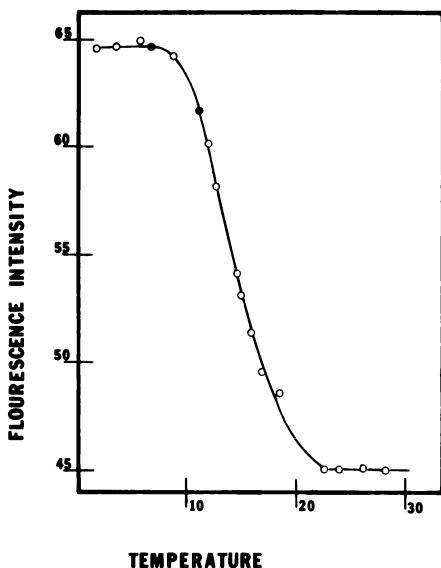


FIGURE 14. The fluorescence intensity of aromatic amino acid residues are shown as a function of temperature ($^{\circ}\text{C}$). Excitation at 205 nm and emission recorded at 333 nm. Open circles indicate values taken upon warming the solution and dark circles indicate values taken upon recooling. Note the occurrence of an inflection point near 15°C . Data from Massey, et al. (38).

becomes "orderly", yet differing from the previous vicinal water structure.

D. Current Research

As discussed above, the present authors have investigated the growth of *Cyanidium caldarium* as a function of temperature. Recall that three optima were observed with minima in growth rates near 31 and 43° . Nitrate was metabolized only above 30° . It is conjectured that a nitrate reductase (or other enzyme) is affected by the vicinal water transition near 30° , accounting for the change in growth. Further investigation into the role of vicinal water in metabolic processes as outlined here is presently underway in the Laboratory for Water Research. It appears at this time that at least some metabolic pathways may be "regulated" by structural changes in proteins induced by vicinal water structures.

If, as envisioned here, water possesses the ability to alter the metabolic properties of organisms, then vicinal water structure must certainly play a great role in biogeography by virtue of its temperature dependence. Indeed, this thesis is developed in some detail in the article by Fisher in this volume.

IV. EVIDENCE FOR PROTEIN TRANSITIONS NEAR VICINAL WATER TRANSITION TEMPERATURES

A considerable amount of evidence exists in the literature for the occurrence of protein transitions near the vicinal water transition temperatures. Hydrodynamic, kinetic and thermodynamic methods seem particularly sensitive for the detection of these transitions. On the other hand, spectroscopic methods generally appear relatively insensitive to these transitions. The water-induced transitions apparently result only in rather minor conformational changes, but ESR, C¹³, NMR and fluorescence spectroscopy appear to the present authors to possess the greatest potential - as far as spectroscopic methods are concerned - for detecting the subtle transitions induced by vicinal water.

A few examples of protein transitions which appear related to vicinal water structure changes will be discussed. For example, Massey, *et al.* (38) studied the rates of D-amino acid oxidase catalyzed-reactions as discussed previously and obtained evidence for an abrupt change in the energy of activation in the vicinity of 14° (see Figures 12, 14, 15). Grande and co-workers (47) have also observed a transition in D-amino acid oxidase near 15° using ESR methods (see Figure 16). Furthermore, Henn and Ackers (48) noted that D-amino acid oxidase apoenzyme underwent thermal transition in its subunit dissociation constant near 12 to 14° (see Figure 17). Thus, D-amino acid oxidase provides an excellent example in which a break in an Arrhenius graph is correlated with a protein transition detected by other independent methods. Indeed, the transition temperature for D-amino acid oxidase is very close to the temperature of transition for vicinal water, as determined from experiments on simple, physico-chemically well defined systems. The present authors thus conclude that the protein transition is most likely induced by vicinal water.

Somewhat similar to the changes reported above, Kayne and Shelter, (49) reported a transition in pyruvate kinase

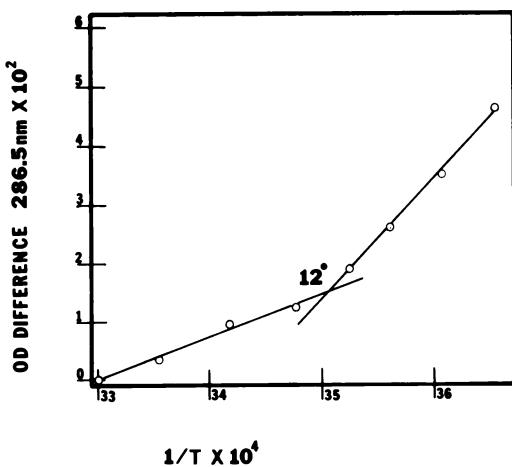


FIGURE 15. The effect of temperature on ultraviolet absorbance for D-amino acid oxidase. Note the transition near 12°C . Data from Massey, et al. (38).

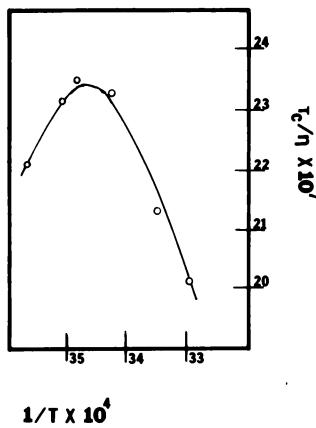


FIGURE 16. The ratio of the average, rotational correlation time (τ_c) to viscosity of the medium as a function of reciprocal absolute temperature ($^{\circ}\text{K}^{-1}$). Note that τ_c/η passes through a maximum near 15°C . Data by Grande, et al., 1972.

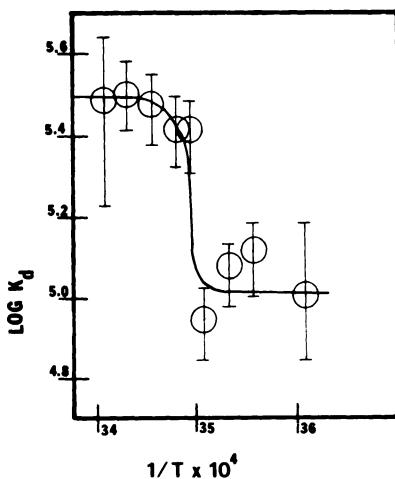


FIGURE 17. The dissociation constant for D-amino acid oxidase apoenzyme subunits as a function of reciprocal absolute temperature. A thermal transition occurs near 12-14°C. Data by Henn and Ackers (48).

centered around 16° (see Figures 18 and 19) using ultraviolet spectroscopy. While this transition is quite broad, it does seem to be centered around the temperature of the vicinal water transition and yields Arrhenius graphs which may be drawn to exhibit a rather abrupt change in activation energy near this temperature. Kayne and Shelter speculated that the transition may be related to changes in the nature of solvation! Specifically, they believe the transition involves the transfer of certain chromophores from an aqueous environment to a hydrophobic environment. In addition, from sedimentation and ORD data, it was noted that the transition only produced small structural changes in the protein! These observations hence appear to be consistent with the views of the present authors.

Otieno, *et al.* (72) have very recently studied the activity of certain sulphhydryl groups on yeast hexokinase-B. They found that only one of the sulphhydryl groups was essential for hexokinase function. This essential sulphhydryl was found, in temperature studies, to "flip" out into the medium near 31°C. The movement of the sulphhydryl group was studied via alkylation and it was observed that a sudden increase in the rate of alkylation occurred near 31°. These results can very readily be accounted for on the basis that vicinal water imposes subtle but important conformational or configurational

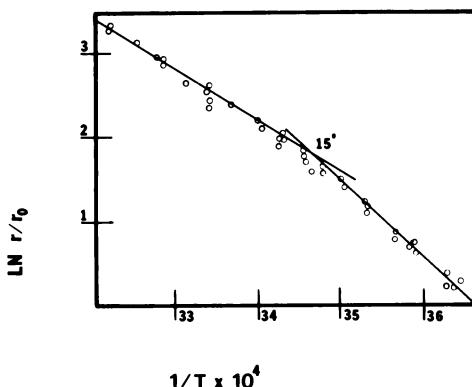


FIGURE 18. Arrhenius plot for pyruvate kinase. An abrupt change in activation appears to occur near 13°C . Data by Kayne and Shelter (49), plotted by the present author.

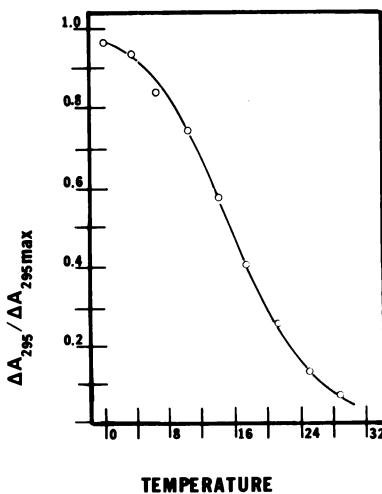


FIGURE 19. The ultraviolet difference spectrum for pyruvate kinase at 295 nm. The reference maintained at 25°C ; sample varied from .5 to 28.9°C . From the inflection point observed it is suggested that a transition centered near 16°C exists. (These data appear to correlate with the data presented in Figure 18). Data by Kayne and Shelter (49).

changes upon hexokinase. As far as the present authors are aware, this is the first time a specific group or residue within a protein has been shown to undergo some geometrical change near a vicinal water transition temperature.

Other enzyme systems and proteins which apparently exhibit transitions near the vicinal transition temperatures are given in Figure 20 to 33. The transitions near 32° observed by Privalov and co-workers (62-67) for tropocollagen are of particular interest: only above 32° is tropocollagen capable of forming supramolecular structures that are associated with the biological roles of this protein.

The above examples illustrate that a great variety of proteins undergo transitions at the vicinal water transition temperatures. Indeed, if water plays the role we have proposed for it in the determination of macromolecular conformation, a more careful literature search, as well as tedious data taking, may be expected to reveal many more examples.

At present, it appears evident that proteins do exhibit structural transitions near the vicinal water transition temperatures. The present authors believe that these transitions are indeed induced by the changes in vicinal water structures. No doubt, the interplay between macromolecule and solvent is complex. Apparently, within any given temperature interval (for instance, between 15 and 30°), only one major type of vicinal water structure is stabilized. This structure, therefore, may impose specific conformational or configurational constraints on the macromolecule in solution. At the same time, the macromolecule in solution - apart from stabilizing the vicinal water structure - obviously also imposes some structural effects on the solvent by short-range, localized (high energetics) interactions, specifically ion hydration.

V. COMMENTS ON ABRUPTNESS OF THERMAL TRANSITIONS

It is well known that pure anhydrous lipids may undergo abrupt changes at well defined temperatures. As discussed, more or less abrupt changes in biochemical reaction rates have also been reported from time to time as well as relatively abrupt changes in physiological parameters. These observations have not failed to attract attention from theoreticians, but generally without consideration of the possible role of vicinal water structure. Classically,

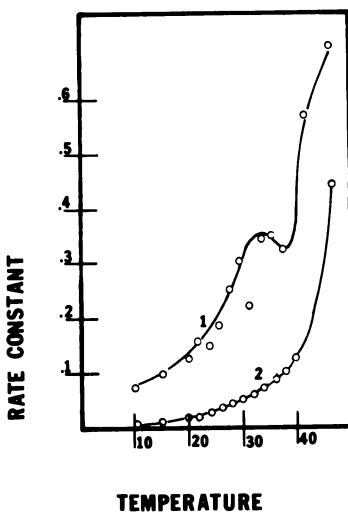


FIGURE 20. The mutarotation of glucose as a function of temperature. Curve 1 shows the rate of mutarotation in the presence of green pepper mutarotatase. Curve 2 represents the spontaneous mutarotational rate. The anomaly near 30°C takes place only in the presence of an enzyme. Apparently large molecules are required to provide sufficient surface for the existence of vicinal water. Data by Bailey, et al. (50).

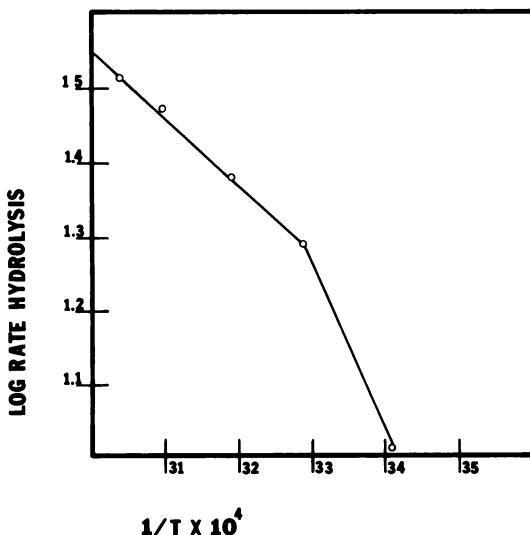


FIGURE 21. The effect of temperature on the rate of hydrolysis of urea by urease. An abrupt change in the activation energy appears to occur near 30°C - if one may rely on just one data point for the low temperature region. Data by I. Magana-Plazer, et al. (51).

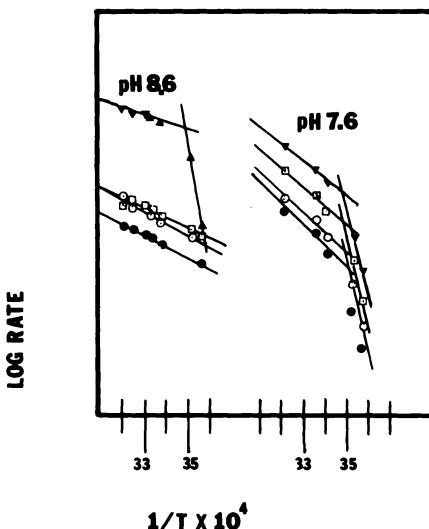


FIGURE 22. The effect of temperature on the kinetic rates of 4 allotypes of *Rana pipiens* (H_4LDH). Pyruvate was used as the substrate. Note that where abrupt changes in activation occur they are found in the 12-13°C interval, suggesting a role for vicinal water! It is presumed that transitions do not occur for some enzyme forms at certain pH values for energetic reasons. Data by Levy and Salthe (52).

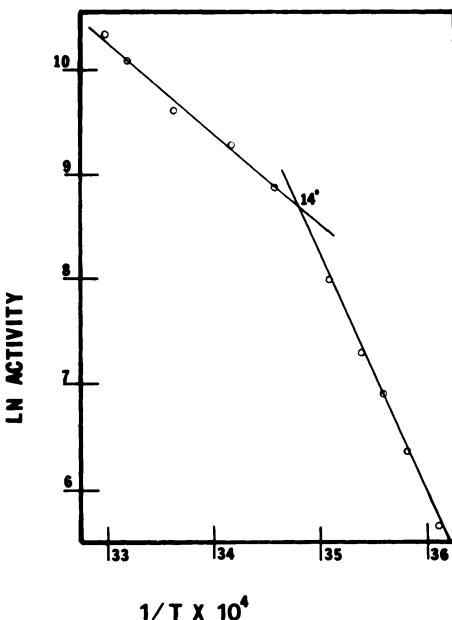


FIGURE 23. Arrhenius graph for phosphorylase. An abrupt change in activation energy is seen near 14°C. Data by Graves, et al. (53).

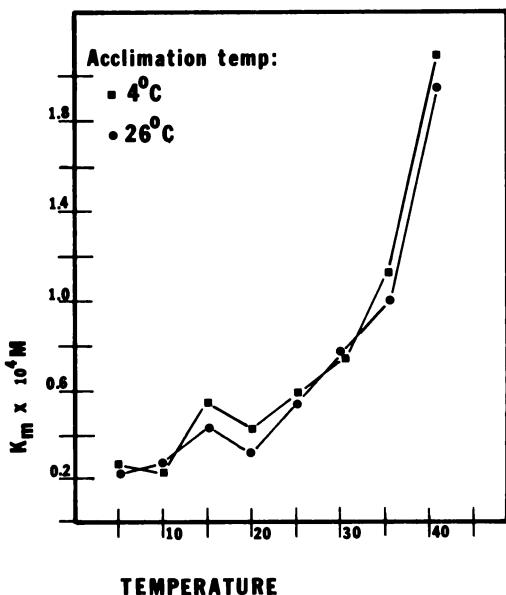


FIGURE 24. The Michaelis-Menton constant (K_m) for oxaloacetate for S-MDH (malate dehydrogenase) enzymes isolated from Thamnophis sirtalis parietalis (a cold climate reptile), as a function of assay temperature for two acclimation temperatures, $4^\circ C$ and $26^\circ C$. Anomalous behavior is seen near $15^\circ C$. Data by Anne, Hoskins and Aleksink (54).

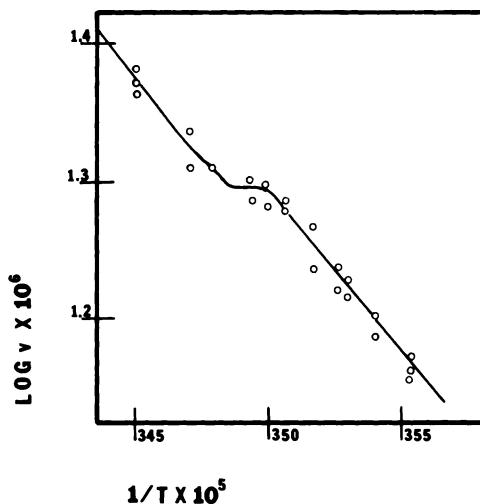


FIGURE 25. The rate of reaction between N^α -benzoyl-*L*-arginine ethyl ester and trypsin as a function of reciprocal absolute temperature. Anomalous behavior is observed in the $12\text{--}15^\circ\text{C}$ range. This type of transition suggests that entropic consideration plays a more important role in the transition than enthalpic. Data by Talsky, et al. (55).

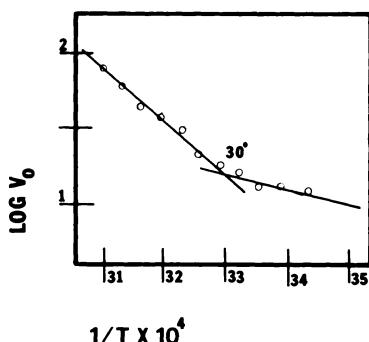


FIGURE 26. Arrhenius graph for the transamination of L-alanine with α -ketoglutarate by aspartate transaminase. The activation energy shows anomalous behavior near 30°C . Data by Polyanovskii and Makarova (56).

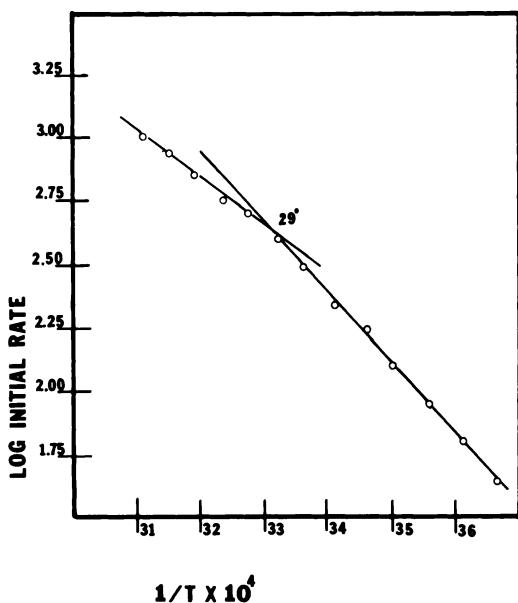


FIGURE 27. Arrhenius graph of inorganic pyrophosphatase activity. A break in the activation appears to occur near 29°C. Data by Pynes and Younathan (57).

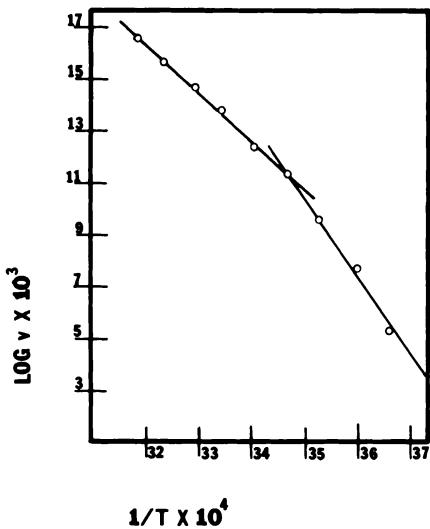


FIGURE 28. Arrhenius graph of α -amylase from *Pseudomonas saccharophila*. An abrupt change in activation energy is seen near 140°C. Data by Markovitz, et al. (58).

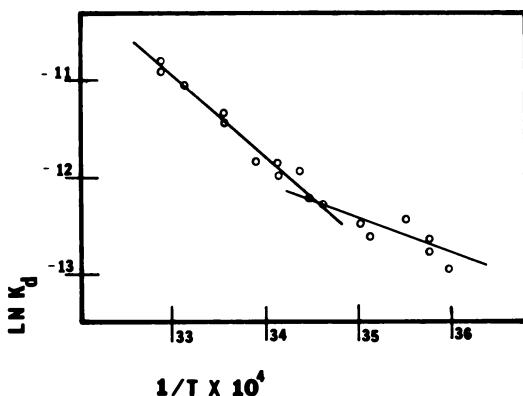


FIGURE 29. *Van't Hoff plot of the equilibrium constant for the dissociation of osimate from the binary LDH-NADH complex. Anomalous behavior appears to occur near 15°C. These data have been reinterpreted by the present authors from data by Schmidt, et al. (59).*

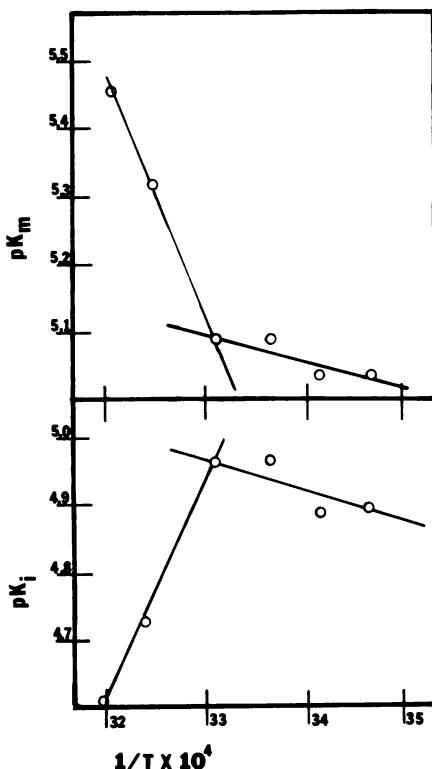


FIGURE 30. *The effect of temperature on the Michaelis constant (K_m) and the inhibition constant (K_i) of AMP for the reaction of 5-phosphoribosyl pyrophosphate with phosphoribosyl-transferase. Note the anomalous behavior near 30°C. Data by Murray (60).*

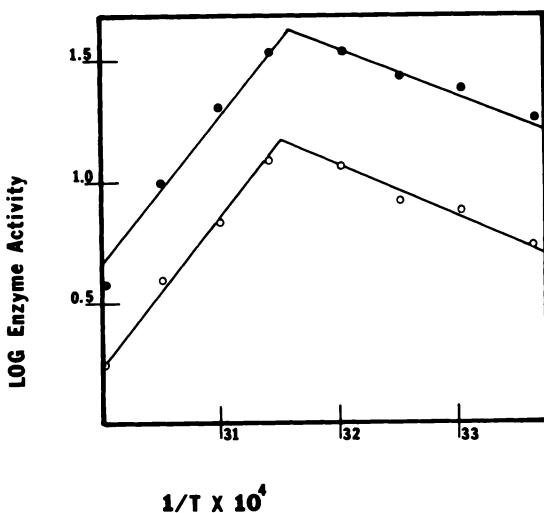


FIGURE 31. The effect of temperature on the utilization of orotic acid (solid circles) and orotidine-5'-phosphate (open circles) by calf thymus preparation. Data by Kasbeker (61).

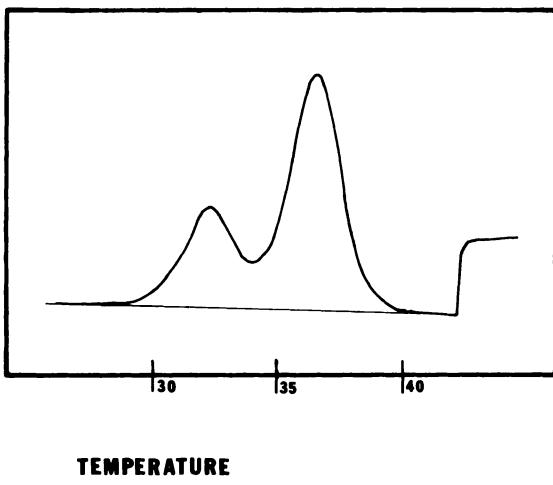


FIGURE 32. Thermal absorption of tropocollagen in a salt solution (.1 M). The first transition, near 32°C, represents a functional transition. Only above 32°C can tropocollagen form supramolecular structures necessary for its biological function. The transition near 37°C likely represents thermal denaturation. The present authors believe that vicinal water plays a major role in the first (32°C) transition. Data by Privalov (62).

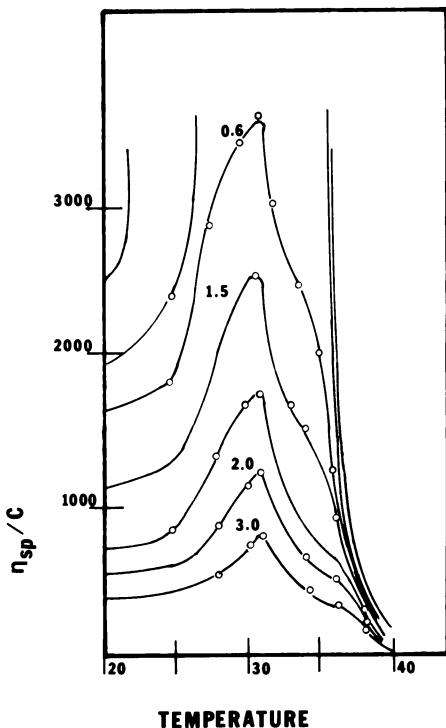
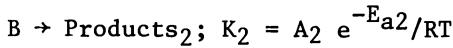


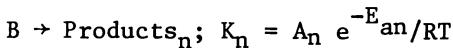
FIGURE 33. The temperature dependence of the characteristic viscosity of procollagen in the presence of 0.1 M NaCl for various shear rates. Values of shear rates (sec^{-1}) are given alongside their respective curves. A maximum in viscosity occurs near 30°C . Data by Privalov (63).

kineticists have considered the possibility of explaining more or less abrupt changes in reaction rates through suitable combinations of parallel and consecutive reactions in terms of classical kinetics.

Giralt and Missen (68) have derived theoretically the temperature dependence for a set of competing parallel reactions. Thus, these authors consider a set of reactions as indicated in the equations below:



$$\begin{matrix} \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot \end{matrix}$$



In these equations, B represents the reacting species, k the rate constant, and A and E the Arrhenius parameters. The overall rate expression then simplifies to the following:

$$A_{\text{obs}} = \sum_{i=1}^n A_i$$

$$E_{\text{obs}} = RT \ln [\sum A_i / \sum A_i e^{-E_i/RT}]$$

$$K_{\text{obs}} = A_{\text{obs}} e^{-E_{\text{obs}}/RT}$$

Figure 34 shows an Arrhenius graph of an arbitrarily chosen set of rate constants. This graph is constructed using the equations given above. Note that no "kinks" are observed. This approach does not provide an explanation for observed anomalies in biological systems unless vastly differing energies of activation are assumed - values that are well outside the realm of physical reality.

In 1936, Burton (69) showed how apparent (false) critical temperatures may be obtained from two consecutive reactions:

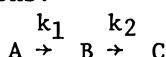


Figure 35 shows an example of Burton's calculation. He has constructed an apparent anomaly in the energy of activa-

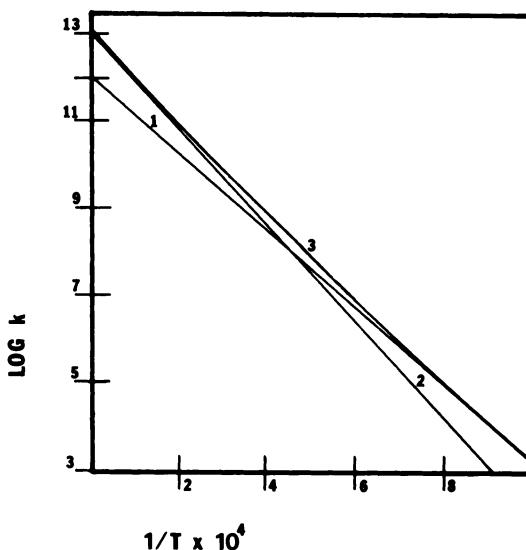


FIGURE 34. Arrhenius plots for arbitrary rate constants for a set of 2 parallel reactions.

$$\text{Line 1: } \log k_1 = 12 - 8742/T$$

$$\text{Line 2: } \log k_2 = 13 - 10927/T$$

$$\text{Line 3: } \log k_{\text{obs}} = \log (k_1 + k_2)$$

$$A_1 = 10^{12}, A_2 = 10^{13}, E_1 = 40,000 \text{ and } E_2 = 50,000 \text{ cal/m}$$

No Arrhenius graph anomalies arise.

tion near 17° using two reactions, the first of which has an apparent energy of activation of 16 kilocalories and the second with an apparent energy of activation of 8 kilocalories. Kistiakowsky and Lumry (70) have also advanced similar views. In other words, apparent thermal anomalies were introduced by selecting suitably large differences in the energies of activation for the participating reactions. However, the present authors believe that this type of approach cannot account for the unusually high frequency of anomalous changes in physiological and biochemical systems near 15, 30 and 45°C. Table I shows a few examples of unanticipated anomalous behaviors of physiological systems at the vicinal water transition temperatures. See also the table in (10). The present authors find it truly amazing that other authors, in the past, have made little or no comment as to why such a great variety of physiological and biochemical systems exhibit anomalous properties near the

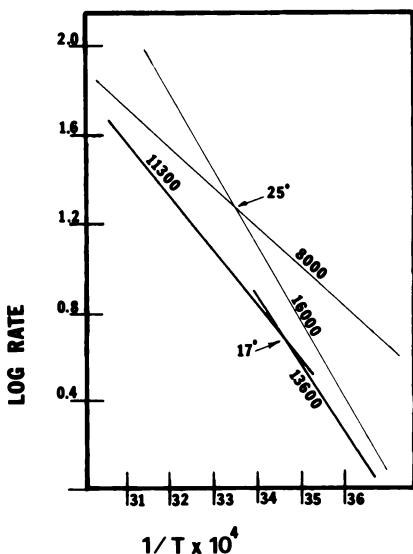


FIGURE 35. An Arrhenius graph for two consecutive reactions has been constructed from the two components (thin line): one with an activation energy of 8,000; the other with 16,000. In addition, both steps have equal rates at 25°C. The resultant Arrhenius graph (thick line) shows an anomaly near 17°C. Data by Burton (69).

TABLE I. Examples of Physiological Properties Which Show Anomalies Near 15, 30, 45°C

-
1. Viscosity of Cumingia egg cytoplasm, $T_k = 15, 30^\circ\text{C}$
 2. Total storage life of cucumbers, $T_k = 13^\circ\text{C}$
 3. Germination of Ajuga reptans, $T_k = 30^\circ\text{C}$
 4. K-uptake by wheat seedlings, $T_k = 16, 30^\circ\text{C}$
 5. Potential across Valonia utricularis membrane, $T_k = 15, 29^\circ\text{C}$
 6. Average generation time for Euglena in batch culture, $T_k = 28^\circ\text{C}$
 7. Flowering percentage and yield of Allium cepa, $T_k = 17^\circ\text{C}$
-

NOTE: T_k is the temperature of the anomaly

vicinal water transition temperatures. In fact, in general, it seems that most authors have avoided this issue altogether. The only hypothesis to date which is able to account for the anomalous properties of physiological systems at the temperatures of 15, 30, 45 and 60°C appears to be the one presented in this chapter, in other portions of this volume, and in writings of one of us (W. Drost-Hansen). It is for these reasons that we suggest that water-induced transitions are superimposed on proteins, macromolecules, and supramolecular structures. In other words, we believe that a "master reaction", as such, does not exist; rather, that selective, cooperative effects amongst loosely bound enzyme complexes may lead to the observed anomalies. The mechanism most likely consists of conformational or configurational changes of the appropriate macromolecules, induced by changes in vicinal water structure. It should also be noted that vicinal water appears to be able to affect the configuration of single proteins in solutions. This effect is illustrated, for example, in the case of D-amino acid oxidase discussed in a previous section.

While the idea that vicinal water phase transitions cause abrupt physiological changes was essentially first formulated in its present form by one of the authors of this article (W. Drost-Hansen), it appears that other authors were significantly close to the idea that water near interfaces could be involved in physiological anomalies. These authors include Crozier (22-31) who thought transitions in a "surface film" might be a controlling factor behind the "critical temperatures" and Goddard (71) who concluded in 1935 that some "unknown transitions" were responsible for anomalies in *Neurospora* ascopore activation. Because these "unknown transitions" were not causally assigned to protein denaturation, Goddard left open the possibility that the solvent might be the determining factor.

VI. SUMMARY

A brief outline has been presented concerning our views on the role of water in biological systems. The involvement of water in various rate processes has been stressed. While it is quite obvious that water is extremely important to biological systems - after all, water is the "mother liquor" of life - very few attempts have been made to incorporate a specific role for water in models of the living state. The present authors have presented a model, which appears to be

at least partially testable, for the role of water in metabolism. The model is based on the idea that vicinal water structures are able to impose small, low energy transitions upon proteins. These transitions alter the physico-chemical properties of these proteins which, in turn, affects their biological activity. The hypothesis presented in this chapter can account for the anomalous behavior of many biological systems at the temperatures of 15, 30, 45 and 60°C. We intend to continue to refine and expand this hypothesis.

NOTE ADDED IN PROOF

Since the original manuscript was prepared, work has continued on the question of possible different metabolic pathways in different temperature intervals. Thus, the nitrogen metabolism of *Cyanidium caldarium* has been studied in some detail. It was found that while NH_4^+ may serve as sole nitrogen source over the entire interval of growth studied (18 to 53°C), replacement of NH_4^+ by NO_3^- leads to cessation of growth above 45°C. In other words, the growth optima occurring near 48°C is eliminated. This result is in agreement with the proposal that different metabolic pathways are employed in different temperature intervals (separated by one of the vicinal water transition temperatures). Note for comparison that Rigano and Violante (17) observed that NO_3^- was not being metabolized below 30°C. For a recent discussion of these studies, see (73).

In connection with the seed germination studies discussed in our paper, see also the paper by Roberts and Smith (74). These authors observed remarkably abrupt (almost discontinuous) changes in the breaking of dormancy of barley seeds around 16°C. Somewhat similar results have subsequently been observed also for the two weed species of the common dock (E. H. Roberts, personal communication).

For recent general discussions of interfacial water, see for instance, references 75, 76, 77, 78, 79, 80, 81.

Finally, a wealth of papers continues to appear dealing with effects of temperature on aqueous lipid systems. The main thermal effects of the lipids are related to the phase transitions observed in the pure (anhydrous) lipids. A number of authors have also discussed in some detail water/lipid interactions; however, very few attempts have been made to consider specifically the (mutual) vicinal water/lipid inter-

actions. Because most of the studies reported were not intended to test for the influence of vicinal water on the aqueous lipid systems, it is often difficult to extract relevant information on the subject from the published data. For this reason, attention is called only to a few papers dealing with aqueous lipid systems. In considering these papers the reader is encouraged to bear in mind the vicinal water transition temperatures and to consider the possibility that (mostly subtle) effects of vicinal water structure may be reflected (superimposed) in the available thermal data (82,83,84,85,86,87,88,89).

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A VIEW OF THE SIGNIFICANCE AND
UNDERSTANDING OF THE PHYSICAL
PROPERTIES OF CELL-ASSOCIATED WATER¹

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I. INTRODUCTION

Although water constitutes 60–90% of the weight of most biological tissues, little unambiguous knowledge is available on its physical nature within the cell. Because of this lack of understanding, all models of the living cell describing the unequal distribution of ions between the inside of the cell and the outside, the dynamic aspects of solute and water movement into and out of cells, and the bioelectric phenomena of cells require fundamental assumptions concerning the physical state of cellular water. Therefore, ultimately, an understanding of cellular function will require concrete knowledge of the physical state of water.

Conventionally, biologists assume that a living cell is equivalent to a dilute aqueous solution surrounded by a semi-permeable membrane (1–4). Bioelectric phenomena of cells are also described classically by equations evolving from dilute solution theory. The application of the theory of

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osmotic regulation in biological systems dates from the 18th century (1). The greatest impetus to the application of osmotic theory to biological cells came, however, near the turn of the 20th century, when the chemists van't Hoff and Arrhenius laid much of the foundation for dilute solution theory. The biologists de Vries and Pfeffer capitalized on the theories of van't Hoff and Arrhenius (1). Bernstein developed a detailed theory of bioelectric phenomena which evolved from the ideal solution theory (5). For example, Katz (6) states that "...in 1902 he [Bernstein] proposed an important theory which applied the physico-chemical concepts of Nernst and Ostwald to bioelectric phenomena".² The findings of Hodgkin and Huxley (7), in 1939, conflicted with the Bernstein theory which argued for the impermeability of the cell to sodium. Later, Hodgkin and others modified and extended the Bernstein theory to what is essentially the present concept of the excitable cell, yet the theory of dilute solutions remains fundamental to the membrane concept (8).

It is also known that, since the middle of the 19th century, theories have existed which maintain that water in cytoplasm is not free as in dilute solutions but bound to macromolecular constituents (9). The concept of bound water in living tissues was pursued actively during the 1920's and 1930's. Between 1934 and 1965, a small but persistent group of biological scientists presented evidence that a large part or all of the cellular water exists in a physical state significantly different from ordinary water (10-14). Thus, two schools of thought concerning the physical state of cellular water emerged and, hence, two schools of thought concerning transport and accumulation of solutes in living cells developed. A review of this fundamental controversy was presented by Oscar Hechter in 1965 (15) whereupon he concluded the following:

"We have discussed the two opposing classical concepts of transport. Upon analysis, both ideas are shown to be right in part; and both partially wrong. The proponents of the plasma membrane thesis of transport were wrong in that they neglected the role of the cell interior, the holists were wrong

²The generalizations of Ostwald stemmed largely from the work of van't Hoff and Arrhenius (1).

in their de-emphasis of the plasma membrane and of intracellular membranes generally. If one considers that membrane systems throughout the cell are involved in transport, and that cells exhibit diversity as well as uniformity, a pluralistic resolution is achieved."

To date, considerable evidence has been gathered by proponents of both views, but little has been done to bring about the pluralistic resolution proposed by Hechter. It does appear clear that the divergent views concerning cellular function (i.e., the absorption theories or the dilute solution theory of cellular function) are based on different assumptions concerning the physical state of solutes and water. It is the author's contention, therefore, that a fundamental understanding of the physical state of cellular water will lead to a resolution of these divergent views.

II. EXPERIMENTAL EVIDENCE SUPPORTING THE VARIOUS VIEWS ON THE PHYSICAL STATE OF WATER IN THE LIVING CELL

A. *The Membrane Pump or Ionic Theory*

In 1902, Bernstein (5) published a general theory to explain the electrolyte distribution and the cellular voltage. Bernstein's theory utilized the ideal solution concepts that had been proposed by van't Hoff and Arrhenius. Additional assumptions employed by Bernstein³ were as follows:

1. The existence of a discrete cellular membrane separating two solutions.
2. The cellular membrane was absolutely impermeable to sodium and cellular proteins.
3. The voltage across the cell membrane could be described as a concentration cell (implicit in this assumption is the idea that the activities of internal and external electrolytes were the same).

³More detailed explanations of Bernstein's hypothesis may be found elsewhere (5,6,16,17).

It was discovered in the late 1930's and 1940's that the resting⁴ cell was permeable to sodium and chloride ions. These observations disproved the assumption above, that the membrane was absolutely impermeable to sodium. Hodgkin and Katz (18), in 1949, utilizing Goldman's constant field equation (19) developed a general equation to describe the resting potential. Assumption 2 above was replaced by the following assumptions, labelled 2a and 2b.

2a. The membrane exhibits a different permeability to potassium and sodium such that, in the resting state, the permeability of the membrane to potassium (P_K) is much greater than that for sodium (P_{Na}); and during excitation $P_{Na} > P_K$.

2b. Chloride is distributed across the membrane at equilibrium with the resting potential.

The two assumptions, plus the other assumptions outlined above, led to the description of the resting potential by equation (1).⁵

$$E_m = \frac{RT/F}{P_K[K]_o + P_{Na}[Na]_o} \ln \frac{P_K[K]_o + P_{Na}[Na]_o}{P_K[K]_i + P_{Na}[Na]_i} \quad (1)$$

where R, T, and F have their usual meaning; P_K and P_{Na} are the membrane permeabilities to potassium and sodium, respectively; and []_o and []_i refer to the concentrations of the ions in the external and internal solutions. A fourth assumption must be added to account for the ability of the cell to exclude sodium - the existence of a membrane-situated, energy-requiring pump ... the sodium pump.

1. *The Sodium Pump.* Beginning with the ideal solution theory and the postulation of a cell membrane, absolutely impermeable to sodium, the discovery that sodium did exist

⁴The word "resting" is used in this case to refer to cells that are not excited electrically.

⁵The most general form of equation (1) (see references 8 and 19) is often referred to as the Goldman-Hodgkin-Katz (GHK) equation. Equation (1) is referred to as the modified GHK equation because it takes into account only the involvement of sodium and potassium ions and their membrane permeabilities.

within the cell in low concentration required that, in order for sodium to leave the cell, some energy-requiring process be involved. Thus, Dean (20), in 1941, concluded that "... There must be some sort of a pump, possibly located in the fiber membrane, which can pump out the sodium, or what is equivalent pump in the potassium." The early emergence of the concept of active transport of inorganic ions in biological systems was reviewed by Krogh in 1946, yet he was not aware, for reasons stated in his Croonian lecture, of Dean's postulation of the pump (21). It was not until 1957 (22) that a membrane-associated enzyme (the Na^+-K^+ ATPase), which was to become synonymous with "The Pump", was isolated (23).

The discovery of an enzyme system associated with isolated membrane fractions served as an important boost to those investigators proposing the existence of a membrane pump. The transport of sodium and other ions was linked with cellular metabolism by way of the discovery of the Na^+-K^+ ATPase and by transepithelial transport (24). Skou established a minimum number of criteria that must be met to demonstrate active transport and the involvement of the Na^+-K^+ ATPase in solute transport (25).

Recently, it appears that the definition of active transport, which was consistent with ideal solution theory, used by Dean (20), Krogh (21), Skou (25), and Ussing (24) has been changed: "... *Thus an active transport process is defined, not by demonstrating that flux is thermodynamically uphill, but only by demonstrating that flux is coupled to metabolism.*" (See reference 26, pages 1-23 for quote and pages 1-21 for discussion of active transport; and reference 27 suggesting that the definition has been changed). If such a change in the definition is indeed accepted, then it is not clear what fundamental assumptions are basic to the definition. If it is a definition by statement, it may be impossible to test. An attempt to clarify further the basic differences between the membrane pump and the adsorption views of the cell is given in Section II-C-4.

2. *The Cell Membrane.* The postulation that a plasma membrane exists dates back to the 19th century; however, the acceptance of the existence of plasma membranes was not firm until the late 1950's (28-31). Robertson, in 1957, demonstrated a specialized structure at the surface of the cell (28). This very important observation was considered as unequivocal evidence for the existence of a discrete membrane and consistent with the many speculations concerning the various structures proposed for the plasma membrane (see

above references plus references 32 and 33). In general, the plasma membranes of cells were thought to have a distinct and finite thickness (i.e., an outer and inner demarcation separating two aqueous media).

3. *The Cellular Cytoplasm.* According to Katz (reference 6, page 42), a cellular cytoplasm is considered to be an aqueous protein gel. He further states that many investigators take the view that the structural proteins of the cellular aqueous gel with their fixed electric charges and counter ions make only a small contribution to the osmotic balance of cells. It appears that Katz (6) and many other investigators view the publication of Hodgkin and Keynes (34) as definitively proving that the diffusion coefficient of potassium (in squid axon) is equal to that of aqueous potassium chloride solution. The measurements of the diffusion coefficient of potassium in skeletal muscle made by Kushmerick and Podolsky (35) seem also to have heavily reinforced the notion that the mobility of cellular potassium is equivalent to that in a dilute solution. In addition, the vapor pressure measurements of cellular water, made by A. V. Hill (36) in 1930, are viewed as definitely favoring the view that cellular water and electrolytes obey the laws of ideal solutions. Major review articles concerning the physical state of cellular water and, to a lesser extent, electrolytes may be found in references 2-4, 9, 37 and 38. These authors conclude that the majority of the cellular water conforms to the demands of dilute (ideal) solution theory. The small quantity of water and electrolytes that may be adsorbed to cellular macromolecules can be considered for the most part as trivial. (The notable exception of potassium binding to myosin is mentioned by Dick in reference 2, page 41).

B. Adsorption Theories of the Cell

Various investigators have presented interpretations of data that require adsorption of water and electrolytes to cellular macromolecules in various degrees (9-14,39-48). Of these theories, the association-induction hypothesis of Ling, however, has been developed in the most detail (9,11,12,49-57). Ling has derived two general equations based on adsorption to describe the distribution of cellular solutes and the cellular resting potential. An overview of Ling's hypothesis is presented below and the crucial differences between his view and that of the membrane theory are stressed.

1. *Role of the Cellular Surface.* In Ling's view, the cellular surface is not considered to be a membrane as defined in the classical sense (i.e., a distinct inside and outside boundary separating two solutions). Instead, the cellular surface is considered to have a specialized organization with complex macromolecular structures extending both outward and inward from the surface. The normal surface of an inactive (resting) cell is hypothesized to have fixed charges that selectively adsorb potassium over sodium and that a finite number of vacant negative sites exist. In short, the cellular potential is considered to be a phase boundary (or contact) potential derived from the assumptions of equilibrium and adsorption, and described by equation (2).

$$\psi = \text{constant} - \frac{RT}{F} \ln(\overset{\sim}{K_K} [K]_{\text{ex}} + \overset{\sim}{K_Na} [Na]_{\text{ex}}) \quad (2)$$

where R, T, and F have the usual meaning; $\ln \overset{\sim}{K_K}$ and $\ln \overset{\sim}{K_Na}$ are the association energies for potassium and sodium; and $[K]_{\text{ex}}$ and $[Na]_{\text{ex}}$ are the external concentrations of potassium and sodium, respectively. The derivations of this equation may be found in Chapter 10 and Appendices A and B of reference 11. Note that the potential ψ depends on the external sodium and potassium concentrations. Internal ionic concentrations do not enter into the equation.

Edelmann (58,59) put both equation (1) and equation (2) to experimental test and concluded that only equation (2) could describe the data (58,59). Edelmann did indicate that the discrepancy between theory and experiment using equation (1) may be overcome provided the permeability coefficient of sodium changes in the correct direction. He concluded that such a change was unlikely but suggested further investigations should reveal the influence of K^+ , Rb^+ , and Cs^+ on the permeability coefficient of sodium. Unfortunately, to the author's knowledge, no critique of Edelmann's experiment has been published by the proponents of the membrane theory.⁶

It should be added that equation (2), derived by assuming an equilibrium condition, can explain the overshoot of the action potential and the resting potential provided

⁶Edelmann has published a short review entitled "Resting Potential: Membrane or Phase Boundary Potential" (*Physiol. Chem. & Physics*, 5:449, 1973) which summarizes this controversy and places the problem in perspective. Additional comments on this controversy may be found in Ling, G. N.; *Physiol. Chem. & Physics*, 7:91, 1975.

there is a change in the relative values of \tilde{K}_K and \tilde{K}_{Na} . No time dependence of ψ has yet been worked out. Karreman, assuming adsorption but ignoring the presence of vacant sites, has derived equations similar to equation (2); yet, no time dependence has been worked out (61).

One implication of equation (2) concerns the inward cellular diffusion of potassium and sodium which, according to Ling (11), the inward diffusion of potassium should be surface-limited and that of sodium should be bulk-phase limited. Experimental tests of this notion have confirmed these predictions (62,65).

The surface of the cell is thought to possess cardinal sites (receptors) which, when associated with bioregulants or cardinal adsorbants, induce changes in macromolecules which extend along the cellular surface and some of which may extend deep within the cell. These macromolecular extensions within the cell can influence the physical state of water and the ionic selectivity of the macromolecules. (See references 11, 17, 58, and 64 for further discussions of this subject).

2. *The Cellular Cytoplasm.* The tertiary structures of the cellular proteins are considered to determine the physical state of the cellular water and the selectivity of the protein. Their tertiary structure is argued to be determined by cardinal adsorbants such as ATP. Hence, the cytoplasm is considered to be a proteinaceous fixed-charged system.

Under normal physiological conditions, the macromolecular structures are assumed to prefer specifically the adsorption of potassium over sodium; however, some sodium could be adsorbed. This proposed mechanism would lead to an accumulation of potassium in excess of sodium. In addition, the cytoplasmic water is considered to exist in a state such that sodium is excluded (9).

The previous discussion may be summed up in the following manner: the distribution of cellular electrolytes according to the association-induction hypothesis is described by equation (3).

$$[S]_{in} = \alpha [S]_{int} + \sum_{L=1}^N [S]_{ad}^L \quad (3)$$

where $[S]_{in}$ is the intracellular concentration of a given

solute; α is the percentage of intracellular water; $[S]_{int}$ is the concentration of solute dissolved in the cellular water and is termed interstitial water; and $[S]_{ad}^L$ are the fractions of adsorbed solutes on different types of sites.

The ratio of the interstitial solute concentration to the external solute concentration is the distribution coefficient (defined as q_s in equation 4).

$$\frac{[S]_{int}}{[S]_{ex}} = q_s \quad (4)$$

Substituting equation (4) in equation (3), and assuming only one type of site, we have

$$[S]_{in} = \alpha q_s [S]_{ex} + [S]_{ad} \quad (5)$$

The q value (q_s) varies for different solutes, and as a function of the physical state of the cellular water.

In 1964, Ling developed a cooperative adsorption isotherm to describe the accumulation of the adsorbed fraction of cellular solutes which is given in equation (4) for potassium (65).

$$[K^+]_{ad} = \frac{[F^-]}{2} \left\{ 1 + \frac{\xi - 1}{[(\xi-1)^2 + 4\xi \exp(\gamma/RT)]^{1/2}} \right\} \quad (6)$$

where

$$\xi = \frac{[K^+]_{ex}}{[Na^+]_{ex}} \cdot \frac{\bar{K}_{Na}^O}{\bar{K}_K^O}$$

In equation (6) $[F^-]$ is the number of adsorbing sites. \bar{K}_{Na}^O and \bar{K}_K^O are the intrinsic adsorption constants of sodium and potassium, respectively, and γ/RT is the free energy of nearest neighbor interaction. Where $\exp(\gamma/RT)$ is less than one (i.e., nearest neighbor interaction favoring adsorption of like charges), equation (6) predicts that the cellular uptake of potassium as a function of $[K^+]_{ex}$ is sigmoid (65). (For a more complete bibliography on this subject, the reader is referred to an independent derivation of a cooperative adsorption isotherm by Karreman [66,67]). The first experimental findings demonstrating that the potassium uptake (as a function of $[K^+]_{ex}$) is best fit by a sigmoid curve is that of Ling (51) followed by a detailed report by Jones and Karreman (45,46) on isolated dog carotid strips. Later in 1969, a detailed account of similar findings on frog sar-

torius muscle came from Ling's laboratory (54). Ling's co-operative adsorption isotherm was rapidly demonstrated to describe the cellular regulation of solutes and small molecules in a variety of tissues (45,46,55,64,68-77). Ling further developed the concept of cardinal adsorbants which would influence the accumulation of cytoplasmic electrolytes by changes in the parameters \bar{K}_K^o and/or \bar{K}_{Na}^o in equation (6). Examples of cardinal adsorbants are ouabain and ATP. For example, Ling and Bohr (68) and Gulati and Jones (69) demonstrated that ouabain shifts the cooperative adsorption isotherm to the right (i.e., decreased potassium preference) in frog skeletal muscle and dog carotid smooth muscle, respectively. Gulati (75) reported shifts of the potassium co-operative adsorption isotherm in response to varying levels of external calcium (see also reference 77) and sodium; these findings are consistent with the association-induction hypothesis. Ling has demonstrated that equation (6), which is a general equation based on adsorption and a one-dimensional Ising model, phenomenologically, describes quite well not only the oxygenation of hemoglobin (64), but also the adsorption and desorption of sodium and potassium.

The effects of temperature on the cooperative adsorption isotherm have been evaluated by Reisin and Gulati (74,76). They have shown that, with increasing temperature, the co-operative adsorption isotherms for both potassium and sodium are shifted to the left. Above 8°C, the data were fit best by sigmoid curves with $\gamma/2RT > 0$ (equation 6), whereas, for temperatures 8°C or less, nearest neighbor interaction was not significant, and the data were fit best by a hyperbola. All of the above findings are consistent with the association-induction hypothesis. It should be noted that the effects of temperature are not predicted explicitly within the formalized equations of the adsorption isotherm (equation 6) of Ling. That is, additional assumptions are required (see page 366 of reference 76). Huang, however, has derived equations describing cooperative phenomena in biological systems in which temperature appears explicitly (78). His theory is applicable to all sigmoid-type cooperative phenomena.

The fact that the uptake of cellular potassium and the depletion of cellular sodium can be fit best by cooperative adsorption isotherms does not, in itself, constitute proof of the cellular binding of ions. It certainly is consistent, however, with the specific prediction of adsorption made by the association-induction hypothesis and with hypotheses that, in general, argue for ionic association of ions within

cells. In addition to the above studies, the nuclear magnetic resonance studies of the potassium nucleus constitute further evidence in favor of the pairing of potassium ions to cellular fixed charges (47,79,80-80b). The work of Ling and Cope (81) also supports the notion that a large fraction (virtually all) of the cellular potassium is associated with fixed charges within the cell.

C. *Major Points of Difference Between the Membrane Pump and Adsorption Views*

1. *The Cell Membrane.* The proponents of the membrane theory consider the discovery of a specialized structure at the cellular surface as unequivocal evidence favoring their view. Certainly, if the type of discrete membrane envisaged by Bernstein, Davson, and Danielli, or even Singer were actually discovered, then the finding might be of unequivocal nature. Recent findings, however, show that extensive internal and external extensions of molecular structures are attached to the plasma membrane of many cells (82,83) and, therefore, it becomes difficult to know where the internal surface of any plasma membrane ends and where the aqueous cytoplasmic phase begins. Furthermore, the reports of extensive microfilaments and microtubular structures in cells and the attachments of some of these structures (at least in the case of actin) with plasma membrane (84-90) "create" conceptually a "membrane" completely different from that of a simple sheet separating two solutions. Such integration of the structures of the cellular surface with the cytoplasm makes the following a very relevant question: Is the specialized structure observed at the cellular surface really a membrane as perceived in the classical membrane theory? Also, we must ask: What are the consequences of cytoplasmic and extracellular extensions of the "cell membrane" to the two divergent models of the living cell?

2. *Membrane Pump(s)*

a. *Red blood cell ghosts.* The results of experiments with red blood cell ghost preparations have been interpreted as unequivocal demonstrations of active cellular transport. A review of the literature by Freedman (27), however, led to the conclusion that all the experiments were at best equivocal. Furthermore, Ling (91) has demonstrated that the red blood cellghost preparations, when viewed by electron microscopy, are not merely red blood cell membranes with all the

cytoplasmic material removed but contain considerable cytoplasmic material. In fact, the "ghost" preparations are essentially solid.⁷

b. *Energy requirements of the pump(s).* Several investigators have evaluated the energy requirements of the sodium pump. Keynes and Maisel (92), who probably were the first to study this problem, concluded that only 10% of the total metabolic energy was required to operate the sodium-pumping mechanism. Harris reports that the work required for pumping sodium amounts to 20% of the resting heat production of skeletal muscle (93). However, Ling reported in 1962 that the energy requirements of the sodium pump actually exceeded the cellular supply (reference 11, page 211). The reason for the difference between these two conclusions is quite clear and will now be discussed. The equation used by the above investigators to compute the energy requirements is as follows:

$$W = k[Na]_i F(E_{Na} + E_r) \quad (7)$$

where W is the secretory work; k is the rate constant of sodium efflux; $[Na]_i$ is the internal sodium concentration; F is the Faraday; E_{Na} , the sodium equilibrium potential; and E_r the measured resting membrane potential.

For the most part, both groups of investigators agree on the intracellular concentration of sodium (except for the influence of the extracellular space determination on this number - to be discussed below), the cellular voltage, and the oxygen consumption (i.e., a measure of the available energy) of the skeletal muscle preparations studied. They differ, however, in the efflux rate used in equation (7). Ling, in 1962, found that the efflux of sodium from single muscle fibers is a complex curve which can be decomposed into two exponential components (11). He argues that the faster efflux rate is the one characterizing efflux from the cytoplasmic water and that the slower rate represents the efflux of sodium from fixed sites within muscle fibers (i.e., a desorption rate). In the Keynes and Maisel (92) study, a two component efflux curve was observed in whole muscle preparation. They considered that the fast efflux rate which originated from the extracellular space of muscle could be

⁷The author has confirmed Ling's observation; however, the data have not yet been published.

ignored and, in their view, only the slower efflux arose from the cytoplasmic water. Later, Hodgkin and Horowitz (94) reported a single-muscle-fiber-efflux study in which a single exponential efflux rate was observed. If this flux rate is substituted in equation (7), then approximately 10-30% of the total available energy is required for the sodium pump. Ling has re-investigated this problem in preparations of single muscle fibers, several muscle fiber preparations, and in whole muscles (63,95). Again, he observed the two component efflux curve. He also observed certain fundamental differences between his studies and those of Hodgkin and Horowitz (95). Ling correctly points out that the study by Hodgkin and Horowitz (94) did not include data in the first 10 minutes of the efflux curve. Therefore, when Ling plotted his efflux data in the same time domain as Hodgkin and Horowitz, he also obtained a single exponential curve (95). If this slow efflux rate is now used to compute the "energy of pumping", between 16-30% of the total energy is required (i.e., Ling's "slow sodium efflux" energy calculation essentially agrees with the other investigators). If, however, the faster efflux rate observed by Ling is used to compute the "energy of pumping", it is found that the energy requirement exceeds that produced by the skeletal muscle - an obvious violation of thermodynamic laws. The question is: Which interpretation of the sodium-efflux curve is correct?

There appear to be three major interpretations to explain the fast efflux rate for sodium in skeletal muscle. First, that it arises from the extracellular space (see reference 92 and pages 202-213 of reference 96). Second, that the fast efflux rate arises from the sarcoplasmic reticulum (97-99). Third, that the fast efflux rate arises from the cytoplasmic water which excludes sodium entropically (95).

The first interpretation requires that the extracellular space for frog muscle be as high as 23% (see page 204 of reference 96). This requirement far exceeds the values reported for the frog sartorius muscle by Conway (100) and other workers (see pages 202-207 of references 96, 101). Harris argues in favor of the larger value for the extracellular space (see page 204 of reference 96) because of: 1) the phenomenon of exchange diffusion, and 2) considering the sarcoplasmic reticulum to be a part of the extracellular space. Unfortunately, neither of these arguments are soundly supported by experimental evidence. Exchange diffusion cannot account for the sodium ion transfer in skeletal muscle (102), and recent electron microprobe analysis (discussed in the next paragraph) does not support the idea that the sarco-

plasmic reticulum is a part of the extracellular space.

The second interpretation suggests that, although the sarcoplasmic reticulum does not appear to be anatomically continuous with the extracellular space, it is physiologically continuous with that space. Along with this argument the suggestion is made that the fluid within the sarcoplasmic reticulum is similar to that in the extracellular space. The indirect analyses of Rogus (98), Rogus and Zierler (97), and Neville (99) are reported to be consistent with this interpretation. The direct electron microprobe determination of chloride in the terminal cistern of the sarcoplasmic reticulum made by Somlyo, *et al.* (103) does not support the idea that the sarcoplasmic reticulum is physiologically continuous with the extracellular space. In fact, the latter findings (103) support the idea that chloride is excluded from both the sarcoplasm and the terminal cisternae of skeletal muscle (i.e., consistent with Ling's interpretation).

The third interpretation does appear to be consistent with the data reviewed by the author. Ling's interpretation that the slow efflux curve is a measurement of sodium desorption is consistent with data obtained by nuclear magnetic resonance (NMR) spectroscopy indicating that 60% of the muscle sodium is adsorbed (81,104-107). Berendsen and Edzes, however, have questioned this interpretation of NMR data (108). Cope (109), in response to Berendsen and Edzes' findings, offered a re-interpretation of the sodium NMR data. (This re-interpretation was presented in the discussion of the Berendsen and Edzes paper (108); however, in press, Cope's remarks were included in his general paper (110 - see page 427). Additional discussion of this subject may be found beginning on page 480 of reference 108). In considering the sodium NMR data, it should be realized that interpretations other than that of Berendsen and Edzes are equally plausible (110-112). Although further studies by NMR techniques are needed to assure unequivocal interpretation, the bulk of the non-NMR data strongly support the notion that a significant fraction of cellular sodium is adsorbed and, hence, support Ling's interpretation of the sodium efflux curve (45,46,49,51,52-58,65,68,69,71-77). Furthermore, Ling and Ochsenfeld (113) have demonstrated, in skeletal muscle, that the sodium efflux rate is little affected by low temperature and is disassociated from the cellular concentration of sodium and potassium. That is, the efflux rate of muscle sodium is essentially unaffected by temperature and metabolic poisons even though the cells accumulate sodium and lose potassium. This latter work,

along with the observation that membraneless preparations of muscle can exclude sodium (114), appears to prove conclusively that no membrane-situated, energy-requiring pump is required or necessarily involved in the exclusion of sodium from the cell.

Minkoff and Damadian have conducted an experiment with *E. coli* which permitted a determination of the energy requirements for active transport of six solutes (115,116). Specifically, they studied a phage resistant, non-pathogenic, wild strain of *E. coli*, which was grown in fresh medium and, when in the logarithmic phase of growth, transferred to a medium lacking citrate and supplemented with a marginal supply of glucose. Triphosphate concentration, oxygen consumption, efflux rates of solutes, and ATP turnover were determined. Cellular voltage was computed from chloride distribution data.

The following quote summarizes their conclusions:

"On the basis of the measurements and calculations set forth in this paper, we must conclude that these models of ion transport, the only models thus far proposed, are thermodynamically untenable.

Consequently, by exclusion, it can be said that the rate of energy delivery calculated by the traditional bioenergetics utilizing the P:O ratio is insufficient to operate membrane-situated pumps. The degree of disparity for the experimentally determined solutes was severe: a deficit of 24.08 cal/g dried cells over a period of 340 minutes. Only 14.8% of the necessary energy was available from the hydrolysis of ATP.

In addition, our studies have only accounted for the transport requirements of six solutes. The amino acids arginine, histidine, tyrosine, phenylalanine, methionine, threonine, valine, glutamine, asparagine: the organic acids acetate, pyruvate, succinate, α -ketoglutarate, malate, lactate, fumarate, formate, bicarbonate, and the inorganic ions sulfate, manganese, and phosphate, all of which

accumulate in *E. coli* (see Table 4 in reference 35), are likely to compound this discrepancy when their caloric requirements are known. On the basis of the measurements and calculations in this paper, we find membrane models of solute transport dependent on ATP for energy to be thermodynamically untenable. We therefore conclude that the present pump models as constituted pose a serious dilemma, a caloric catastrophe, so to speak."

The reader is referred to the comments of White and Ibsen (117), Raven (118), Essig (119), and Edelman (120) for criticism of the study by Minkoff and Damadian. The response to those criticisms may be found in references 121 and 122.

3. *Physical State of Ions and Water in Living Cells.* The controversy over the physical state of solutes (particularly, sodium and potassium) in living cells has waxed and waned since the turn of the century (11,15,123-125). It is generally accepted, however, that potassium is not significantly associated with cellular macromolecules (126,127).

The major piece of work cited as proof that potassium is free in the cell is that of Hodgkin and Keynes (34). Ling discussed these findings and offered an alternative explanation in 1969 (53). Also, in 1969, Jones and Karreman (45,46) reported that the cellular uptake of potassium in carotid artery smooth muscle could be described by a cooperative adsorption isotherm derived by Ling and Yang (65,51). In addition, Ling and Bohr (54) and Gulati and Reisin (74) have shown that potassium uptake in frog muscle and *taenia coli* can be described by the same adsorption isotherms. Gulati and Jones demonstrated that the potassium cooperative adsorption isotherm could be altered by varying concentrations of ouabain (69). These studies were extended to include a number of metabolic poisons and it was shown that cellular potassium is directly proportioned to the ATP content of skeletal muscle (55). More importantly, Ling (57) has reported a detailed study of the diffusion coefficient of potassium (in a "membraneless" preparation) and found that the diffusion coefficient of potassium in the cytoplasm is significantly reduced in a viable cell. As the muscle preparation deteriorates, the value for the potassium diffusion coefficient approaches those values reported by Hodgkin and Keynes (34).

and Kushmerick and Podolsky (35). (It may be of interest to note that Hodgkin and Keynes used the electrical activity as an index of the viability of the axoplasm of the squid axon, yet in 1962, Baker, Hodgkin and Shaw [128,129] demonstrated that electrical activity of the membrane was independent of the axoplasm).

The diffusion coefficient measurement of Ling (57) and the adsorption isotherms for potassium uptake quoted above offer strong evidence that intracellular potassium cannot be considered to have unrestricted motional freedom as in a dilute solution. In addition, the recent NMR studies of potassium (79-80b) support the general interpretation that the potassium ion interacts significantly with, or is adsorbed to, cytoplasmic proteins. These studies, however, do not provide information as to the mechanism of adsorption⁸ or to the localization of potassium within the cell. Within the context of the association-induction hypothesis, it is proposed that a large portion of the cytoplasmic potassium should be associated with β and γ carboxyl groups of the cellular proteins. In skeletal muscle, this means that the bulk of the potassium should be localized in the A band (i.e., the myosin fraction of skeletal muscle). Actually, evidence has been reported as early as 1905 showing that significant quantities of cellular potassium are localized in the A band of skeletal muscle (123, pages 150-151 of reference 124, 131-138). (For additional references published on this subject during the first half of the 20th century, see reference 124, Section C, pages 98-184).

Very recently, utilizing new freeze drying techniques and electron microscopy, Edelmann has found that the potassium is indeed localized in the myosin or A band of skeletal muscle (139). Edelmann, in order to obtain the needed resolution, replaced the sarcoplasmic potassium with cesium and

⁸Ling did provide a theoretical mechanism for selective ion-adsorption in a hypothetical fixed-charged system (Chapter 4 of reference 11). His cooperative adsorption isotherm provides a description of the uptake of potassium and desorption of sodium in a potassium-depleted and sodium-loaded muscle. Taken together, these fundamental, theoretical, and experimental studies indicate that the concept of selective adsorption as a general mechanism for the accumulation of potassium is reasonable. Furthermore, the findings on intracellular ionic conductivity (130), diffusion (57), and NMR relaxation times (79-80b) for potassium are consistent with the adsorption concept.

with thallium. Both cesium and thallium could be identified within the A band. Edelmann now has confirmed these findings by direct analysis of potassium with electron microprobe techniques (140). Ling has confirmed the findings of Edelmann utilizing autoradiograph techniques: potassium and substituted solutes such as cesium and thallium are localized in the A band of skeletal muscle (141).

The proponents of the two major concepts of the living cell have amassed considerable evidence in support of their ideas. Numerous articles and reviews on this subject may be referred to by the reader (2,3,4,8,9,11,13,14,15,17,38-40, 44,48,124). Understanding the physical state of water in biological systems, at least in a qualitative way, is essential if we are to know from which fundamental theoretical base to work. Obviously, the description of cellular water in terms of dilute solution (and, hence, osmotic) theory is attractive because it has been worked out extensively: it is difficult, however, to visualize cellular water as being essentially the same as water in dilute solution. Ambiguous terms such as "bound", "ice-like", and "ordered" water lack quantitative description. Furthermore, most scientists (particularly, physicists) find it hard to conceive that electrostatic forces on macromolecular surfaces can influence water molecules over distances greater than a few Angstroms. Even greater frustration may appear when experimental evidence suggests large numbers of water molecules are influenced by surfaces in general, and no physical model can be provided to explain the putative long-range order. Indeed, the complexity of the problem can be appreciated fully when it is realized that we have no clear-cut physical model for pure water.

In an attempt to provide a model of cellular water that could be subjected to experimental scrutiny, Ling, in 1965, developed the polarized multilayer concept (12). He gave, in 1972, a more complete account of the mechanism for the build-up of multilayers of water in living cells (9). Furthermore, in his equations describing cellular electrolyte concentrations (see equations 3-6, pages 9-10) he postulates a role for cellular water. This appears in the q value which is simply the partition coefficient for a given solute between the intra- and extra-cellular water (see equation 4). Ling, again in 1972, defined the q value in thermodynamic terms. He proposes that exclusion of certain cellular solutes is modulated by the physical state of the water which, in turn, is controlled by the macromolecular structures within the cell. The following is a summary of Ling's hypothesis (in regard to the q value) taken from his 1972 publication (see reference 9, pages 688-690).

"The theory was based on the concept that in those systems water exists as partially immobilized, polarized multilayers. Since, as a rule, water-soluble substances interact with water through hydrogen bonds or ionic bonds, the varying degree of fixation of the water can then impede the normal freedom of motion of the solute. However, since the excess energy due to polarization is not very high, the degree of immobilization will depend on how many hydrogen bonds the solute makes with the surrounding medium.

The transfer of a solute from normal water to water existing as polarized multilayers involves the following enthalpy and entropy changes.

1. To accommodate the solute, a hole must be dug in the water. Since the average water-water bound in the multilayer is higher than in normal water, this enthalpy change is unfavorable and thus tends to produce exclusion.

2. To varying degrees this exclusion is offset by the stronger hydrogen bonds the sucrose molecules make with surrounding water molecules in the polarized multilayers.

3. There is an immobilization and, hence, a decrease in the translational freedom of motion, hence translational entropy. This gain of negative entropy varies with the number of hydrogen bonds the solute forms with the water.

4. There is immobilization that decreases rotational-motion freedom, hence the rotational entropy. This gain of negative entropy should also increase with the increase in the number of hydrogen bonds the solute can form with the surrounding water molecules.

From these considerations one may derive theoretical premises.

a. For small molecules with no hydrogen-bonding groups, there will be little or no exclusion ($q \sim 1$).

b. Large, bulky molecules with few hydrogen-bonding groups on the surface, such as certain globular proteins, may be excluded for both enthalpic and entropic reasons.

c. Molecules that are not too bulky but do possess hydrogen-bonding groups, will be excluded according to the number of hydrogen-bonding groups they possess, and this exclusion occurs primarily for entropic reasons.

From these criteria, the theory predicts that many biologically important solutes such as sucrose and hydrated ions such as Na^+ should be excluded from polarized multilayered water on account of an unfavorable entropy."

Experimental testing of Ling's theory for cellular water can be approached from at least two major directions: 1) determining the q value for various solutes, and 2) certain physical properties of cellular water should be distinguished from those of pure water of dilute solutions. In regard to studies of the solvent properties of water in living and model systems (i.e., q values), Ling has demonstrated solute exclusion. For example, in sulfonate ion-exchange resins, the q values for methanol and D-glucose were found to be 0.89 and 0.25, respectively. The equilibrium distribution (q value) coefficients of methanol and D-glucose in the water of insulin-depleted frog-muscle fibers were found to be 0.9 and 0.25, respectively. Ling proposes that little or no "non-solvent" water (as defined by the classical osmotic view [2-4]) exists in living cells. In the case of solute exclusion in skeletal muscle, membrane theory would have to propose the existence of specific pumps which would require energy to maintain the unequal distributions. Ling has tested further the generality of his q value concept for charged solutes in exchange resins (53,142), rubidium in sheep's wool (53), and alcohols and sugars in exchange resins (143,144). Wiggins also has presented evidence that the structure of cellular water is enhanced relative to that of extracellular water (145). Furthermore, she has demonstrated that cellular water in cortical kidney slices and water in

silica gels will exclude sodium (146). In general, these findings are consistent with the part of Ling's hypothesis concerning cytoplasmic water.

In regard to Ling's hypothesis that the physical properties of cellular water and water in model systems are different from those of pure water, the evidence is substantial (9,12,53,143-150). Discussions of this evidence will be included in Section III of this paper.

4. Summary. The evidence supporting both views of the living cell are thought to be substantial and even unequivocal, depending on the position of a given investigator. Two major generalizations appear to the author to be clear.

(A) Virtually all of the so-called "unequivocal evidence" that has been presented in support of the classical membrane pump view of the cell has been challenged seriously by the proponents of the adsorption views in general and by the proponents of the association-induction hypothesis in particular. The proponents of the membrane pump view probably would admit that the application of dilute solution theory to describe such a complex system as the living cell will not be exact. That is, some of the cellular solutes will be adsorbed or even bound, and some of the cytoplasmic water may exhibit peculiar properties, but, for the most part, the amount of solutes and water that are involved in a non-ideal manner is small and can be either neglected or that corrections can be made for the non-ideal behavior. The question then becomes "how small is small", and at what point does "small" become too large. (B) The most fundamental difference between the two views is that they evolve from different thermodynamic bases. The membrane pump view is a steady-state concept. That is, the chemical potentials for solutes inside and outside the cell are not equal, and that the resultant unequal distributions are maintained by a continuous supply of energy through some mechanism within the membrane. The alternate view argues that the chemical potentials inside and outside the cell are the same. In this model, the unequal distribution of solutes is determined by altered physical states of the water and ions within the cell. These may be generalized in the following way.

$$\mu_j^i = \mu_{oj}^i + RT \ln a_j \quad (8)$$

and,

$$\mu_j^e = \mu_{oj}^e + RT \ln a_j \quad (9)$$

where $a_j = \gamma_j []_j$ and γ_j and $[]_j$ are the activities and the concentrations of the j th ion; μ_j^i and μ_j^e are the chemical potentials of the j th ion inside and outside the cell; μ_{oj}^i and μ_{oj}^e are the standard chemical potentials of the j th ion inside and outside the cells, respectively; and a_j is the activity of the j th ion. In the membrane pump view, $\mu_j^i \neq \mu_j^e$ and $\mu_{oj}^i \sim \mu_{oj}^e$, whereas the adsorption views regard the chemical potential within the cytoplasm to be entirely different from that of a dilute solution. Thus, $\mu_j^i = \mu_j^e$ and does not require that $[j]_i = [j]_e$. Thus, it becomes imperative that we know more about the physical state(s) of the ions and water for this provides one concrete way ultimately to distinguish between the two views.

The studies already referred to above provide near unequivocal evidence that potassium and sodium are associated in some way with cellular macromolecules. It is virtually certain that potassium is associated with myosin molecules and perhaps with the Z line in skeletal muscle. Indeed, potassium is not homogeneously distributed throughout the skeletal muscle. Unfortunately, the nature of the adsorption is not known at this time; however, extensive analysis of the relaxation time of the potassium nucleus by NMR techniques will provide needed information about the nature of potassium ion. In the author's view, the technique of extended X-ray adsorption fine structure (EXAFS) will provide the quantitative microscopic information concerning potassium adsorption in biological systems (152). At the present time, there are only a few facilities in the world in which synchrotron radiation and EXAFS analysis can be accomplished, but studies of potassium are already underway (153). In regard to water, considerable evidence of kinetic and non-kinetic nature has already been amassed permitting significant conclusions concerning its physical state in biological systems. Therefore, the remainder of this review will be concerned with reviewing our current understanding of the physical state of water in biological systems.

III. RECENT ADVANCES IN THE UNDERSTANDING OF THE PHYSICAL STATE OF WATER IN LIVING CELLS

Because of the enormous literature on water, it would be impossible for this review to be complete without constructing a book of several volumes. This section will be divided into two parts under the broad headings: *non-nuclear magnetic resonance investigations* and *nuclear magnetic resonance*

investigations. In general, the bulk of the references will date from 1965; however, earlier work will be referred to occasionally. Several references to broad reviews of this subject have already been cited (2-4,9,37,38,48,125,126). Perhaps the most comprehensive treatise on water is now up to five volumes and the sixth volume is being prepared by Felix Franks (154-158). It is the understanding of the author that the sixth volume by Franks will emphasize water in biological systems.

Reviews on water at various interfaces, including biological systems, have been published by Drost-Hansen (148, 149,159). In these reviews, considerable data indicating that surfaces of various types do influence the physical properties of water over large distances have been compiled. (See also chapter by Etzler and Drost-Hansen of this volume for a brief review of some aspects of this subject).

A. Non-nuclear Magnetic Resonance Investigations

1. *Thermal Anomalies.* Drost-Hansen (148) has championed the idea that the physical properties of water near virtually any interface are altered. In his 1971 review of *Structure and Properties of Water at Biological Interfaces*, he states:

"Most previous authors have not challenged the traditional view of aqueous interfacial structures; that is, they have ignored the possible existence of structural ordering in water near an interface. Among those who have taken issue with this over-simplified view are notably Derjaguin (1965)[60] in Russia and Henniker (1949)[161], Low (1961) [162], and the present author (1969b)[159] in this country. The traditional evidence for structuring in liquids, particularly for water adjacent to an interface, has come from measurements of viscosity of liquids in narrow pores, anomalous diffusion coefficients or energies of activation for ionic conduction in capillaries, etc. Recently, the present author (Drost-Hansen) has added to this type of evidence an additional, independent set of arguments for demonstrating the probable structuring of water near interfaces."

He then proceeds to offer evidence derived by various techniques (disjoining pressures, low-frequency mechanical damping, surface entropies and diffusion data) in support of the occurrence of "higher-order phase transitions which, in turn, are taken as evidence for the existence of large, ordered, cooperative arrangements of water molecules". The evidence reviewed by Drost-Hansen does appear to support the view that surfaces, in general, may alter the physical properties of water over quite large distances. No quantitative physical models, however, are presented to describe the enhanced order given to water molecules near interfaces.

2. *Dielectric Measurements.* Schwan and Foster have concluded recently from dielectric measurements that most of the tissue water has rotational mobilities identical to those in pure liquid water (163). Their work, however, does not discuss the publications of Fricke and Jacobsen (164,165) which contain interpretations that, in macromolecular solutions, significant quantities of water are bound to have perturbed molecular motions. Data from other laboratories are not consistent with the conclusions of Schwan and Foster (166,167). It seems, at the present time, that the interpretations of dielectric measurements of cellular water are, at best, equivocal. Perhaps, when measurements are made in the 20 gigahertz range, more definitive conclusions can be reached. (See chapter by Clegg in this volume).

3. *Viscosity Measurements.* Crick and Hughes (168), using a magnetic particle method, reported the modulus of rigidity of chick fibroblast cytoplasm to be on the order of 10^2 dynes/cm². They also reported evidence of this thixotropic behavior. Later, Wilson and Heilbrunn (169), using a centrifugation method, reported the viscosity of the immature egg of *Spisula* as 4.3 centipoises. These pioneering studies suggest that the cytoplasm of living cells is different from that of pure water.

More recently, electron spin resonance techniques have been used to measure cytoplasmic viscosity (170-175) and water in membrane vesicles (176). The results of these investigations indicate that the microviscosity of cytoplasm is between 3-10 centipoises compared with one centipoise for pure water.

These investigations utilizing ESR techniques measure primarily rotational correlation times from which viscosity of the surrounding fluid is measured. Keith, *et al.* (175, 177), however, have attempted to measure the translational

diffusion of probes in biological systems. Most interpretations of these data favor the idea that the two fractions of water (i.e., small fraction of hydration water and majority of free water) exist within the cell and that the physical state of cellular water is changed very little. We should be reminded that ESR provides information which permits one to make inferences about water through the molecular motions of the probe. An additional potential problem that clouds unambiguous interpretations of ESR data is the possibility of intracellular probe binding (171,173). It appears that additional studies utilizing this technique are needed to permit definitive conclusions concerning cellular water to be made.

4. *Calorimetry.* The use of microcalorimeters to obtain direct thermodynamic details of water in biological systems has, by necessity, been limited primarily to purified macromolecular solutions (178-187). (For additional references on this subject, see chapter by Clegg in this volume). The works of Andronikashvili and Mrevlishvili (188) were extended to include normal and cancerous tissue. They found that the relative amount of unfrozen water and the heat capacity of that fraction of water were greater than normal in several cancerous tissues. It also appears that the heat capacity of the unfrozen (so-called "bound") water is less than one. (It is not clear if this is the case for several macromolecular solutions - see reference 186). Above 0°C, the heat capacities of the water appear to be greater than one. This latter observation is consistent with the observations of Hoeve and Kakivaja (183) in which the heat capacity of water was determined as a function of the hydration of collagen. Hoeve and Kakivaja report the heat capacity of water in a water-collagen system to be $23 \text{ cal deg}^{-1} \text{ mol}^{-1}$ at 30°C over a wide range of water concentrations (0.008-0.95 gms H₂O/gm collagen). Clegg (personal communication; also see chapter by Clegg in this volume) has reported a similar value for the heat capacity of water in cells of the brine shrimp (*Artemia*) over a wide range of hydrations. The full significance of these observations of the heat capacity of water in biological systems must await further investigation; however, it appears obvious that the heat capacity of water in macromolecular systems and in living cells is different from that of pure water.

5. *Freezing Experiments.* Ice crystal formation of pure water is characterized by considerable branching taking on a "feather-like" appearance (143,189). In addition, pure water cannot be supercooled if ice crystals are present (i.e., nucleation occurs throughout). On the other hand, skeletal

muscle may be supercooled when the external solution is frozen and, when the muscle fibers are seeded with a micro-pipette with an iced tip, the ice crystals grow in a longitudinal direction without branching. For additional information on this subject, the reader is referred to several references concerned with the growth of ice crystals in living cells (143,190-192).

Chambers and Hale, as early as 1932 (190), and later Miller and Ling (192) demonstrated that the ice crystals in skeletal muscle grow in the longitudinal direction (appearing as a spicule) following the orientation of the protein filaments therein, even when the muscles were twisted or bent. These very simple experiments demonstrating the lack of lateral growth of the ice crystal, even though one might argue that they are too macroscopic to be definitive, do suggest strongly that cellular proteins (particularly in skeletal muscle) interact with cellular water so that the water is in a different physical state. Miller and Ling (192) have shown further that, with local muscle contraction, the structure of cellular water can be altered in the area of the contraction.

6. Bradley Adsorption Isotherm. In 1936, Bradley (193) attempted to explain the adsorption of inert gases on salt crystals. It was considered possible that inert gases, at low temperatures, might donate electrons to ions with incomplete electronic shells. He developed a theory to explain the formation of thick films, beginning with the assumption that the first layer of adsorbed atoms is polarized by the positive ions of the salt. The first layer polarizes the second and this, in turn, can attract a third layer, and so on until a thick film is built up with gradually decreasing polarization as the distance from the surface increases. This theoretical development resulted in the following equation.

$$T \log_{10} (P_o/P_a) = K_1 K_3^a \quad (10)$$

where T is temperature; P_o and P_a are the saturation pressures of the liquid and the adsorbed film, respectively; K_1 and K_3 are constants and a is the amount of gas adsorbed. Equation 10 requires a flat S-shaped curve which actually was observed. Later, in the same year, Bradley published another equation to describe the adsorption of molecules possessing a permanent dipole on finely-divided solids (194). This latter work resulted in equation (11).

$$\log (P_s/P) = K_1 K_3^a + K_4 \quad (11)$$

where P and P_s are the partial pressure and saturation pressures, respectively; a is the amount of adsorbed molecules; and K_1 , K_3 and K_4 are constants.

Ling, in 1965, applied equation (11) to several biological systems and found good agreement between experiments and theory (12; see reference 9 for additional publications utilizing the Bradley adsorption isotherm). Later, Ling and Negendank (195) published an extensive study of the hydration of skeletal muscle testing the utility of the Bradley adsorption isotherm. Again, theory and experiments were in accord. Cope, in 1965, derived equations, based on the Bradley adsorption isotherm, which accurately describe cellular volume as a function of external electrolyte concentration (196). Clegg also has tested the Bradley adsorption isotherm in the hydration of cells of the brine shrimp, *Artemia* (197; see chapter by Clegg in this volume). Clegg's data on the hydration of the entire cellular component of the cyst could not be described by a single isotherm; however, the possibility of the existence of more than one isotherm could not be ruled out for fully hydrated cells.

Although the Bradley adsorption isotherm can be used successfully to describe cellular hydration and volume, this does not by itself prove that cellular water exists in polarized multilayers. It does, however, strongly suggest that the physical state of water within cells and water associated with macromolecules is different from that of ordinary bulk water. In the case of most cellular systems tested, the results of these studies (12,195,196) imply that virtually all of the water experiences some restriction of motional freedom. Furthermore, the implied involvement of such a large amount of cellular water suggests that one might expect the solvent properties of the cellular water for various solutes to be altered. The exclusion of certain solutes by water in physical as well as biological systems (i.e., q values less than 1 - see Section II-C-3), and the fact that the Bradley adsorption isotherm can describe cellular hydration provides substantial circumstantial evidence that Ling's hypothesis may be correct. This subject will be discussed again in Section III-B.

7. *Density.* Ernst, et al. (198) proposed that there is an increase in water structure when the water content of skeletal muscle is reduced to approximately 0.3 gm of H₂O/gm of dry weight. Pocsik later found the fully hydrated muscle

(4 gms H₂O/gm dry weight) to have a water density of 1.012 gm/cm³ and that there was an increase in the density as muscle was dehydrated to 0.05 gms H₂O/gm dry weight. He further reported that the density increases substantially below 0.3 gm H₂O/gm dry weight. In the experiments the simple removal of water could have caused solute concentration and, thereby, increased density of the water associated with different materials within the muscle. This inference was met somewhat when Poosik determined the density of muscle water and of muscle in the frozen and the non-frozen state (200). He found that, upon freezing, the density of whole muscle and muscle water decreased upon freezing and that the density of frozen muscle water was slightly less than one. Additional discussion of these data may be found elsewhere (5,201,202). It is interesting that, when frozen, approximately 75% of the muscle water has a density less than one as expected for ice; yet, in native muscle, the density of this same volume of water is greater than one.

B. Nuclear Magnetic Resonance (NMR) Investigations of Water in Biological Systems

1. *Relaxation Times and the Diffusion Coefficient.* It appears that the first experiments published on live tissues, using high resolution NMR techniques, were those of Odeblad and his co-workers in 1956 (203). Later, in 1962, Berendsen (204) determined the water proton signals in collagen-water systems using broad-line NMR techniques. Bratton, Hopkins and Weinberg (205) used pulsed (spin-echo) NMR techniques to determine the relaxation times of water hydrogens in normal and contracted skeletal muscle. Since these early investigations, many papers have appeared and the author has attempted to review much of the literature and reduce this review to three tables. The data on normal tissues are listed in Table I. Table II contains a review of NMR data on tissues exposed to altered physiological conditions *in vivo* or *in vitro*. Table III attempts to summarize the data obtained on various isolated cellular systems.

The author is aware of the dangers of generalization, particularly when data on such a wide range of tissue and cellular types have been collected; however, such generalizations become necessary in order to formulate physical models that can be tested experimentally. In addition, it is good to begin model building at points of general agreement. It now appears that virtually all of the investigators involved in this area of research are in general agreement that the

TABLE I. Normal Tissues

<u>Tissue</u>	<u>Animal</u>	<u>Nucleus</u>	<u>Freq.</u> <u>(MHz)</u>	<u><i>T</i>₁</u> <u>(msec)</u>	<u><i>T</i>₂</u> <u>(msec)</u>	<u>D'a</u>	<u>Additional</u> <u>Information</u>	<u>Reference</u>
<u>BRAIN</u>								
Rat		H	24.0	595 \pm 7	--	--	A, H	206
Rat (cortex)		H	14.0	405 \pm 11	83.4 \pm 1.9	--	A	207
Rat (white matter)		H	14.0	347 \pm 10	79.5 \pm 1.5	--	A	207
Rat		H	25.0	472	--	--	A, H	208
Mouse (³ C ₃ H/He/Jms)		H	100.0	840	88	--	A	209
Rat		H	60.0	866 \pm 42	71 \pm 7	--	A, G, H, N	210
Rat		² H	4.0	131	22	--	C	211
Rat		² H	6.0	181 \pm 5	33 \pm 1	--	A	212
Rat		H	6.0	--	--	0.05-0.45	I, J	212
Rat		H	13.56	474 \pm 58	--	--	A, E, K, N	213
Mouse		H	30.0	646 \pm 4	45	--	A, H, L	214
Mouse		H	24.0	526	--	--	A, H	215
Mouse		H	2.7	284.8 \pm 1.0	75.21 \pm 0.38	--	A, E, N	215a
Human (gray matter)		H	60.0	435	105	--	A	216
Human (white matter)		H	60.0	--	65	--	C	216
Human		H	100.0	998 \pm 16	--	--	A, H	217
<u>LIVER</u>								
Rat		H	24.0	293 \pm 10	52 \pm 3	--	A	206
Rat		H	60.0	467	--	--	A, G	218
Mouse (living)		H	8.0	233 \pm 8	--	--	B, H, M	219
Mouse (5-15 minutes after death)		H	8.0	227 \pm 20	--	--	B, H, M	219
Mouse (1-3 hours after death)		H	8.0	250 \pm 8	--	--	A	219
Rat		H	25.0	273	--	--	A, H	208
Rat		H	100.0	420	37	--	A	209
Rat		H	60.0	340	30	--	A	210
Rat		H	8.0	163 \pm 9	--	--	A, G	220

TABLE I (continued)

<u>Tissue</u> <u>Animal</u>	<u>Nucleus</u>	<u>Freq.</u> <u>(MHz)</u>	<u>T₁</u> <u>(msec)</u>	<u>T₂</u> <u>(msec)</u>	<u>D'a</u>	<u>Additional</u> <u>Information</u>	<u>Reference</u>
<u>LIVER (con't.)</u>							
Chicken	H	32.0	208 \pm 16	--	--	A	221
Rat	H	38.0	--	--	0.30	--	222
Rabbit (10 day)	H	38.0	--	--	0.25 \pm 0.04	--	222
Rabbit (2 year)	H	38.0	--	--	0.195 \pm 0.03	--	222
Mouse	H	24.3	350 \pm 47	51	--	A	223
Rabbit	H	24.3	353 \pm 45	--	--	A	223
Hamster	H	24.3	303 \pm 45	--	--	A	223
Golden Hamster	H	25.6	--	53 \pm 3	--	A, G	240a
Dog	H	24.3	301	--	--	A	223
Human	H	24.3	383 \pm 48	--	--	A	223
Rat	H	13.56	238 \pm 32	--	--	A, E, K, N	213
Mouse	H	30.0	386 \pm 13	25	--	A, G, H, L, N	214, 226, 226a
Frog	H	20.0	270	46	0.42	A, N	224
Mouse	H	24.0	263	--	--	A, G, N	215, 227
Mouse	H	2.7	137.9 \pm 2.0	36.7 \pm 0.52	--	A, E, N	215a
Mouse (C ₃ H)	H	30.3	438 \pm 36	32.5 \pm 1.7	--	A, G, H	225
Human	H	100	570 \pm 29	--	--	A, H	217
<u>HEART</u>							
Rat	H	25	518	--	--	A, H	208
Rat	H	60	873 \pm 27	46 \pm 11	--	A, N	210
Rat	H	38	--	--	0.36 \pm 0.02	--	222
Rabbit	H	38	--	--	0.26 \pm 0.03	--	222
Human	H	24.3	873 \pm 118	--	--	A	223
Mouse	H	30	650 \pm 6	45	--	A, H, L	214
Mouse	H	24.0	490	--	--	A, G, H	215, 227
Human	H	100.0	906 \pm 46	--	--	A, H	217
<u>KIDNEY</u>							
Human	H	100.0	862 \pm 33	--	--	A, H	217
Mouse	H	30.3	503 \pm 11	47 \pm 2.4	--	A, G, H	225

KIDNEY (con't.)

<i>Mouse</i>	H	2.7	214.0	\pm 2.1	56.19	\pm 0.71	--	A, E, N	215a
<i>Rat</i>	H	24.0	480	\pm 26	--	--	--	A	206
<i>Rat</i>	H	60.0	668	--	--	--	A, G	218	
<i>Rat</i>	H	25.0	577	--	--	--	A, H	208	
<i>Rat</i>	H	60.0	685	--	56	--	A, N	210	
<i>Chicken</i>	H	32	403	\pm 37	--	--	A	221	
<i>Mouse</i>	H	24.3	396	\pm 43	52	--	A	223	
<i>Rabbit</i>	H	24.3	363	--	--	--	A	223	
<i>Hamster</i>	H	24.3	398	\pm 38	--	--	A	223	
<i>Dog</i>	H	24.3	534	--	--	--	A	223	
<i>Human</i>	H	24.3	770	--	--	--	A	223	
<i>Rat</i>	H	13.56	410	\pm 41	--	--	A, E, K, N	213	
<i>Mouse</i>	H	30.0	470	\pm 6	42	--	A, G, H, L, N	214, 226, 226a	
<i>Mouse</i>	H	24.0	370	--	--	--	A, G, H	215, 227	
<i>Human</i>	H	24.0	459	--	--	--	A, G, H	215, 227	

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SKELETAL MUSCLE

(a) Rectus Muscle

<i>Rat</i>	H	24.0	538	\pm 15	55	\pm 5	--	A	206
<i>Rat</i>	H	13.56	404	\pm 48	--	--	--	A, E, K, N	213

(b) Muscle (specific type not mentioned)

<i>Rat</i>	H	60.0	850	--	--	--	A, G	218	
<i>Cow</i>	H	25.6	\sim 500	\sim 50	--	--	A, G, H, I	240	
<i>Mouse</i>	H	100.0	850	57	--	--	A	209	
<i>Rat</i>	2H	4.0	92	9	--	--	C	211	
<i>Mouse</i>	H	2.7	184.7	\pm 1.9	48.52	\pm 0.43	--	A, E, N	215a
<i>Mouse</i>	H	30.0	615	\pm 10	37	--	A, G, H, L, N	214, 226, 226a	
<i>Human</i>	H	100.0	1023	\pm 29	--	--	A, H	217	
<i>Rat</i>	H	30.0	720	\pm 50	44.75	\pm 0.08	0.51	C, H	236, 237
					195.65	\pm 34.2			251, 252

TABLE I (continued)

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<u>Tissue</u>	<u>Animal</u>	<u>Nucleus</u>	<u>Freq.</u> <u>(MHz)</u>	<u>T_1</u> <u>(msec)</u>	<u>T_2</u> <u>(msec)</u>	<u>D'a</u>	<u>Additional</u> <u>Information</u>	<u>Reference</u>
<u>SKELETAL MUSCLE</u>								
(b) <u>Muscle</u> (specific type not mentioned--con't.)								
Frog		H	60.0	--	--	0.7-0.4	F	269
Frog (<i>Rana pipiens</i>)		H	24.0	400	40	--	A	205
Human		H	24.3	807	--	--	A	223
(c) <u>Gastrocnemius muscle</u>								
Frog		H	27.5	700	44	--	A,D,E	228
Frog (muscle excitable, relaxed)		H	30.0	--	48 \pm 3	0.46 \pm 0.03	A,N	229
Rat (muscle excitable, relaxed)		H	30.0	--	52 \pm 4	0.45 \pm 0.03	A,N	229
Frog		H	8.1	423 \pm 12.8	--	--	A,G,H,N	230,231
Frog		2H	8.1	143 \pm 5.5	--	--	A,G,H,N	230,231
Frog		170	8.1	1.86 \pm 0.041	--	--	B,G,H,N	230,231
Frog		H	20.0	658	47	0.65	A,N	224
Frog		H	60.0	--	168	--	C	232
					38			
					10			
Rat		H	60.0	850	--	--	A,D,E,H,I,N	233
Toad (<i>Bufo marinus</i>)		H	30.0	\sim 730	--	--	A,D,E,H,I,N	234
Frog		H	10.0	\sim 520	\sim 30	--	A,I,N	235
Mouse		H	24.0	471	--	--	A,G,N	215,227
(d) <u>Porcine muscle</u>								
Pig (post rigor)		H	10.7	405 \pm 10	$\frac{53 + 2}{140 + 10}$ }	0.45 \pm 0.05	C,D,E,H,N	238 239
(e) <u>Thigh muscle</u>								
Rat		2H	6.0	112 \pm 2	18 \pm 0.5	--	A	212
Rat		H	6.0	--	--	0.7-0.3	I,J	212

SKELETAL MUSCLE (con't.)(f) Sartorius muscle

Frog	H	60.0	1400 ± 100 1000 ± 50	44 \pm 3	--	A, F, Q	269
Toad	H	20.77	230 ± 20	37 \pm 3	0.47	A, N	253

(g) Semi-tendinous muscle

Frog	H	60.0	1100 ± 200 1350 ± 200	55 \pm 15	--	A, F, Q	269
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(h) Peronius muscle

Frog	H	60.0	1100 ± 100	50 \pm 10	--	A, F, Q	269
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(i) Red muscle

Chicken	H	32.0	642 ± 22	--	--	A	221
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(j) White muscle

Chicken	H	32.0	598 ± 20	--	--	A	221
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(k) Breast muscle

Chicken (male)	H	30.0	661	39	--	A, G, R	268
Chicken (female)	H	30.0	644	39	--	A, G, R	268

(l) Adductor muscle

Toad	H	20.77	125 ± 5	29.7 ± 2	0.5	A, N	253
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HEART MUSCLE

Rat	H	38.0	--	--	0.4-0.3	--	222
Rat (myocardium)	H	30.0	725 ± 122	51 \pm 2	--	A, H	241

TABLE I (continued)

<u>Tissue</u>	<u>Animal</u>	<u>Nucleus</u>	<u>Freq.</u> <u>(MHz)</u>	<u>T_1</u> <u>(msec)</u>	<u>T_2</u> <u>(msec)</u>	<u>D'a</u>	<u>Additional</u> <u>Information</u>	<u>Reference</u>
<u>SPLEEN</u>								
	Rat	H	60.0	582	--	--	A, G	218
	Rat	H	25.0	457	--	--	A, H	208
	Rat	H	8.0	273 \pm 8	--	--	A, G	220
	Chicken	H	32.0	596 \pm 22	--	--	A	221
	Mouse	H	24.3	571 \pm 45	61	--	A	223
	Rabbit	H	24.3	614 \pm 47	--	--	A	223
	Golden Hamster	H	25.6	--	72 \pm 3	--	A, G	240a
	Hamster	H	24.3	600	--	--	A	223
	Dog	H	24.3	539	--	--	A	223
	Mouse	H	30.0	571 \pm 8	45	--	A, G, H, L, N	214, 226, 226a
198	Mouse	H	24.0	458	--	--	A, G, H	215, 227
	Mouse	H	2.7	262.7 \pm 1.8	65.76 \pm 0.97	--	A, E, N	215a
	Human	H	24.3	543	--	--	A, P	242
	Human	H	100.0	701 \pm 45	--	--	A, H	217
	Mouse	H	30.3	737 \pm 6	55 \pm 1.7	--	A, G, H	225
	Human	H	24.3	680 \pm 177	--	--	A	223
<u>LUNG</u>								
	Rat	H	25.0	487	--	--	A, H	208
	Chicken	H	32.0	670 \pm 70	--	--	A	221
	Mouse	H	30.0	641 \pm 9	47	--	A, G, H, L, N	214, 226, 226a
	Mouse	H	24.0	491	--	--	A, H	215
	Human	H	24.0	404	--	--	A, H	215
	Human	H	100.0	788 \pm 63	--	--	A, H	217
<u>STOMACH</u>								
	Rat	H	24.0	270 \pm 16	--	--	A, H	206
	Human	H	24.3	541	--	--	A	223
	Mouse	H	30.0	294 \pm 23	35.0	--	A, H, L	214
	Human	H	100.0	765 \pm 75	--	--	A, H	217
	Dog (gastric mucosa-fundus)	H	30.0	684	74	0.353	A, F, G, H, V	214a

SMALL INTESTINE

Rat	H	24.0	257	\pm 30	--	--	A, H	206
Mouse	H	30.0	366	\pm 19	35.0	--	A, H, L	214
Mouse	H	24.0	255		--	--	A, H	215
Human (small bowel)	H	100.0	641	\pm 80	--	--	A, H	217

SKIN

Rat	H	25.0	272		--	--	A, H	208
Mouse	H	30.0	390	\pm 39	35.0	--	A, H, L	214
Mouse	H	24.0	199		--	--	A, H	215
Golden Hamster	H	25.6	--		72 \pm 3	--	A, G	240a
Human	H	24.3	423		--	--	A, P	242
Human	H	100.0	616	\pm 19	--	--	A, H	217

TAIL

Rat	H	25.0	258.0		--	--	A	208
Mouse	H	18.0	300.0		--	--	A	243
Mouse	H	24.0	218.0		--	--	A	215

THYROID GLAND

Human	H	100.0	882	\pm 45	--	--	A, H	217
Human	H	32.0	< 700		--	--	A	244
Human	H	24.3	586		--	--	A	223
Human	H	24.3	410		--	--	A, P	242
Rat	H	32.0	552	\pm 57	37 \pm 5.9	--	R, S	245

BONE MARROW

Chicken	H	32.0	408	\pm 53	--	--	A	221
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GINGIVIAL TISSUE

Human	H	16.7	--		6.8	--	A, K	246
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TABLE I (continued)

<u>Tissue</u>	<u>Animal</u>	<u>Nucleus</u>	<u>Freq.</u> <u>(MHz)</u>	<u>T_1</u> <u>(msec)</u>	<u>T_2</u> <u>(msec)</u>	<u>D'a</u>	<u>Additional</u> <u>Information</u>	<u>Reference</u>
<u>BREAST TISSUE</u>								
Mouse (pregnant)		H	30.3	357 \pm 47	47.1 \pm 0.9	0.155	A, G, H	225
Human		H	100.0	367 \pm 79	--	--	A, H	217
Human		H	24.3	191	--	--	A, P	242
Human		H	30.3	682 \pm 32	35.5 \pm 3.5	--	A, H	250
Rat (lactating mammary)		H	13.56	337 \pm 26	--	--	A, E, K, N	213
<u>COLON</u>								
Human		H	24.0	552	--	--	A, P	215, 242
Human		H	100.0	641 \pm 43	--	--	A, H	217
<u>ESOPHAGUS</u>								
Human		H	100.0	804 \pm 108	--	--	A, H	217
<u>LYMPHATIC TISSUE</u>								
Human		H	100.0	720 \pm 76	--	--	A, H	217
Mouse		H	24.3	867	--	--	A	223
<u>BONE TISSUE</u>								
Human		H	100.0	554 \pm 27	--	--	A, H	217
<u>BLADDER</u>								
Human		H	100.0	891 \pm 61	--	--	A, H	217
<u>NERVE</u>								
Human		H	100.0	557 \pm 158	--	--	A, H	217
Frog (polarized nerve trunk)		H	60.0	--	260 34	--	C	247
Frog (depolarized nerve trunk)		H	60.0	--	180 24	--	C	247

<u>OVARY</u>							
<i>Human</i>	H	100.0	989 ± 47	--	--	A,H	217
<u>UTERUS</u>							
<i>Human</i>	H	100.0	924 ± 38	--	--	A,H	217
<u>CERVIX</u>							
<i>Human</i>	H	100.0	827 ± 26	--	--	A,H	217
<u>TESTES</u>							
<i>Human</i>	H	100.0	1200 ± 48	--	--	A,H	217
<i>Human</i>	H	24.3	1010	--	--	A	223
<u>PROSTATE</u>							
<i>Human</i>	H	100.0	803 ± 14	--	--	A,H	217
<i>Human</i>	H	24.3	767	--	--	A	223
<u>ADRENAL</u>							
<i>Human</i>	H	100.0	608 ± 20	--	--	A,H	217
<i>Human</i>	H	24.3	585	--	--	A	223
<u>PERITONEUM</u>							
<i>Human</i>	H	100.0	476	--	--	A,H	217
<u>PANCREAS</u>							
<i>Human</i>	H	100.0	605 ± 36	--	--	A,H	217
<i>Human</i>	H	24.3	320 ± 15	--	--	A	223
<u>PLACENTA</u>							
<i>Human</i>	H	24.3	1000	--	--	A	223

TABLE I (continued)

<u>Tissue Animal</u>	<u>Nucleus</u>	<u>Freq. (MHz)</u>	<u>T_1 (msec)</u>	<u>T_2 (msec)</u>	<u>D'a</u>	<u>Additional Information</u>	<u>Reference</u>
<u>THYMUS</u>							
Human	H	24.3	809	--	--	A	223
<u>EGGS</u>							
Frog	H	30.0	216	36	0.28	A, N	248
<u>EMBRYONIC CYSTS</u>							
Brine shrimp (<i>Artemia salina</i>)							
(a) Dry cysts	H	30.0	178	67.9	0.035	A, G	248a
(b) Cysts (fully hydrated; i.e., 1.4 gm/gm of dry solids)	H	30.0	255	54.2	0.16	A, G	248a
<u>EYE LENS</u>							
Rabbit (whole lens)	H	25.0	490	29.3	0.37	C	249
Rabbit (cortex)	H	25.0	551	40.0	0.436	C	249
Rabbit (nucleus)	H	25.0	268	7.7	0.40	C	249
<u>SLIME MOULD</u>							
	H	20.77	131 \pm 13	30.7 \pm 2.2	0.49	A, N	253
<u>BLOOD</u>							
Chicken	H	32.0	920 \pm 20	--	--	A	221
Human (oxy blood)	H	6.0	559	--	--	A, E, N	261b
<u>PLASMA</u>							
Chicken	H	32.0	1580 \pm 110	--	--	A	221
Human	H	38.0	--	--	0.86 \pm 0.02	--	222
Human	170	10.0	3.9	--	--	N	261
Human	H	9.0	--	540	--	A	261a
Human (oxyplasma)	H	6.0	1098	--	--	A, E, N	261b

TABLE II. Tissues - Altered Physiological State

A. Development

<u>Tissue Animal</u>	<u>Nucleus</u>	<u>Freq. (MHz)</u>	<u>T_1 (msec)</u>	<u>T_2 (msec)</u>	<u>D'a</u>	<u>Additional Information</u>	<u>Reference</u>
<u>IMMATURE SKELETAL MUSCLE</u>							
Rat	H	30.0	1206 \pm 55	127 \pm 9	--	A, H	251
<u>IMMATURE BRAIN</u>							
Rat	H	60.0	1361	149	--	A, H, N	210
<u>IMMATURE HEART</u>							
Rat	H	60.0	936	69	--	A, H, N	210
<u>IMMATURE LIVER</u>							
Rat	H	60.0	527	43	--	A, H, N	210
<u>B. Pathology</u>							
<u>BRAIN</u>							
<u>Vasogenic Edema of Brain</u>							
Rat (cortex)	H	14.0	651 \pm 47	115 \pm 3.9	--	A	207
Rat (white matter)	H	14.0	591 \pm 22	131.5 \pm 7.4	--	A	207
<u>Osmotic Edema of Brain</u>							
Rat (cortex)	H	14.0	463 \pm 23	91.6 \pm 3.1	--	A	207
Rat (white matter)	H	14.0	392 \pm 24	91.2 \pm 3.1	--	A	207

TABLE II (continued)

<u>Tissue</u> <u>Animal</u>	<u>Nucleus</u>	<u>Freq.</u> <u>(MHz)</u>	<u>T_1</u> <u>(msec)</u>	<u>T_2</u> <u>(msec)</u>	<u>D'a</u>	<u>Additional</u> <u>Information</u>	<u>Reference</u>
<u>TRIETHYLTIN EDEMA</u>							
Rat (cortex)	H	14.0	428 \pm 35	82.5 \pm 2.2	--	A	207
Rat (white matter)	H	14.0	455 \pm 35	90.3 \pm 6.8	--	B,C	207
				389 \pm 77			
<u>HEART</u>							
Mouse (infected with salmonella)	H	30.0	643 \pm 7	--	--	A	214
<u>LIVER</u>							
Chicken (infected with erythroleukosis)	H	32.0	370 \pm 36	--	--	A	221
Mouse (infected with salmonella)	H	30.0	371 \pm 7	--	--	A,H	214
<u>SPLEEN</u>							
Chicken (infected with erythroleukosis)	H	32.0	641 \pm 19	--	--	A	221
Mouse (infected with salmonella)	H	30.0	589 \pm 13	--	--	A,H	214
<u>KIDNEY</u>							
Chicken (infected with erythroleukosis)	H	32.0	481 \pm 38	--	--	A	221
Mouse (infected with salmonella)	H	30.0	484 \pm 12	--	--	A,H	214
<u>LUNG</u>							
Golden Hamster (oedema)	H	25.6	--	106 \pm 3	--	A	240a

LUNG (con't.)

<i>Chicken (infected with erythroleukosis)</i>	H	32.0	735 \pm 50	--	--	A	221
<i>Mouse (infected with salmonella)</i>	H	30.0	644 \pm 1	--	--	A, H	214

RED MUSCLE

<i>Chicken (infected with erythroleukosis)</i>	H	32.0	665 \pm 48	--	--	A	221
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WHITE MUSCLE

<i>Chicken (infected with erythroleukosis)</i>	H	32.0	1320 \pm 130	--	--	A	221
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BREAST MUSCLE

<i>Dystrophic chicken (male)</i>	H	30.0	700	53	--	A, G, R	268
<i>Dystrophic chicken (female)</i>	H	30.0	678	53	--	A, G, R	268

MUSCLE

<i>Mouse (infected with salmonella)</i>	H	30.0	625 \pm 10	--	--	A, H	214
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BONE MARROW

<i>Chicken (infected with erythroleukosis)</i>	H	32.0	717 \pm 42	--	--	A	221
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WHOLE BLOOD

<i>Chicken (infected with erythroleukosis)</i>	H	32.0	1320 \pm 130	--	--	A	221
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TABLE II (continued)

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<u>Tissue Animal</u>	<u>Nucleus</u>	<u>Freq. (MHz)</u>	<u>T_1 (msec)</u>	<u>T_2 (msec)</u>	<u>D'a</u>	<u>Additional Information</u>	<u>Reference</u>
<u>PLASMA</u>							
Chicken (infected with erythroleukosis)	H	32.0	1810 \pm 300	--	--	A	221
<u>SMALL INTESTINE</u>							
Rat (cholera exposed)	H	30.0	668 \pm 119	80.4 \pm 21.5	--	A, G	254
Mouse (infected with salmonella)	H	30.0	329 \pm 19	--	--	A	214
<u>STOMACH</u>							
Mouse (infected with salmonella)	H	30.0	399 \pm 26	--	--	A	214
<u>NODULE LINE D₁</u>							
Mouse (hyperblastic alveolar nodule outgrowth)	H	30.3	657 \pm 68	54.7 \pm 1.8	0.3105	A, G, H	225
<u>NODULE LINE D₂</u>							
Mouse (hyperblastic alveolar nodule outgrowth)	H	30.3	709 \pm 29	54.1 \pm 1.2	0.273	A, G, H	225
<u>C. Cancerous Tissues</u>							
<u>WALKER SARCOMA</u>							
Rat	H	24.0	736 \pm 22	100	--	A, H	206
Rat (Walker-256)	H	60.0	1093	77	--	A, G, N	210
<u>HEPATOMA</u>							
Rat	H	24.0	826 \pm 13	118 \pm 2	--	A, H	206
Rat (Dunning hepatoma)	H	13.56	422 \pm 18	--	--	A, E, K, N	213

FIBROADENOMA (Benign)

Rat	H	24.0	492	--	--	A,H	206
Human (breast)	H	30.3	980 \pm 51	62.5 \pm 7.3	--	A,H	250
Human (breast)	H	24.3	496	--	--	A,H,P	242

BRAIN TUMOR

Human (malignant)	H	60.0	588	108	--	C	216
Human (benign)	H	60.0	526	87	--	C	216

ADENOCARCINOMA

Rat	H	60.0	1113	--	--	A,G	218
Rat (mammary adenocarcinoma)	H	13.56	560 \pm 32	--	--	A,E,K,N	213
Mouse	H	24.0	798	--	--	A,H,P	215
Human (lung)	H	24.3	555	--	--	A,H,P	242
Human (colon)	H	24.3	595	--	--	A,H,P	242
Human (stomach)	H	24.3	535	--	--	A,H,P	242
Human (breast)	H	30.3	874 \pm 28	68.6 \pm 2.3	--	A,H	250

FIBROSARCOMA

Mouse	H	25.0	749	--	--	A,H	208
Mouse	H	24.0	662	--	--	A,H,P	215
Mouse (MC-1)	H	30.0	853 \pm 19	47 \pm 3	--	A,G,H,L,N	214, 226, 226a

MELANOMA

Golden Hamster (melanotic melanoma tumor)	H	25.6	--	67 \pm 2	--	A,G	240a
Golden Hamster (amelanotic melanoma tumor)	H	25.6	--	128 \pm 3	--	A,G	240a
Mouse	H	24.0	708	--	--	A,H,P	215
Mouse (amelanotic melanoma tumor)	H	25.6	\sim 725	\sim 82	--	A,G,H,I	240
Human	H	100.0	724 \pm 147	--	--	A,H	217

TABLE II (continued)

208

<u>Tissue Animal</u>	<u>Nucleus</u>	<u>Freq. (MHz)</u>	<u>T_1 (msec)</u>	<u>T_2 (msec)</u>	<u>D'a</u>	<u>Additional Information</u>	<u>Reference</u>
<u>RETICULUM SARCOMA</u>							
Rat	H	60.0	1138	--	--	A, G	218
<u>RHABDOMYOSARCOMA</u>							
Rat	H	60.0	1150	--	--	A, G	218
<u>LEIOMYOSARCOMA</u>							
Human	H	24.3	793	--	--	A	223
<u>EHRLICH SOLID TUMOR</u>							
Rat	H	60.0	1025 \pm 7	38.2 \pm 6	--	A, G, H	210
<u>PERODONTITIS CHRON</u>							
Human	H	16.7	--	9.6	--	A	246
<u>LYMPHOSARCOMA</u>							
Mouse	H	24.3	669 \pm 63	--	--	A	223
<u>ASCITES SARCOMA</u>							
Mouse	H	2.7	362.2 \pm 5.5	82.02 \pm 1.6	--	A, E, N	215a
<u>BREAST</u>							
Human	H	100.0	1080 \pm 80	--	--	A, H	217
Human (adenocarcinoma)	H	30.3	874 \pm 28	68.6 \pm 2.3	--	A, H	250
Human (fibrocystic)	H	30.3	655 \pm 21	37.0 \pm 3.0	--	A, H	250
Mouse (mammary tumor)	H	30.3	920 \pm 47	91 \pm 8	0.33	A, H	255
Human (carcinoma)	H	25.0	819	--	--	A, G, H, P	256, 257
Human (metastatic carcinoma)	H	24.3	575	--	--	A, P	242
Human (fibrocystic)	H	24.3	450	--	--	A, P	242
Human (lobular carcinoma)	H	24.3	658	--	--	A	223

LIVER

<i>Human</i>	H	100.0	832 + 12	--	--	A, H	217
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SPLEEN

<i>Human</i>	H	100.0	1113 + 6	--	--	A, H	217
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KIDNEY

<i>Human</i>	H	24.0	638	--	--	A, H	215
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LUNG

<i>Human</i>	H	100.0	1110 + 57	--	--	A, H	217
<i>Human (adenocarcinoma)</i>	H	24.3	555	--	--	A, H, P	242
<i>Human (carcinoma)</i>	H	25.0	1003	--	--	A, G, H, P	256, 257

SKIN

<i>Human</i>	H	100.0	1047 + 108	--	--	A, H	217
<i>Human</i>	H	25.0	1037	--	--	A, G, H, P	256, 257

MUSCLE

<i>Human (malignant)</i>	H	100.0	1413 + 82	--	--	A, H	217
<i>Human (benign)</i>	H	100.0	1307 + 154	--	--	A, H	217

ESOPHAGUS

<i>Human</i>	H	100.0	1040	--	--	A, H	217
<i>Human</i>	H	25.0	895	--	--	A, G, H, P	256, 257

STOMACH

<i>Human</i>	H	100.0	1238 + 109	--	--	A, H	217
<i>Human</i>	H	25.0	997	--	--	A, G, H, P	256, 257
<i>Human (adenocarcinoma)</i>	H	24.3	535	--	--	A, H, P	242

TABLE II (continued)

<u>Tissue</u>	<u>Animal</u>	<u>Nucleus</u>	<u>Freq.</u> <u>(MHz)</u>	<u>T_1</u> <u>(msec)</u>	<u>T_2</u> <u>(msec)</u>	<u>D'a</u>	<u>Additional</u> <u>Information</u>	<u>Reference</u>
<u>INTESTINAL TRACT</u>								
	<i>Human</i>	H	100.0	1122 \pm 40	--	--	A, H	217
<u>COLON</u>								
	<i>Human (adenocarcinoma)</i>	H	24.3	595	--	--	A, H, P	242
<u>RECTUM</u>								
	<i>Human</i>	H	25.0	1270	--	--	A, G, H, P	256, 257
<u>THYROID</u>								
210	<i>Human</i>	H	100.0	1072	--	--	A, H	217
	<i>Human</i>	H	25.0	669	--	--	A, G, H, P	256, 257
	<i>Human</i>	H	32.0	> 700	--	--	A	244
	<i>Human</i>	H	24.3	679	--	--	A, H, P	242
<u>ADRENAL</u>								
	<i>Human</i>	H	100.0	683	--	--	A, H	217
<u>PROSTATE</u>								
	<i>Human</i>	H	100.0	1110	--	--	A, H	217
<u>CERVIX</u>								
	<i>Human</i>	H	100.0	1101	--	--	A, H	217
	<i>Human</i>	H	25.0	1089	--	--	A, G, H, P	256, 257
<u>UTERUS</u>								
	<i>Human (malignant)</i>	H	100.0	1393	--	--	A, H	217
	<i>Human (benign)</i>	H	100.0	973	--	--	A, H	217
<u>BLADDER</u>								
	<i>Human</i>	H	100.0	1241 \pm 165	--	--	A, H	217

TESTES

<i>Human</i>	<i>H</i>	100.0	1223	--	--	<i>A,H</i>	217
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OVARY

<i>Human</i>	<i>H</i>	100.0	1282 \pm 118	--	--	<i>A,H</i>	217
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NERVE

<i>Human</i>	<i>H</i>	100.0	1204	--	--	<i>A,H</i>	217
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BONE

<i>Human</i>	<i>H</i>	100.0	1027 \pm 152	--	--	<i>A,H</i>	217
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<i>Human (osteogenic sarcoma)</i>	<i>H</i>	25.0	754	--	--	<i>A,G,H,P</i>	256, 257
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<i>Human (osteogenic sarcoma)</i>	<i>H</i>	24.3	1140	--	--	<i>A</i>	223
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TONGUE

<i>Human</i>	<i>H</i>	100.0	1288	--	--	<i>A,H</i>	217
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CHEEK

<i>Human</i>	<i>H</i>	25.0	1189	--	--	<i>A,G,H,P</i>	256, 257
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TAIL

<i>Mouse (in vivo)</i>	<i>H</i>	18.0	700	--	--	<i>B</i>	243
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*D. Tissues from Tumor-Bearing Animals*LIVER

<i>Golden Hamster (bearing melanotic melanoma)</i>	<i>H</i>	25.6	--	57 \pm 3	--	<i>A,G</i>	240a
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TABLE II (continued)

<u>Tissue</u> <u>Animal</u>	<u>Nucleus</u>	<u>Freq.</u> <u>(MHz)</u>	<u>T_1</u> <u>(msec)</u>	<u>T_2</u> <u>(msec)</u>	<u>D'a</u>	<u>Additional</u> <u>Information</u>	<u>Reference</u>
<u>LIVER</u> (con't.)							
Golden Hamster (bearing melanotic melanoma)	H	25.6	--	55 \pm 3	--	A, G	240a
Mouse (bearing mammary tumor)	H	30.3	468 \pm 35	33.7 \pm 2.9	--	A, G, H	225
Mouse (with large fibrosarcoma tumor)	H	30.0	461 \pm 9	33.0 \pm 2	--	A, H, L	214
Rat (with reticulum sarcoma)	H	60.0	652	--	--	A, G	218
Rat (with adeno-carcinoma)	H	60.0	628	--	--	A, G	218
Rat (with Rhabdomyo-sarcoma)	H	60.0	556	--	--	A, G	218
Mouse (bearing MH 134 solid tumor)	H	100.0	480	35	--	A	209
Mouse (bearing ascites tumor)	H	2.7	161.3 \pm 3.6	41.01 \pm 0.94	--	A, E, N	215a
<u>KIDNEY</u>							
Mouse (bearing mammary tumor)	H	30.3	577 \pm 14	51.4 \pm 2.7	--	A, G, H	225
Mouse (bearing large fibrosarcoma tumor)	H	30.0	601 \pm 19	40 \pm 2	--	A, H, L	214
Rat (bearing Rhabdomyo-sarcoma)	H	60.0	811	--	--	A, G	218
Mouse (bearing ascites sarcoma)	H	2.7	236.9 \pm 2.4	61.71 \pm 0.77	--	A, E, N	215a
<u>SPLEEN</u>							
Mouse (bearing mammary tumor)	H	30.3	734 \pm 12	52.8 \pm 1.4	--	A, G, H	225

SPLEEN (con't.)

Mouse (bearing large fibrosarcoma tumor)	H	30.0	731	<u>±</u> 8	43	<u>±</u> 2	--	A,H,L	214
Rat (bearing Rhabdomyo-sarcoma)	H	60.0	686	--	--	--	A,G	218	
Golden Hamster (bearing melanotic melanoma)	H	25.6	--	69	<u>±</u> 3	--	A,G	240a	
Golden Hamster (bearing amelanotic melanoma)	H	25.6	--	70	<u>±</u> 3	--	A,G	240a	
Mouse (bearing ascites sarcoma)	H	2.7	271.2	<u>±</u> 2.3	67.84	<u>±</u> 0.54	--	A,E,N	215a

BRAIN

Mouse (bearing large fibrosarcoma tumor)	H	30.0	681	<u>±</u> 41	43	<u>±</u> 2	--	A,H,L	214
Mouse (bearing ascites sarcoma)	H	2.7	288.3	<u>±</u> 1.6	76.09	<u>±</u> 0.25	--	A,E,N	215a
Mouse (bearing MH 134 solid tumor)	H	100.0	930	--	88	--	A	209	

HEART

Mouse (bearing large fibrosarcoma tumor)	H	30.0	705	<u>±</u> 5	35	<u>±</u> 1	--	A,H,L	214
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LUNG

Mouse (bearing large fibrosarcoma tumor)	H	30.0	665	<u>±</u> 47	49	<u>±</u> 3	--	A,H,L	214
Golden Hamster (bearing amelanotic melanoma)	H	25.6	--	75	<u>±</u> 3	--	A,G	240a	

TABLE II (continued)

214

<u>Tissue Animal</u>	<u>Nucleus</u>	<u>Freq. (MHz)</u>	<u>T_1 (msec)</u>	<u>T_2 (msec)</u>	<u>D'a</u>	<u>Additional Information</u>	<u>Reference</u>
<u>SKIN</u>							
Mouse (bearing large fibrosarcoma tumor)	H	30.0	442 \pm 37	40 \pm 1	--	A,H,L	214
<u>MUSCLE</u>							
Mouse (bearing large fibrosarcoma tumor)	H	30.0	643 \pm 10	38 \pm 2	--	A,H,L	214
Rat (bearing reticulum sarcoma)	H	60.0	921	--	--	A,G	218
Rat (bearing adeno-carcinoma)	H	60.0	628	--	--	A,G	218
Rat (bearing Rhabdomyo-sarcoma)	H	60.0	882	--	--	A,G	218
Mouse (bearing MH 134 solid tumor)	H	100.0	880	57	--	A	209
Mouse (bearing ascites sarcoma)	H	2.7	195.8 \pm 2.5	51.61 \pm 0.61	--	A,E,N	215a
<u>INTESTINE</u>							
Mouse (bearing large fibrosarcoma tumor)	H	30.0	399 \pm 24	40 \pm 4	--	A,H,L	214

STOMACH

Mouse (bearing large fibrosarcoma tumor) H 30.0 317 \pm 29 30 \pm 1 -- A,H,L 214

E. Other AlterationsTHYROID

Rat (treated with propyl thiouracil [PTU]) H 32.0 698 \pm 54 61 \pm 4.5 -- R.S. 245

THYROID (con't.)

Rat (treated with NaClO₄) H 32.0 717 \pm 42 62 \pm 11.3 -- R.S. 245

MUSCLE

Calf (muscle tissue dehydrated) H 25.6 310 0.142 -- A,D,F 258

TUMOR (Dehydrated)

Mouse (amelanotic melanoma - B16) H 25.6 190 0.575 -- A,D,F 258

TABLE III. Isolated Cellular Systems

<u>Tissue Animal</u>	<u>Nucleus</u>	<u>Freq. (MHz)</u>	<u>T_1 (msec)</u>	<u>T_2 (msec)</u>	<u>D'a</u>	<u>Additional Information</u>	<u>Reference</u>
BACTERIA							
<i>Halobacterium</i> (Dead Sea)	H	14.0	40.0	21.0	--	<i>D,I,M</i>	259
<i>Halobacterium</i> (Dead Sea)	2H	14.0	83.0	14.0	--	<i>D,I,M,T</i>	259
ASCITES CELLS							
<i>Rat (Yoshida ascites)</i>	H	25.0	1911	--	--	<i>A</i>	208
<i>Rat (Ehrlich ascites)</i>	H	60.0	1150	--	--	<i>A,H,N</i>	210
HEPATOMA							
<i>Mouse</i>	H	24.0	600	--	--	<i>A,H</i>	215
MELANOMA							
<i>Mouse</i>	H	24.0	620	--	--	<i>A,H</i>	215
FIBROSARCOMA							
<i>Mouse</i>	H	24.0	619	--	--	<i>A,H</i>	215
ANAPLASTIC CARCINOMA							
<i>Mouse</i>	H	24.0	697	--	--	<i>A,H</i>	215
ERYTHROCYTIS							
<i>Human (normal-deoxy)</i>	H	24.3	--	260	--	<i>A</i>	260
<i>Human (normal-oxy)</i>	H	24.3	--	301	--	<i>A</i>	260
<i>Human (normal-oxy)</i>	H	6.0	382	--	--	<i>A,E,N</i>	261b

ERYTHROCYTIS (con't.)

<i>Chicken (normal)</i>	H	32.0	470 \pm 20	--	--	A	221
<i>Human (normal)</i>	H	38.0	--	--	0.86 \pm 0.02	--	222
<i>Human (normal)</i>	H	25.0	570	--	--	N	261
<i>Human (normal)</i>	170	10.0	1.7	--	--	N	261
<i>Human (normal)</i>	170	8.13	1.6	--	--	A, I, N	231a
<i>Human (sickle cell-deoxy)</i>	H	24.3	864	68.0	--	A	260
<i>Human (sickle cell-oxy)</i>	H	24.3	879	282	--	A	260
<i>Chicken (infected with erythroleukosis)</i>	H	32.0	560 \pm 50	--	--	A	221

LEUKEMIC CELLS

<i>Human (new case)</i>	H	25.0	1031	--	--	A	262
<i>Human (after treatment of patient)</i>	H	25.0	612	--	--	A	262

AMNIOTIC

<i>Human</i>	H	25.0	749	--	--	A	257
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HUMAN LIPOGENIC SARCOMA

<i>Human</i>	H	25.0	1003	--	--	A	257
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HELA

<i>Human</i>	H	25.0	990	--	--	A	257
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HELA (Cell Cycle Stages)

<i>M₀ (0 min.)</i>	H	30.0	1020	130	--	A, G, H, U	263, 265
<i>M₃₀ (30 min.)</i>	H	30.0	817	127	--	A, G, H, U	263, 265

TABLE III (continued)

<u>Tissue</u> <u>Animal</u>	<u>Nucleus</u>	<u>Freq.</u> <u>(MHz)</u>	<u>T_1</u> <u>(msec)</u>	<u>T_2</u> <u>(msec)</u>	<u>D'a</u>	<u>Additional</u> <u>Information</u>	<u>Reference</u>
<u>HELA (Cell Cycle Stages--con't.)</u>							
G_1 (early) (4 hrs.)	H	30.0	638	110	--	A, G, H, U	263, 265
G_1 (late) (8 hrs.)	H	30.0	570	117	--	A, G, H, U	263, 265
S (12 hrs.)	H	30.0	534	100	--	A, G, H, U	263, 265
G_2 (18 hrs.)	H	30.0	621	96	--	A, G, H, U	263, 265
G_2 (19 hrs.)	H	30.0	690	--	--	A, G, H, U	263, 265
<u>HELA CELLS (Treated)</u>							
Random Hela cells in NaCl (0.15 M)	H	30.0	651	116	--	A, G, H, U	263, 265
S -phase cells (treated with spermine-0.02 M for 2 hrs.)	H	30.0	701	--	--	A, G, H, U	263, 265
<u>HUMAN BREAST CANCER</u> ^{aa, bb}							
MB-MDA-231	H	30.0	934 \pm 78	123 \pm 31	--	A, F, G, H, U	264, 265, 266
MB-MDA-157	H	30.0	907 \pm 10	135 \pm 4	--	A, F, G, H, U	264, 265, 266
MB-MDA-361	H	30.0	849	--	--	A, F, G, H, U	266
MB-MDA-134	H	30.0	717 \pm 64	145 \pm 39	--	A, F, G, H, U	266
MB-MDA-330	H	30.0	752 \pm 39	--	--	A, F, G, H, U	264, 265, 266
SW-627	H	30.0	729 \pm 26	110 \pm 10	--	A, F, G, H, U	266
MB-MDA-231 (slow)	H	30.0	662 \pm 70	104 \pm 18	--	A, F, G, H, U	266
MB-MDA-157 (slow)	H	30.0	669 \pm 52	102 \pm 18	--	A, F, G, H, U	266
SW-613	H	30.0	606 \pm 26	104 \pm 15	--	A, F, G, H, U	266
MB-MDA-331	H	30.0	549 \pm 136	75 \pm 20	--	A, F, G, H, U	265, 265, 266
MB-MDA-431	H	30.0	521	126	--	A, F, G, H, U	266
MB-MDA-436	H	30.0	499 \pm 49	100 \pm 20	--	A, F, G, H, U	266
MB-MDA-415	H	30.0	260 \pm 50	69 \pm 4.5	--	A, F, G, H, U	266

CHINESE HAMSTER OVARY CELLS

Chinese Hamster (random
ovary cells treated
with 0.15 M NaCl) H 30.0 689 -- -- A, G, H 265

CHINESE HAMSTER LUNG FIBROBLASTS

Chinese Hamster (V-79-S 171
with NaCl concentrations
of 0.155 to 0.3 M) H 30.0 ~ 820 -- -- A, G, H, I, N 267a

PRIMARY MAMMARY CELLS

Mouse (normal cells)	H	30.0	847.2 \pm 71.3	134.8 \pm 20.3	--	A, G, H	267
Mouse (pre-neoplastic HAN-nodule cells)	H	30.0	989.4 \pm 12.3	179.4 \pm 19.4	--	A, G, H	267
Mouse (malignant adenocarcinoma cells)	H	30.0	1090.6 \pm 87.3	183.0 \pm 24.7	--	A, G, H	267

FOOTNOTES FOR TABLES I-III

aa: MB-MDA series established from breast cancer metastasis.

bb: SW series established from solid tumors.

a: D' is the ratio of the self-diffusion coefficient of water protons in the tissue to that of pure water.

A: Initial values for relaxation times or single relaxation times or mostly single relaxation times reported.

B: Multiple relaxation reported in T₁.

C: Multiple relaxation reported in T₂.

D: Temperature dependence of relaxation times studied.

FOOTNOTES FOR TABLES I-III (continued)

- E: Frequency dependence of relaxation times studied.
- F: Unpublished data or to be published.
- G: Determined T_1 , T_2 dependence on (a) hydration and/or (b) development.
- H: Additional publication by author(s) on this topic.
- I: Value obtained from graph.
- J: D' varies as a function of the 90° - 180° pulse spacing.
- K: T_1 , T_2 measured using c.w. techniques.
- L: T_1 has also been measured.
- M: Values quoted are the average values in the multi-component relaxation.
- N: Used two-fraction fast-exchange model with a single correlation time for each fraction or used two-fraction fast-exchange model with a distribution of correlation times for bound water.
- P: Mean values calculated and given in the table from the individual values given by authors.
- Q: Two different samples.
- R: Investigated dependence of T_1 , T_2 on protein content or other constituent of the cell.
- S: Treated as single exponential but found that the decaying magnetization is a composite curve.
- T: T_1 , T_2 values in partially deuterated systems.
- U: Studied T_1 and T_2 as a function of cell cycle and/or division rate.
- V: T_1 , T_2 , D' are dependent on hydration and the values reported are the average values for a hydration of 4.4 gm of H_2O per gm of dry solids.

three most frequently measured NMR parameters of water molecules are decreased in cells and tissues. Three rather striking generalizations can be made concerning the data presented in Tables I-III, provided one ignores the observation of multiple relaxation times reported in several investigations. (Multiple relaxation times will be discussed later in the text). Allowing for the wide range of sample types, all of the investigations report values of T_2 (spin-spin) relaxation times to be reduced by a factor of 5.8 to 221, T_1 (spin-lattice) relaxation times to be reduced by a factor of 2.5 to 23, and the diffusion coefficient (D) to be reduced by a factor of approximately 2 when compared to the values of these parameters obtained for pure water or dilute electrolyte solutions. The interpretations of these findings lead to two general schools of thought and this will be discussed in the next section.

2. *Interpretations.* In general, there are two interpretations of the data presented in Tables I-III. The most widely held view utilizes one limit of a phenomenological model first presented by Zimmerman and Brittin in 1957 - specifically, the fast-exchange limit of the Zimmerman-Brittin model (270). In this limit, the relaxation times may be described as average values due to the rapid exchange between two populations of water molecules (bound and free) with vastly different correlation times. This model, although not offering a mechanism for relaxation, does provide explicit predictions concerning tissue hydration and relaxation times. Implicitly, the diffusion coefficient should be equivalent to that of pure water.

The most widely held alternative hypothesis used to interpret the data in Tables I-III is the ordered water hypothesis proposed by the author and his colleagues and by Cope (211) in 1969. In this general interpretation, the word "order" implies the reduction of mobility (increased correlation time) for some degree of freedom. Furthermore, the ordering of water molecules may include longer rotational and/or translational correlation times or preferential orientation of water dipoles due to the influence of macromolecular surfaces. Obviously, this interpretation does not offer formalized mechanisms for the relaxation processes. On the other hand, Ling's polarized multilayer concept and his more generalized association-induction hypothesis do provide some formalization concerning the order of water molecules in biological systems. In its most general conceptualization, the ordered water concept requires that both the relaxation times and diffusion coefficient of molecules be reduced in biological systems.

The author realizes that, ultimately, all hypotheses and theories in science fail. As experimental investigations proceed in a given area of research, data accumulate which clearly prove that one theory (or hypothesis) is incorrect. Even though one may be fortunate enough to prove one theory incorrect, it does not follow that the alternate theory (or hypothesis) is correct. Such discovery does, however, provide considerable insight into which direction of inquiry one might more fruitfully take. Therefore, the remainder of this section will be concerned with determining which of the two interpretations given above more accurately explains the data acquired thus far.

a. *Two-fraction fast-exchange model.* In an attempt to describe adsorption phenomena by NMR pulse techniques, Zimmerman and Brittin (270) developed a stochastic theory of relaxation for multiphase behavior which describes the essential features of the relaxation processes encountered in adsorption phenomena. In their general theory, the spin system is assumed to be composed of a finite number of phases, and each phase is characterized by a single relaxation time. In the limit of fast exchange, their solution resulted in equation (12).

$$(1/T)_{av} = \sum_{i=1}^n P_i/T_i \quad (12)$$

where $(1/T)_{av}$ is the weighted average of the reciprocals of the relaxation times; P_i is the population of spins in each phase; and T_i is the relaxation time of the spin in the i th phase. $\sum P_i$ is equal to 1. It should be noted that equation (12) may apply to either T_1 or T_2 relaxation times. If the system under investigation is comprised of only two phases (e.g., a and b), equation (12) becomes

$$(1/T)_{av} = P_a/T_a + P_b/T_b \quad (13)$$

Bratton, *et al.* (205) and Clifford, *et al.* (271) were the first to use equation (13) to explain the shortened relaxation times in skeletal muscle and erythrocyte membranes. Bratton, *et al.* (205) concluded that if a very small fraction of water was bound to the macromolecules and was exchanging rapidly with the bulk of the cytoplasmic water (which was considered equivalent to pure water), the shortened relaxation times in skeletal muscle could be explained. Clifford, *et al.* (271), however, tested equation (13) further. For example, equation (13) may be rewritten (using the notation of Clifford, *et al.*) as

$$1/T_{av} = a/T_b + (1-a)/T_f \quad (14)$$

where a is the fraction of bound water in the system; T_b and T_f are the relaxation times of the bound and free fractions of water, respectively.

If x is the dry weight cells or tissues per gram of sample, then

$$(1-x)/T_{av} = cx/T_b + (1-x-cx)/T_f \quad (15)$$

where c is a constant. From equation (15) it follows that

$$1/T_{av} = [cx/(1-x)][1/T_b - (1/T_f)] + 1/T_f \quad (16)$$

Equation (16) explicitly shows that $1/T_{av}$ should be a linear function of $x/(1-x)$ which is the inverse of the concentration of water in the system. Clifford, *et al.* were (as far as the author can determine) the first to test the two-fraction fast-exchange limit of the Zimmerman-Brittin model (hereinafter referred to as the two-fraction model) as a function of hydration. They found, in erythrocyte membranes and in whole erythrocytes, that $1/T_{av}$ was not a strict linear function of hydration as predicted by the two-fraction model. The estimate of c in equation (16) which is the amount of bound water per unit weight of dry solids, was on the order of 30-36%.

Cooke and Wein (272,273) also tested the two-fraction model as a function of hydration. Their conclusion as stated on pages 202-203 of reference 273 is as follows:

"We have investigated the intracellular water proton relaxation times in glycerinated muscle fibers and in partially dried muscle fibers. We conclude that

⁹The conclusions reached by Cooke and Wein were challenged by Chang in the discussion that followed their paper (273). Chang pointed out that the two-fraction model as applied to their data was insensitive to the fraction of bound water chosen to fit their data (*i.e.*, ten times more water could be bound and the model would describe their data). Cooke agreed with Chang but argued that the important point was that 0.2 gm of bound H₂O/gm of protein could also describe the data and that this prevented one from concluding from NMR measurements that the bulk of water in skeletal muscle suffers restrictions in rotational freedom.

there are two phases of muscle water: a small phase, less than 4-5% of the total water, which interacts strongly with the proteins and has short relaxation times; and a major phase, which comprises the remainder of the observable water and has longer relaxation times than those observed in the whole muscle.¹⁰

Attempts to explain the reduction of the relaxation times in cellular systems and the dependence of the relaxation times on hydration (as demanded by the two-fraction model) have, for the most part, involved measuring only one relaxation time. As pointed out above, the two-fraction model is general and holds for both T_1 and T_2 relaxation times. Because of this, another method for testing the model as a function of hydration is open to the investigator. The author and his colleague have determined the hydration and the relaxation times in a wide variety of tissues and over a wide range of hydrations (274,275). A summary of these findings follows.

Equation (13) applies to both T_1 and T_2 relaxation times and the T_1/T_2 ratio, in terms of the two-fraction model, yields the following solution:

$$\frac{T_1}{T_2} = \frac{\frac{1}{T_{2f}} + \frac{W_d}{W_t} Z \left(\frac{1}{T_{2b}} - \frac{1}{T_{2f}} \right)}{\frac{1}{T_{1b}} + W_d Z \left(\frac{1}{T_{1b}} - \frac{1}{T_{1f}} \right)} \quad (17)$$

where $T_{1,2f}$ = relaxation times of free water; W_d = dry weight of sample; W_t = concentration of tissue water; Z = fraction of bound water; $T_{1,2b}$ = relaxation time in bound water phase.

Equation (17) may be reduced to this form:

$$\frac{T_1}{T_2} = a_1 + \frac{a_2}{a_3 x + 1}$$

where,

$$a_1 = \left(\frac{1}{T_{2b}} - \frac{1}{T_{2f}} \right) \left(\frac{1}{T_{1b}} - \frac{1}{T_{1f}} \right)$$

$$a_2 = \frac{T_{1f}}{T_{2f}} - a_1$$

$$a_3 = Z \left(\frac{T_{1f}}{T_{1b}} - 1 \right)$$

$$x = \frac{W_d}{W_t}$$

Equation (17) is the general form for a hyperbola, and the plot of T_1/T_2 vs. x should reveal such a relationship provided this model is applicable. Figure 1 is a plot of data obtained in our laboratory on variations of tissue hydration. Data in developing muscle are also included. The solid line in Figure 1 was determined from equation (17) using the T_1 and T_2 data of mature and immature muscle, and of pure water (taking $T_1 = 3.0$ seconds and $T_2 = 1.6$ seconds for pure water). The hyperbolic curve does not fit the data of tissues with different hydration. The reason for this poor fitting may be that the parameters Z and $T_{1,2b}$ are different from different tissues, but it is clear from Figure 1 that the hyperbolic relationship does not hold for data from a single tissue type. In addition, it has been shown that like tissues may not have distinguishable percentages of water, yet significantly altered relaxation times (255,240, 249,263,264). It appears that the relaxation times of water protons may be altered by chemical and physical events other than hydration and that the changes in hydration may not be solely responsible for the observed changes.

Several variations of the two-fraction model have been applied in attempts to explain the relaxation times of water molecules in biological systems. One such variation is that the bound fraction of water is not defined best by single correlation times (234,235,253,276). The general conclusions of these studies are that, within the bound fraction, only a small fraction of water is relatively immobile and that the water molecules within this fraction cannot be characterized by a single correlation time. Outhred and George (234) report that their evaluation of the relaxation data of water protons in skeletal muscle indicate that not more than 2% of the protons have correlation times greater than 10^{-9} second compared with a free water value of 10^{-11} sec. They further conclude that it is not impossible that a significant fraction of the water exists in a state with correlation times equal to or greater than 10^{-10} .

Although no data that prove paramagnetic impurities to be involved in the relaxation processes of water protons in cells and tissues have been reported, the possibility concerns virtually all of the investigators. This concern

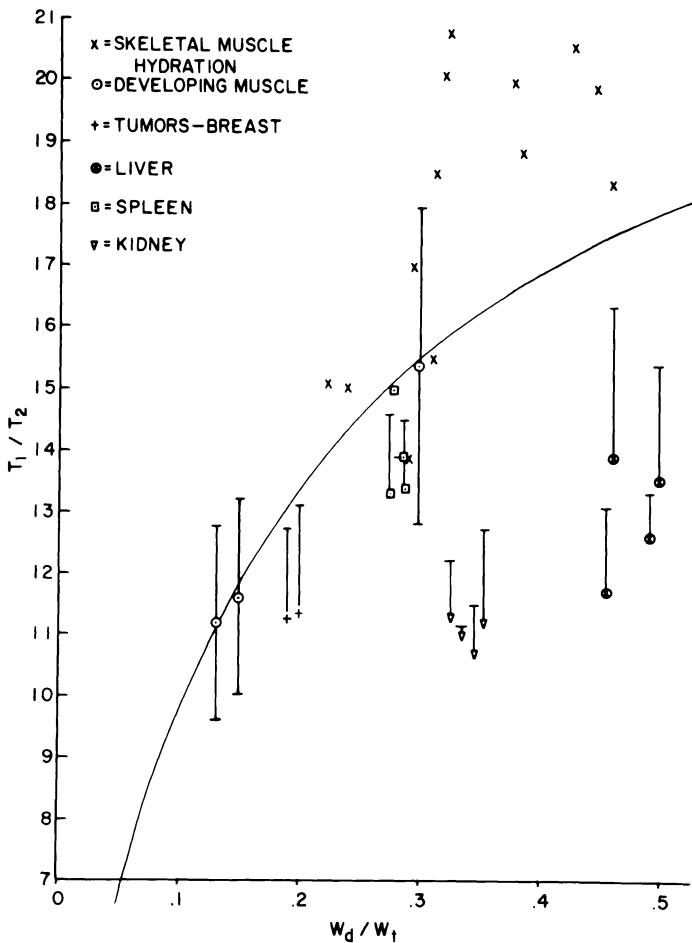


FIGURE 1. Plot of the T_1/T_2 ratio of various tissues against the fraction of water in each tissue type. The maximum error bars are presented on the data of the tissue groups. Individual skeletal muscle values for different levels of hydration are presented as X's. The solid line was derived from equation 17 and using T_1 and T_2 values of pure water, two-day old muscle, and mature muscle.

persists even though there is no direct evidence for this mechanism or considerable circumstantial evidence against it (211, 215, 229, 277, 278). Hollis, *et al.* (215), however, have suggested that an enhancement effect of paramagnetic ions could be operative in cells and tissues. In this case, also, there is no experimental evidence that such a mechanism operates in biological systems; however, it does present a specific alternative worth discussing.

The effects of paramagnetic impurities were discussed in the theories of Solomon (279) and Bloembergen (280), and will be summarized here. Since the strength of the dipole-dipole relaxation is, in general, inversely proportional to the sixth power of the distance between the two nuclei, only the protons within the first hydration sphere of a paramagnetic ion need to be considered. The contribution of paramagnetic ions to relaxation rates of these protons are summarized in equations (18) and (19) (281).

$$\frac{1}{T_{1M}} = \frac{1}{15} \frac{S(S+1)\gamma_I^2\gamma_s^2}{r^6} \left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2} \right) \\ + \frac{2}{3} \frac{S(S+1)A^2}{\hbar^2} \left(\frac{\tau_e}{1+\omega_s^2\tau_e^2} \right) \quad (18)$$

$$\frac{1}{T_{2M}} = \frac{1}{15} \frac{S(S+1)\gamma_I^2\gamma_s^2}{r^6} \left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_s^2\tau_c^2} \right) \\ + \frac{1}{3} \frac{S(S+1)A^2}{\hbar} \left(\tau_e + \frac{\tau_e}{1+\omega_s^2\tau_e^2} \right) \quad (19)$$

where, T_{1M} and T_{2M} are the paramagnetic contributions to the T_1 and T_2 , respectively; S is the electron spin quantum number; γ_I and γ_s are the gyromagnetic ratio of the proton and the electron spin, respectively; r is the proton-ion internuclear distance; \hbar is Planck's constant divided by 2π ; ω_I and ω_s are the Larmor frequency of the proton and the electron spin, respectively; τ_c is the correlation time for the dipole-dipole interaction; τ_e is the correlation time for the scalar interaction; and A is the hyperfine structure coupling-constant. ω_s is always much larger than ω_I , and τ_e is in general longer than τ . Under most of the experimental magnetic fields, $\omega_s^2\tau_e^2 \gg 1$. Thus, terms in equations (18) and (19) involving $\frac{1}{1+\omega_s^2\tau_e^2}$ may be neglected. In aqueous

solution, $\omega_I^2 \tau_c^2$ usually is much smaller than one. Then,

$$\frac{\tau_c}{1+\omega_I^2 \tau_c^2} \approx \tau_c \quad (20)$$

When the paramagnetic ions are bound to some macromolecules, the rotation of the ions are restricted. As a result, τ_c tends to be lengthened and, consistent with relations (18), (19), and (20), the paramagnetic relaxations rates increase. This is generally known as the enhancement effect.

It was proposed that the enhancement effect might account for the short relaxation times in tissue water (215), but this view has not been supported by experimental evidence. Published data, in fact, suggest that the enhanced paramagnetic relaxation effect is relatively unimportant. Blicharska, *et al.* (282) have measured the proton relaxation time, T_1 , in 5% protein solutions and in pure water as a function of concentration of added Ni^{++} . They found: (1) that the original amount of paramagnetic ions present in their samples could not be responsible for the observed decrease in T_1 in protein solutions, and (2) for Ni^{++} concentration higher than 10^{-3} molar, the paramagnetic relaxation rate in the protein solution was not larger than in simple water solution. These results indicate strongly that the macromolecular enhancement of paramagnetic relaxation is not sufficient to account for the observed relaxation rates in tissue water.

One further comment is in order relative to paramagnetic impurities as a cause of reduced relaxation times of water protons in living tissues. The inverse of T_{2M} (relaxation times due to paramagnetic impurities) is proportional to γ_I^2 (equations [18] and [19] above). The dipole-dipole contribution is directly proportional to γ_I^2 . The hyperfine structure coupling-constant A is proportional to γ_I (282a) and, thus, the scalar contribution also is directly proportional to γ_I^2 . As γ_I^2 for protons is 42.4 times larger than that for deuterons, the deuteron T_2 would be much longer than the proton T_2 if the paramagnetic effect was responsible for the short T_2 of protons. However, comparison of the data of Cope (211) with others shows that the deuteron T_2 of muscle water is nearly an order of magnitude shorter than the proton T_2 . Furthermore, Civan and Shporer (230) have found that in muscle water the T_1 relaxation times of both the deuteron and ^{17}O T_1 are shorter than the proton T_1 . Hence, the effect of paramagnetic impurities on the relaxation times of muscle water is insignificant.

These proposed explanations of the shortening of the relaxation times of water protons (i.e., the two-fraction model, the two-fraction model with distribution of correlation times for the "bound" fraction, and paramagnetic impurities) can explain the reduction of the relaxation times but require that the diffusion coefficient of water molecules be essentially the same as that of pure water. The reduced diffusion coefficients reported in Tables I-III and again in Table IV are thought to be due simply to compartmentalization or non-specific obstruction. The proponents of the ordered water concept argue, on the other hand, that the reduction of the diffusion coefficient is real and not accounted for by either compartmentalization or non-specific obstruction. In the author's view, the resolution of the meaning of the reduction of the diffusion coefficient in cells and tissues will be decisive in determining which of these general interpretations is more nearly correct. The remainder of this chapter will be addressed to this task.

b. *The self-diffusion coefficient.* The diffusion coefficient (D) of water in cells and tissues has been measured primarily by two methods - isotopic and NMR. A summary of these determinations are presented in Table IV. Several publications have not been included in Table IV; however, they will be discussed in this section (147,212,269).

The data presented in Table IV show that the diffusion coefficient of water molecules in living tissue is reduced by a factor of approximately two compared to pure water. Three explanations for the observed reduction in the diffusion coefficient have been offered: (1) The intracellular membrane systems serve to compartmentalize the cytoplasmic water; (2) intracellular protein structures serve as obstructions to diffusion of cytoplasmic water; and (3) the water-protein interaction induces long-range changes in the water-water interaction in a substantial fraction of the intracellular water.

The possibility that the various membrane systems might serve as impenetrable barriers to diffusion was one of the first explanations offered to explain the NMR observations. The reduction of D due to compartment effects had been examined in considerable theoretical detail by Robertson (291) and tested in a model system by Wayne and Cotts (292). Chang, *et al.* (289) evaluated this mechanism experimentally in the skeletal muscle system and concluded that less than 10% of the observed reduction could be attributed to compartment effects.

TABLE IV. Self-Diffusion Coefficient of Water in Cells and Tissues (D) and in Pure Water (D_w) at Room Temperature

Cells or Tissue	Method	D ($10^{-5} \text{cm}^2/\text{sec}$)	D_w ($10^{-5} \text{cm}^2/\text{sec}$)	Reference
<u>SLIME MOLD</u>	NMR	1.26(1)	2.57(2)	253(3)
<u>EGGS</u>				
Chicken (Yolk)	NMR	0.37-0.60(4)	2.07	278(5)
Chicken (White)	NMR	1.66-1.71(4)	2.07	278(5)
Frog	NMR	0.67	2.07	248(5)
230	Frog	Isotope	0.996	284(6)
<u>SKELETAL MUSCLE</u>				
Barnacle	Isotope	2.42	2.4	285
Barnacle	Isotope	1.35	2.44	286(6)
Barnacle	Isotope	1.34	2.42	287(3,5)
Frog	NMR	1.56	2.4	224(3)
Frog	NMR	1.13(7)	2.43(8)	229(3,5)
Frog	NMR	1.12(9)	2.43(8)	229(3,5)
Rat	NMR	1.09(10)	2.43(8)	229(3,5)

TABLE IV (continued)

<i>Cells or Tissue</i>	<i>Method</i>	D ($10^{-5} \text{cm}^2/\text{sec}$)	D_w ($10^{-5} \text{cm}^2/\text{sec}$)	<i>Reference</i>
<u>SKELETAL MUSCLE (con't.)</u>				
Toad-I	NMR	1.2	2.57(2)	253(3)
Toad-II	NMR	1.5	2.57(2)	253(3)
Rat	NMR	1.43	2.78	288(6)
Rat	NMR	1.5 ⁽¹⁾	2.5	289(6, 11)
Frog	Isotope	1.18	2.07	57(6)
Frog	Isotope	1.43	2.07	57(6)
<u>CARDIAC MUSCLE</u>				
Rat	NMR ⁽¹²⁾	0.9	2.5	222(13)
Rabbit	NMR ⁽¹²⁾	0.65	2.5	222(13)
<u>LIVER</u>				
Rat	NMR ⁽¹²⁾	0.625- .75 ⁽¹⁴⁾	2.5	222(13)
Rabbit	NMR ⁽¹²⁾	0.625 ⁽¹⁵⁾	2.5	222(13)
Rabbit	NMR ⁽¹²⁾	0.49 ⁽¹⁶⁾	2.5	222(13)

TABLE IV (continued)

Cells or Tissue	Method	$(10^{-5} \text{ cm}^2/\text{sec})$	$(10^{-5} \text{ cm}^2/\text{sec})$	Reference
<u>RED BLOOD CELLS</u>				
Human	NMR ⁽¹²⁾	0.2-0.55 ⁽¹⁷⁾	2.5	222 ⁽¹³⁾
Human	NMR ⁽¹²⁾	0.25-0.625 ⁽¹⁸⁾	---	290 ^(13, 19)
<u>LENS</u>				
Rabbit (whole lens)	NMR	0.9	2.95	249
Rabbit (cortex)	NMR	1.06	2.95	249
Rabbit (nucleus)	NMR	0.97	2.95	249

FOOTNOTES TO TABLE IV

1. Temperature dependence determined - approximate room temperature value listed.
2. Quoted from reference 283a.
3. Interpretation involved two-fraction model with obstruction.
4. Range of values for three egg yolks and three egg whites.
5. Interpretation involved two-fraction model permitting adsorption to account for at least some of the reduction of D .
6. Interpretation involved ordered water concept.
7. Average value for frog muscle excitable but relaxed. Computed from Table I of Finch, et al.
8. Quoted from references 287a and 287b.

9. Average value for frog muscle stimulated to exhaustion. Computed from Table I of Finch, et al. (229).
10. Average value for rat muscle excitable but relaxed. Computed from Table I of Finch, et al. (229).
11. Tested for compartmentalization effect.
12. Used pulsed gradient spin-echo NMR technique.
13. Tested for obstructive effect and used theory of Wang (289a). Obstruction effect could not account for the reduction of D over the range of pulse interval studied.
14. Range of D for different pulse intervals.
15. Average value for liver from 10-day old rabbits.
16. Average value for liver from two-year old rabbits.
17. Range of D for different pulse intervals on red blood cells from patient with pulmonary tuberculosis.
18. Range of values obtained from Figure 4 in reference 249.
19. Also used equations for diffusion within impermeable spherical boundaries to evaluate obstruction effect.

It has been proposed that, because of the compartmentalization effect (interpretation 1 above), the observed diffusion will approach that of bulk water when the observation time τ approaches zero. It should be noted that the diffusion of water proton spins is measured along the direction of the static magnetic field by observing the echo amplitude following a $90^\circ-\tau-180^\circ$ pulse sequence as a function of applied field gradient, which is parallel to the static field. The amplitude of this echo is given by the expression:

$$A(\tau, G)/A(\tau, 0) = \exp[-2/3\gamma^2 G^2 \tau^3 D],$$

where $A(\tau, G)$ is the echo amplitude for a $90^\circ-180^\circ$ pulse separation τ with an applied field gradient G , and γ is the gyromagnetic ratio. The total distance that a molecule moves will be less and less as τ is made shorter and shorter. If τ is very short in comparison to the diffusion time, t_D , over a distance d (the distance between barriers), the diffusing molecule will not have time to encounter a barrier. This relationship is as follows:

$$t_D = d^2/D.$$

Actin-myosin filaments, for example, are separated by a distance of approximately 3×10^{-6} cm and t_D would be approximately 3.8×10^{-7} sec.

Various NMR techniques may be used to vary the τ spacing, and the pulsed field gradient technique developed by Stejskal and Tanner (293) permits the shortest τ spacings.

Hansen (212) inferred that the diffusion coefficient of tissue water (D) may approach that of pure water (D_w) at very short τ spacings. He measured D with τ spacings varying from 30 to 100 milliseconds. The author and his colleague (283) measured D over τ spacings of 20-400 msec (Figure 2) and compared the data not only with those of Hansen, but also with the predictions of Robertson's compartment model (291,292, 292a). The compartment model was found inconsistent with the data over the ranges of τ spacings studied (292a).

Cooper, *et al.* (222) have measured the self-diffusion coefficient in several tissues as a function of the observation time, τ , using the technique of pulsed-field-gradient-spin-echo. The measured diffusion coefficient at very short τ did not approach the value for pure water as would be expected if interpretation 1 is correct (290). Tanner (269)

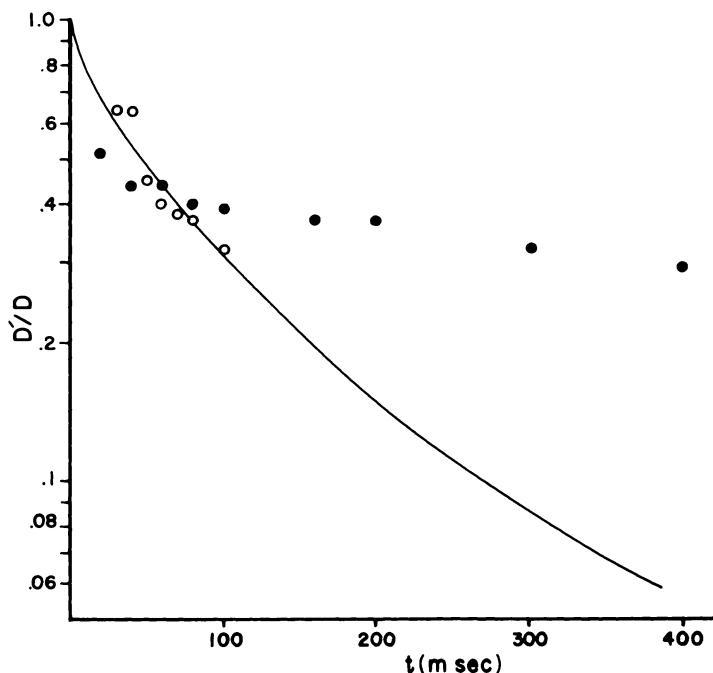


FIGURE 2. The ratio of D'/D as a function of observation time. Open circles are data from Hansen (○). Closed circles are data from Hazlewood, Chang, Nichols, and Woessner (●). Both studies are on rat skeletal muscle. The curve is a best fit of Robertson's compartment model (291).

also using the pulsed-field-gradient-spin-echo technique, has extended the measurement to times sufficiently short that the diffusion length is on the order of 1μ . His results confirm the observations of Cooper, *et al.* and give a value $D/D_w \approx 0.4$ to 0.7. Therefore, the interpretation that the intracellular membrane system serves to compartmentalize the cytoplasmic water is not supported by experimental evidence. However, interpretation number 2 above (i.e., obstruction to diffusion by non-specific barriers such as cytoplasmic proteins) could be operative in living tissues.

The possibility that non-specific barriers could be the cause of the reduction of the diffusion coefficient in tissues

has been tested in skeletal muscle (147). Skeletal muscle serves as an excellent tissue to check the barrier effect on diffusion for two primary reasons: (a) the work of Tanner revealed the D of muscle water was relatively constant down to short T spacings which were equivalent to approximately 1 μm , which is the approximate diameter of skeletal muscle myofibrils; and (b) the myofibrillar structure of skeletal muscle is well known from X-ray analysis and electron microscopic analysis. In addition to these two reasons, skeletal muscle also was considered to be the tissue of choice because Rorschach, *et al.* already had completed theoretical analysis of self-diffusion of water in skeletal muscle and an anisotropy of the D was expected. This could easily be tested. (Finch, *et al.* [229] had attempted this measurement and concluded that the small anisotropy observed was not significant).¹⁰

Experiments were designed to test for the existence of anisotropic diffusion in skeletal muscle, to construct a model based on hydrodynamic principles, and to test for non-specific barrier or obstructive effects (147). A summary of these experiments and the results of their experiments follows.

Diffusion coefficients were determined using standard techniques of pulsed NMR (147,283). Samples in this study were prepared from the tibialis anterior muscle of mature male rats (Texas Inbred) weighing between 350 and 500 grams. A reliable method for insuring muscle fiber orientation was developed and the details of this procedure may be found elsewhere (147). The sample tube was placed in the NMR probe and the orientation of the fiber axis, determined within $\pm 3^\circ$, was noted on the top of the sample tube. The orientation of the fiber axis, relative to the magnetic field and its gradient, was adjusted by rotating a 12 mm sample tube manually.

The results of these experiments are shown in Table V. Results of measurements of the self-diffusion coefficient for pure bulk water and for randomized tibialis anterior also are included in Table V. The value of the self-diffusion coefficient D for randomized muscle was obtained from two

¹⁰In personal communication, Finch indicated that his attempts to measure the anisotropy were hampered by the fact that he could not be certain that he could properly align small pieces of muscle in the NMR sample tubes.

TABLE V. Measurements of the Diffusion Coefficient versus Muscle Fiber Orientation

Sample	Diffusion Coefficients ($\times 10^{-5} \text{cm}^2/\text{sec}$)			D/D_w
	Parallel	45°	Perpendicular	
1	1.366	1.179	1.021	0.748
2	1.389	1.179	1.020	0.735
3	1.400	1.226	1.033	0.738
4	1.410	1.215	0.987	0.700
5	1.400	1.193	0.966	0.690
Average	1.393	1.198	1.005	0.722

Diffusion Coefficient for Pure Bulk Water	$2.283 \times 10^{-5} \text{cm}^2/\text{sec}$
Diffusion Coefficient for Randomized Muscle	$1.189 \times 10^{-5} \text{cm}^2/\text{sec}$

successive measurements on the same piece of muscle for which the fiber orientation had been thoroughly disordered by cutting the muscle into several pieces. This furnishes a point of reference to determine what values might be expected for typical samples for which no care is taken to preserve fiber orientation.

The average value of $D(90)/D(0)$ in this study was 0.72, which represents an anisotropy of 28%. The value of $2.28 \times 10^{-5} \text{cm}^2/\text{sec}$ for the self-diffusion coefficient of pure bulk water compares favorably with other values in the literature. Comparing the average diffusion coefficient for each fiber orientation to the value of D for pure bulk water (D_w), one finds

$$\frac{D(90)}{D_w} = 0.44, \quad \frac{D(45)}{D_w} = 0.53, \quad \text{and} \quad \frac{D(0)}{D_w} = 0.61.$$

Thus, the average diffusion coefficient measured parallel to the fiber axis is still about 40% lower than D_w .

Next, two questions were asked concerning the data presented in Table V: (a) can the obstruction of the non-filament proteins account for the reduction of the self-

diffusion coefficient along the direction parallel to the muscle fiber, and (b) can the actin-myosin filaments account for the observed anisotropy of the self-diffusion of cellular water?

A model often considered to explain the reduction of D due to the obstruction effect in biological systems is that proposed by Wang (289a). This model was intended to apply to capillary flow measurements of D in dilute protein solutions. Wang's model utilizes the steady-state solution of the diffusion equation and assumes that the protein molecules are stationary, impenetrable ellipsoids. The general expression for the effective self-diffusion coefficient D in a given direction is given by

$$D_i = (1 - \alpha_i \Phi) D_0 \quad i = a, b, c$$

where D_0 is the self-diffusion coefficient of the "free" solution, Φ is the volume fraction occupied by the hydrated protein molecules, and α_i is a dimensionless numerical coefficient for diffusion parallel to the i^{th} axis of the ellipsoid. The value of α_i is determined by the dimensions of the principal semi-axes a , b , and c of the ellipsoids. The equation shows that the measured diffusion coefficient D should be less than D_0 if Φ is not negligibly small. In addition, the diffusion of cell water is further reduced if the water molecules are in rapid exchange with a hydration fraction (the "direct hydration" effect). Wang shows that this effect introduces an additional factor $D' = D(1 - f)$, where f is the fraction of the water bound to the proteins. The final result for Wang's theory is

$$D'_i = (1 - \alpha_i \Phi)(1 - f) D_0 \quad (21)$$

A second approach to the problem of reduction of the measured diffusion coefficient perpendicular to the actin-myosin filament network was recently presented by Rorschach, *et al.* (294). In this model, the filaments are approximated by a hexagonal array of impenetrable rods of uniform radius a and lattice spacing $2R$ (see Figure 3). The effective self-diffusion coefficient D is derived from an approximate solution to the steady-state diffusion equation with the appropriate boundary conditions within a hexagonal unit cell. This calculation yields the following expressions for the self-diffusion coefficient D_s :

$$\text{Parallel to fiber axis: } D(0) = D_0 \frac{D_0}{D_0} \quad (22)$$

$$\text{Perpendicular to fiber axis: } D(90) = \frac{1 + .80\Phi}{1 + .80\Phi} \quad (23)$$

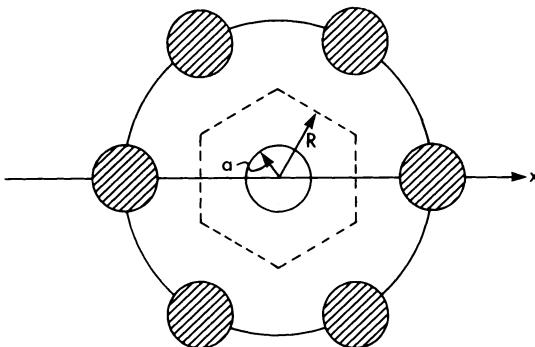


FIGURE 3. A cross-sectional view of the hexagonal lattice of protein filaments. The basic hexagonal cell is shown dotted. For the purposes of the diffusion calculation, the actin and myosin filaments are taken to be cylinders of equal radius a .

where D_w is the self-diffusion coefficient of the solution in the absence of obstructions, and Φ is the volume fraction of the protein.

The reduction in D_s in a cubical array of spheres also has been calculated. The results show that the measured self-diffusion coefficient is:

$$D = \frac{D_w}{1 + .63\Phi} \quad (24)$$

These results differ from those given by Wang for two reasons: (a) the boundary conditions for Wang's calculations are imposed at infinity, and the flow pattern for a random array is determined by superposition (289a). In the present calculation, the influence of the neighbors on the flow is taken into account by imposing the boundary conditions at the surface of the hexagonal cells; (b) the diffusion in a protein solution in a capillary is influenced by the distortion of the flow and also by a geometrical factor that would reduce the transport cross-section, even if all the protein were to be congealed on the walls of the capillary. This geometrical factor does not enter in the spin-echo case. Both of these effects lead to corrections to the relation between D and D_w which are of order Φ .

The experimental results presented here also show that the measured self-diffusion coefficient perpendicular to the muscle fiber is less than that parallel to the muscle fiber. This indicates that the array of the actin-myosin filaments can serve as effective diffusion barriers; however, the self-diffusion coefficient parallel to the muscle fiber still is reduced significantly from that of pure bulk water. According to Rorschach's calculation, this 40% reduction of diffusion coefficient cannot be accounted for by the protein filaments, as D should equal D_w for diffusion parallel to the muscle fiber. The question then is: can the obstruction effect of the non-filament proteins account for this reduction of diffusion?

The obstruction effect of the non-filament proteins also can be estimated from Rorschach's calculation. If we assume that these proteins are spherical, the obstruction effect can be estimated from equation (24). In order to make $\frac{D(0)}{D_w} = .61$, a value for Φ of approximately 1 is required, which is too large: obviously, the cytoplasm is not completely filled with proteins. The alternative is to consider the "direct hydration" effect, which introduced a reduction factor ($1-f$) to the measured diffusion coefficient. (This is true in both Wang's calculation and Rorschach's calculation). By substituting this factor in equation (24), the obstruction effect may be more realistically evaluated. If one assumes the non-filament proteins are dilute ($\Phi \sim 0$), then $f \sim .39$ (i.e., 39% of the cytoplasmic water would be hydration water). If one assumes a more reasonable value for Φ (e.g., the non-filament proteins occupy $\sim 5\%$ of the volume of cytoplasm, and $\Phi = .05$), f will be about .37. The hydration water would have to constitute 37% of the cellular water.

It becomes clear that the obstruction effect of protein filaments and non-filament proteins is not sufficient to account for the reduction in the self-diffusion coefficient. The assumption that the cell water diffuses like free water and that the observed reduction in diffusion coefficient is caused by the "direct hydration" effect requires a large fraction of the cytoplasmic water to be tightly bound with the macromolecules.

Furthermore, the large degree of anisotropy in the self-diffusion coefficient also indicates strongly the inadequacy of the obstruction hypothesis. The anisotropy predicted from the geometrical dimensions of the actin-myosin system ($\Phi = 0.16$) is $\frac{D(0)}{D(90)} = 1.13$. In order to explain the measured

value $\frac{D(0)}{D(90)} = 1.39$, a value of $\phi = 0.49$ is required. This also would imply an enormous sheath of bound water. Therefore, the observed reduction and anisotropy in D requires either a change in the bulk diffusion coefficient of the cellular water, or an effective increase in myosin-actin filament size by a substantial sheath of bound water.

Three potential reasons for the reduction of the self-diffusion coefficient of water have been considered as operative in biological systems. The experiments designed and executed thus far demonstrate that the non-specific compartment effects (by temperature dependent studies and variation of the τ spacing) cannot account for the observed reduction of the self-diffusion coefficient of water in a variety of tissues. The potential effect of non-specific barriers has been evaluated both experimentally and theoretically, and the results indicate that these effects cannot account for the reduction of the self-diffusion coefficient of water in skeletal muscle. These findings lead the author to conclude that the observed reduction of the diffusion coefficient in biological systems is real. It also seems appropriate that models constructed to describe or explain the physical state of water in biological systems must include explanations for the reduced diffusion coefficient.

IV. SUMMARY

1. The fundamental assumptions underlying the membrane pump and adsorption (primarily the association-induction hypothesis) views of the living cell have been reviewed. An attempt also has been made to review the data that proponents of each view consider to be definitive.

2. The most fundamental difference between these two major views is that in the membrane pump model the distribution of solutes in living cells is determined by steady-state processes, energized by membrane-situated, energy-requiring pumps; whereas, in the association-induction hypothesis, cellular macromolecules determine the distribution of solutes by selective adsorption and influence of the physical state of water via a quasi-equilibrium process.

3. The evidence that potassium is adsorbed within cells is supported by isotopic diffusion techniques and NMR data. The non-homogeneous distribution of potassium (i.e., localization within the A band) is supported by chemical extraction,

light microscopic, microincineration, electron microscopic and electron microprobe studies.

4. The data acquired by techniques such as disjoining pressure, low frequency mechanical damping, isotopic diffusion, dielectric measurements and adsorption isotherms indicate that various surfaces (including those of cellular macromolecules) may influence the physical state of water over relatively large distances. Ice crystal formation and measurements of viscosity, heat capacity, and the density of water in cells provide even stronger support to the idea that the physical state of water in cells is different from that of bulk water.

5. The data acquired from NMR techniques has been evaluated by different models, and it is concluded that T_1 , T_2 , and D of water molecules are reduced in biological tissues. It is concluded further that the reduction of the self-diffusion coefficient of water in various tissues (particularly skeletal muscle) cannot be accounted for by compartmentalization, or by non-specific obstructive barriers.

6. It is the author's opinion that the evidence now is sufficient to conclude that the physical state of ions and water in biological systems is different from that in a bulk solution. Therefore, explanations of solute and water distribution in living cells which are based on the assumptions inherent in ideal solution theory should be re-evaluated.

NOTE ADDED IN PROOF

A. *Cooperative Processes in Cells*

1. A brief review by Ling of the association-induction hypothesis in general and a model of cooperative adsorption of cellular potassium and sodium in particular may be found in reference (295). Ling has also written a chapter on his theory in a forthcoming book (296) and presents recent experimental tests of the validity. In addition to the above reports, the association-induction hypothesis has been used to describe cell swelling in high concentrations of KCl and other chloride salts (297).

2. The cooperative adsorption isotherm of Ling and Yang has been applied to the uptake of K^+ and the loss of Na^+ in lymphocytes (298) and the kinetics of potassium uptake have

been evaluated in terms of an adsorption process (299).

3. Huang (300) has presented a new thermodynamic description of allosteric transitions in enzymes. This new method accounts for the effect of temperature on ligand binding affinities as well as the effect of ligand concentration. In addition, the method is successfully applied to the oxygenation of hemoglobin and correctly predicts two aspects of the temperature effect on oxygenation: (1) the shape of a Hill plot is invariant under temperature change; and (2) for a given degree of oxygenation, the oxygen activity is linearly dependent on temperature.

B. Physical Models to Explain the Data on Relaxation Times and the Diffusion Coefficient of Water Molecules

1. Two-fraction fast-exchange model (relaxation times).

a. Effects of hydration. Several publications have appeared since the original draft of this manuscript was completed which should be brought to the attention of those interested in this particular subject. Although changes in tissue hydration may influence the relaxation rates for water protons, the strict linear relationship required by equation (16) in this chapter is not followed (301,302). Bacić, *et al.* (301) made an interesting observation while studying the relaxation times and the water content of Zea mays roots incubated in solutions of varying concentration of LiCl and KCl. Both the relaxation times and the concentration of water go through a minimum value at approximately 10^{-3} molar LiCl or KCl. Again, however, the change in hydration and the changes in relaxation rates of water protons are not described accurately by equation (16) in this chapter.

Additional problems encountered in the use of the two-fraction fast-exchange model to explain the relaxation times of water protons in frozen and non-frozen tissues are reported by Rustgi, *et al.* (303).

b. Correlation times of "bound" water. The author has reread several papers which are referenced in Section III-B of this chapter and thinks the following comments are in order.

Fung and McGaughy (233) have completed a detailed analysis of the T_1 of water protons as a function of both Larmor frequency and temperature. They utilized a two-fraction model and assumed a Log-Gaussian distribution of

correlation times for the small (= 12%) "bound" (non-freezable) fraction of muscle water. The agreement between experiment and theory (i.e., for the T_1 data) is encouraging. They compute a mean correlation time (τ_o) for the "bound" fraction to be on the order of 10^{-10} sec. which appears to be reasonable. This model, however, cannot predict the frequency dependence of T_2 and when studying the frequency dependence of T_1 for ^2H , their τ_o is on the order of 10^{-13} sec. which is shorter than the correlation time of pure water (B. Fung, personal communication).

Held, *et al.* (235) have also examined the frequency dependence of T_1 and T_2 of water protons in skeletal muscle at two temperatures. They applied a "one-state" model assuming a continuous Log-Gaussian distribution of correlation times, but failed to predict the temperature dependence. Another problem exists in that the calculated mean correlation time was on the order of 10^{-14} sec. -- two orders of magnitude less than that of bulk water.

It appears that different modifications of the Zimmerman-Brittin model or completely new approaches are needed before an accurate account of the relaxation times and the relaxation processes will be possible.

2. *Cellular Model.* From equation (24), it is seen that if \emptyset (the volume fraction of obstructing spheres) is as large as 1, the measured diffusion coefficient for water in cells and tissues will be reduced at most by a factor of only two (relative to pure water). In a study of the diffusion coefficient of water as a function of hydration in cysts of the brine shrimp, *Artemia*, Seitz has found the reduction of D to be on the order of 7 times at the highest hydration (304). At lower hydration, D is reduced as much as 20 times. Such reductions cannot be explained by simple obstruction effects according to Wang's model (equation 21) or Rorschach's model (equations 23 and 24) in this chapter. It should be pointed out also that the reductions of D for red cells (Table IV) cannot be explained by the obstruction models.

3. *Compartmentalization Models.* Tanner (305) has now developed a model for evaluation of diffusion in a system of partially permeable barriers which will require consideration in the future.

C. Nuclear Magnetic Resonance Studies of ^1H , ^2D , and ^{17}O Nuclei

Civan, *et al.* (306) have completed a study of ^1H , ^2D , and ^{17}O nuclei in frog muscles. The studies were conducted at room temperature and at 8.1 MHz, and possible molecular mechanisms are presented. Walmsley and Shporer (307) present theory dealing with surface-induced NMR line splittings and augmented relaxation rates in water, and Eisenstadt and Fabry (308) have published a study of the NMR relaxation of the hemoglobin-water proton spin system in red blood cells.

D. Final Note

The author has received, but not yet read, a review paper (in Russian) by I. A. Gamaley, *et al.* entitled "Properties of Cellular Water" (309).

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THE POLARIZED MULTILAYER THEORY
OF CELL WATER ACCORDING TO THE
ASSOCIATION-INDUCTION HYPOTHESIS¹

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I. INTRODUCTION

The crucial role of water in life is well known. It is not only biologists who are aware that without water there is no life. According to a currently widely taught theory of the living cell, the membrane theory, water in living cells is nothing unusual, but merely normal liquid water. The theory implies the existence of a battery of postulated pumps situated in a lipid cell membrane, which are responsible for the unique distribution of ions, sugar, and free amino acids found in all living cells. The difficulties with this theory have been reviewed many times (1-8) and need no further reiteration here. Instead, this article will be devoted entirely to reviewing the new theory of cell water introduced in the association-induction hypothesis (5,9).

II. A LOGICAL SEQUENCE: FROM DNA TO PROTEINS TO WATER

It is now universally accepted that the total aggregate attributes of a living organism are entirely determined by

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the base sequence of DNA molecules constituting the organism's genes. The base sequence in DNA, in turn, determines the sequence of amino acids in the proteins synthesized by the cell. Thus, it is clear that the unique features of living cells are primarily determined by their proteins. Indeed, this is precisely what the association-induction hypothesis contends.

In this theory, certain proteins widely found in all types of living cells serve a key role in delineating the features shared by all living cells through interaction with and control by a small number of molecules of great biological potency called "cardinal adsorbents". These proteins are kept in a conformation so that they can polarize multiple layers of cell water and specifically adsorb ions (2,6,8,10). This whole assembly of protoplasmic protein-water-ion, co-operatively linked, functions as a unit, providing the living cell with the physical basis of functional coherence and synchronized activities. Through this system a whole cell in a multi-cellular organism, or a sub-cellular organelle in a single cell, can be controlled by the addition or removal of cardinal adsorbents. Outstanding cardinal adsorbents include ATP, Ca^{++} , hormones and drugs.

III. THE LIVING STATE - A HIGH ENERGY STATE READY TO BE TRIGGERED INTO ACTIVITY

The association-induction hypothesis, in contrast to the membrane pump theory, sees the living cell, above all, as a highly efficient system, in that it spends little energy when the cell is at rest, but is ever ready to jump into full activity when there is a demand for action.

To achieve both energy economy at rest and promptness in response in activity, the living cell is normally maintained at a high energy state, the resting, living state. Like a cocked gun, the state of preparedness itself does not demand a continuous expenditure of energy; only after the cell, on command, has been triggered into a lower energy state -- the active state, is energy injected into the system to restore it to its resting high energy living state.

The high energy, resting living state represents a particular state of electron distribution in the protein-water-ion systems. To achieve this state of electron distribution in the protein, and its association with water and ions, the

cardinal adsorbent, ATP, plays a key role. By adsorbing onto key cardinal sites, ATP causes a propagated electric polarization (or inductive effects) corresponding to the resting, living state. To understand this we must probe a little deeper into the mechanism whereby proteins can interact with a great number of water molecules and many ions.

IV. THE POLARIZED MULTILAYER THEORY OF LONG-RANGE ORDERING OF WATER

In 1965 the polarized multilayer theory of cell water was explicitly suggested (5) and the details further elaborated in 1972 (9,11).

Being at once highly polarizable and in possession of a strong (permanent) dipole moment, water molecules react strongly with charged atoms or ions. Simple electrostatic considerations lead to the conclusion that a monopolar, uniformly charged surface will not interact with water more than one layer deep, while a surface with fixed positive charges (P), alternating with fixed negative charges (N) in a checkerboard-like arrangement (the N-P system) are more likely to build up multilayers of adsorbed water if the spacings between these fixed charges are correct. The reason for this difference is that in a monopolar uniformly charged surface, water molecules adsorbed on the surface are all oriented in the same direction. As a result, neighboring water molecules repel one another. On the other hand, in the bipolar N-P system², a row of water molecules polarized and oriented by one charged site attracts water molecules in the immediately neighboring rows which are polarized and oriented by fixed charges of the opposite electric sign. The water-water interaction in the polarized multilayers endows the system with the attributes of a three-dimensional cooperative assembly. The stability of such an assembly is further enhanced when one NP surface is juxtaposed to another as in the case of two pieces of polished glass surfaces facing each other, separated by a small space filled with water (12).

²In a picture diagram, water molecules in the state of polarized multilayers are seen as dipoles and are arranged in a cubic lattice. This picture is visualized as dynamic average orientation over long periods of time and should not be construed as that of a fixed crystalline configuration.

An equivalent of the NP-NP system exists in the form of a matrix of linear chains bearing alternating positive and negative fixed charges referred to as an NP-NP-NP system.

Other variations of the NP-NP system and the NP-NP-NP system are the NO-NO, OP-OP, NO-NO-NO and OP-OP-OP systems. In each case, either the fixed positive or negative sites are absent and represented by (0). Yet, charged sites are fixed in space and are separated from each other by a proper spacing. Though less effective as their prototype NP-NP and NP-NP-NP systems, these variants may also function to orient and polarize multilayers of water.

V. THE POLARIZED MULTILAYER THEORY OF CELL WATER

The concentration of free Na^+ in living cells may be as low as 10% of that in the external medium. To explain this effective exclusion, virtually all the water in a living cell must be in a physical state other than normal liquid water. Indeed this is what the association-induction hypothesis has long suggested (5,6,8). Two types of macromolecules most commonly found in living cells are the nucleic acids and proteins. As many types of living cells have comparatively little polynucleotides (e.g., nerve, muscle cells) or none at all (human red blood cells) and yet all possess the same ability to exclude solutes like Na^+ , one must attribute the ultimate source of the NP-NP-NP system to the intracellular proteins. It is also suggested that when a protein chain is stretched out, the exposed NH and CO groups of the backbone serve as the fixed positive and negative sites in an NP-NP-NP system.

Now most living cells contain about 80% water and about 20% proteins. There is thus 44 M water and, assuming an average amino acid residue weight of 110, only 1.8 M of NHCO groups participate, if all cell proteins are involved in polarizing water. More likely, only a portion of the cell protein is so involved. Assuming, say, that one half of the proteins are involved, then there are all told 0.9 M of NHCO groups. Since each NHCO group provides two sites, the total concentration is $0.9 \times 2 = 1.8$ M. Thus, each backbond NHCO site must be able to polarize and orient 44/1.8 or about 25 water molecules. Studies of isolated proteins and their model systems now in progress have suggested that this is indeed feasible.

VI. SOME PHYSIOLOGICALLY IMPORTANT PROPERTIES OF WATER IN THE STATE OF POLARIZED MULTILAYERS

A. Reduced Solubility for Large Solute Molecules

According to the association-induction hypothesis, a major feature of water existing in the state of polarized multilayers is its reduced solubility for larger molecules and ions. The reduced solubility may arise from both entropic and enthalpic causes (5).

Na^+ is an example. In an aqueous medium, Na^+ is surrounded by a layer of intensively polarized water of hydration. The hydrated Na^+ is thus not only much larger than the unhydrated Na^+ , but also has acquired many rotational axes of symmetry and, hence, rotational entropy. In the polarized water the hydrated Na^+ may have less rotational freedom. The consequent loss of rotational entropy then reduces its solubility in the polarized water.

The water-to-water interaction energy in the state of polarized multilayers may be significantly higher than in normal liquid water. In order to excavate a hole in the polarized water, more energy would be needed than the energy gained in filling a hole of the same size in normal liquid water. As a rule, the transfer of a mole of hydrated Na^+ from normal water to the polarized water may also entail an unfavorable enthalpy change.

A second enthalpic mechanism for the reduced solubility of hydrated Na^+ in polarized water may originate from reduced hydrogen-bond energy of the peripheral water molecules in the hydration shell of the Na^+ when the hydrated Na^+ is in the polarized water since the polarized water is less random in orientation and may have less tendency to interact with the water of hydration.

Both the entropic and enthalpic mechanisms of exclusion are greatly reduced for small molecules but increase in effectiveness with molecular sizes of the solutes.

B. Semipermeability

An important corollary of the reduced stability is a reduced permeability. This follows naturally because the permeability is a product of the solubility, or equilibrium distribution coefficient (the q value in the association-

induction theory) multiplied by the diffusion coefficient of the solute. Since the q value for water itself is unity, and the q value decreases with increasing molecular size and complexity, water in the state of polarized multilayers possesses the attributes of living cell membranes: (1) semi-permeability, namely, greater permeability for water than for many solutes; (2) decreasing permeability with increasing solute molecular sizes. Experimental evidence that these fundamental properties of the living cell are not due to lipid layers with pores but are due to water polarized by polar groups on cell proteins, phospholipids, etc. has been presented (13).

VII. COOPERATIVE TRANSITION OF THE STATE OF WATER IN LIVING CELLS BY CARDINAL ADSORBENTS VIA THEIR EFFECTS ON PROTEIN CONFORMATION

Proteins are remarkable in their ability to polarize all the cell's water. It is, however, just as remarkable that a protein can, on demand, promptly and in a clear-cut all-or-none manner change the physical state of the water to that of normal water (14,15,16). This is so because these proteins, like all proteins, can exist in more than one discrete state which, to put it in the simplest terms, is one in which the backbond NH and CO groups are respectively free to interact with deep layers of water, or in which they are not free because they are neutralized by forming H-bonds with NHCO of another part of the same protein molecules (e.g., α -helix) or with NHCO groups of another protein molecule (e.g., β -pleated sheets). Thus, if the proteins involved can be made to switch in an all-or-none manner from one state to the other, we will witness an all-or-none change of the physical state of a large number of water molecules, and with this change of state, the living cell will demonstrate manifestations that have long been known to physiologists: change of cell permeability and electrical excitation (2,9,16).

VIII. THE CARDINAL ADSORBENTS: THE MASTER CONTROL OF THE PROTEIN-WATER-ION SYSTEM OF THE LIVING CELLS

Our final question asks "what then controls the protein conformation?". According to the association-induction hypothesis, these controls are the functions of those agents

which physiologically and pharmacologically have long been known to control biological activity.

Indeed, to pharmacologists and physiologists, the concept that there are proteinaceous receptor sites for all types of hormones and drugs is commonplace (17,18,19). One year after the publication of the association-induction hypothesis - in which the long-range control of enzyme action was discussed in considerable detail and was referred to as an "indirect F-effect" (21) - Monod and co-workers coined the term allosteric control (20).

The association-induction hypothesis has, by invoking the polarized multilayer theory of cell water and the concept of cooperative adsorption and control by cardinal adsorbents of cell protein, provided a reasonable mechanism for how the "reception" with the site of a drug, hormone or other cardinal adsorbent brings about a physiological manifestation in an allosteric manner.

The association-induction hypothesis also offers a solution to the paradox that has long been known but little discussed; namely, that "ATP has no high energy" (21,22,23) and yet it is the "source" of metabolic energy (1,24). The new view is that the energy resides in the whole ATP-protein-water-ion assembly and not in the terminal phosphate bonds of ATP (25). The lability of vulnerability to the hydrolytic enzymes only provides an efficient way for its removal at a time when its removal and the consequent protein conformation and water structure changes are required for the activated cell (6).

NOTE ADDED IN PROOF

Since the original manuscript was submitted a number of other papers have appeared in print. These papers should be consulted for more detailed discussions of the concept of polarized multilayers and their role in cell physiology (26,27,28).

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APPLICATIONS OF CELL BIOLOGY
TO AN UNDERSTANDING OF BIOLOGICAL WATER¹

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I. INTRODUCTION

The purpose of this chapter is to discuss the application of the rapidly advancing technology of cell biology to the study of cell-associated water. As often happens, two independent fields of scientific development have undergone rapid advancement in the same period of time, and are finding mutual applications, with the result that new understanding is generated.

Although many years of hopeful and adventurous trials preceded it, the first cultures of single eucaryotic mammalian cells were established in 1948 in the laboratory of Sanford, Earle and Likely (1). Practical cell culture became possible in the mid-1950's, when Eagle (2) and others determined the minimal nutritional requirements of many types of cells.

During this same period, Bloch (3) and Purcell (4) published their classical papers on nuclear magnetic resonance theory which made it possible to follow the behavior of hydrogen nuclei in water molecules as they accepted and gave off energy in a strong external magnetic field. The time course of energy dissipation through vibrational, transla-

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tional, rotational, and thermal exchange is termed the spin-lattice relaxation time, T_1 , and the exchange of excess energy through spin coupling is termed the spin-spin relaxation time, T_2 . These two variables helped to describe the motional freedom of water molecules, in relation to pure water, where the return to equilibrium of excited hydrogens has a T_1 of ~ 3000 ms for pure water.

The first application of NMR to water in *cells* (not tissue) was published by Odeblad, *et al.* (5) in 1955. Protons of water in packed human red blood cells showed shorter relaxation times than for liquid water, indicating a slower diffusional exchange with extracellular water than had been anticipated. This was confirmed in a study by Odeblad (6) in 1956 of D_2O exchange in red cells by NMR.

In the 1960's, the emphasis in NMR research on biological water centered on explaining the shortened relaxation times for water in biological tissues. Studies on solutions of biological molecules such as DNA (7), proteins (8) including collagen (9), and polysaccharide gels (10) could not fully account for the "ordering" of water observed in living tissues. In 1969, Cope (11) first used NMR to study D_2O in muscle and brain. In 1971, Damadian (12) showed that NMR could distinguish cancerous from normal tissues. From this point on, NMR was used extensively in the study of "biological water".

While the NMR studies of water in biological tissues were progressing, the art of cell culture was also advancing. The establishment by Gey (13) in 1962 of the human cervical cell line called "HeLa", opened up the study of cancer to cell biologists and the field has grown exponentially since that time. The 1960's saw the establishment of many differentiated cell lines (14) and the discovery of techniques for the synchronization of growth in cell cultures (15). Modern techniques include such things as cell hybridization by fusion, transformation by viruses, and DNA-recombinant experiments. Throughout the development of this field, however, little attention has been paid to the 80-90% of the mass of the cell that is water. Even simple desiccation experiments to determine total water content are rare, and osmotic behavior is poorly understood. But no longer can water be ignored, in preference to the more esoterically attractive complex macromolecules of cells, such as enzymes and genetic material. The interdependence of the water-macromolecular lattice is beginning to be recognized. Cell biologists have long needed a tool to better understand the

biophysics of water in cells, and NMR has finally reached the technical level of application required.

One problem inherent in the interpretation of NMR data on whole tissues, organs, or animals is sample heterogeneity. This results not only from intracellular differences, but the whole spectrum of extracellular fluids, such as blood, urine, or lymph. Connective tissue water and fat deposits are also problems. In contrast, the use of essentially identical cells available from cell cultures avoids these problems in NMR research. Cells may even be synchronized so that a very large percentage of them are biochemically similar and in the same phase of the cell cycle. Therefore, the combination of cell biology and magnetic resonance technology is a timely step forward in the understanding of cell-associated water.

II. NUCLEAR MAGNETIC RESONANCE STUDIES OF WATER IN THE SYNCHRONIZED HELa CYCLE

The first comprehensive study of intracellular water in cultured cells has been completed in our laboratory. Nuclear magnetic resonance relaxation times (T_1 and T_2) reveal a reproducible phase-specific pattern of changes in the physical state of water during the synchronized HeLa cell cycle (16).

HeLa cells descended from the line of Gey (13) were grown as monolayer cultures in Falcon plastic dishes at 37°C in Eagle's minimal essential medium (MEM) supplemented with Eagle's non-essential amino acids, fetal calf serum (10%), sodium pyruvate, glutamine, and penicillin-streptomycin (17). Cells were synchronized at eight points in the cell cycle by two methods. A population of cells, partially synchronized by a single excess thymidine block, were treated with either colcemid (0.05 mg/ml) or N_2O gas at 80 psi (18) to yield cells blocked in mitosis (98% mitotic index). Cells just released from the block are called M_0 , and those subsequently grown at 37°C for 30 minutes, M_{30} . Incubation of N_2O -blocked mitotic cells under regular culture conditions for four to eight hours yielded highly synchronous (18) populations of G_1 (4 hr.) and G_1 (8 hr.) cells. The technique of a double excess thymidine block (19) over two days, resulted in synchronized S-phased cells (95% Labeling Index). G_2 cells grown from these S-phase cells were harvested at points equivalent to 18 hours, 19 hours, and 20 hours of the total cell cycle time of 22 hours. Approximately 2×10^7 cells

were required for each sample. Cells were harvested by gentle scraping from the dishes with a teflon policeman and centrifuged at 1000g for 20 minutes in the NMR sample tubes. Excess medium was removed by suction and the samples sealed, placed on ice, and transported to the NMR facility for measurements within one hour.

Pulsed nuclear magnetic resonance relaxation times, T_1 and T_2 , were performed by a $180^\circ-\tau-90^\circ$ pulse sequence (20) and a Carr-Purcell (21) spin echo method with a Meiboom-Gill (22) phase correction on a Bruker SXP NMR spectrometer at 30 MHz and 25°C. Data were analyzed by computer methods. Percent water content was determined by drying to constant weight at 105°C.

Using this combined methodology from cell culture and biophysics, the hour-by-hour progress of changes in the physical state of water was followed (23) in the HeLa cell cycle (see Figure 1). Eight to ten separate experiments showed good reproducibility of this pattern. The maximum motional freedom of water molecules as defined by NMR was found during mitosis with a T_1 of 1020 ms. With the reformation of the nuclear membrane in G_1 , and the decondensation of chromatin, T_1 decreased, indicating more ordered water in the system. Minimum motional freedom of water in the cell cycle was found in S-phase ($T_1 = 534$ ms). During G_2 , the physical state of protoplasmic water slowly changed back to that seen during mitosis.

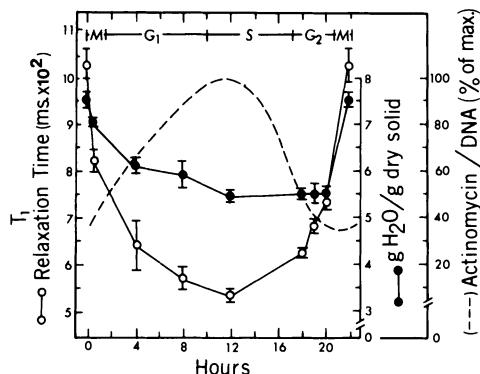


FIGURE 1. (-o-) T_1 of water protons vs. hours of the cell cycle. (-●-) $\text{gms H}_2\text{O/gm dry solids}$ vs. hours of the cell cycle. (---) chromatin condensation cycle.

During the 12 hours of the cell cycle, from S through late G₂ (at 20 hours of the total cycle), the ratio of water to dry solids (hydration) remained constant. However, significant differences in T₁ values occurred that were independent of hydration (Figure 2).

III. EFFECT OF CONFORMATIONAL CHANGES OF CHROMATIN ON T₁ OF WATER IN HELa CELLS

This volume is concerned with the evidence that exists for the restricted behavior of water molecules near surfaces, including macromolecules. If it is true, as documented by Drost-Hansen (24) and others that these influences may extend from 100-1000 Å from a surface, then water in a cell which is within 1000 Å of some structure or macromolecule should exhibit some of the properties of vicinal water. The T₁ values found for water in random and synchronized HeLa cells (< 1000 ms) are not only lower than pure water, they also change in a dynamic way during the cell cycle. We have observed that the T₁ values show an inverse correlation with the chromatin condensation cycle of the cell (dashed line, Figure 1). Therefore, we postulated that since chromatin makes up a large fraction of the macromolecules of the HeLa cell, water-chromatin interaction might account for part of the T₁ pattern changes.

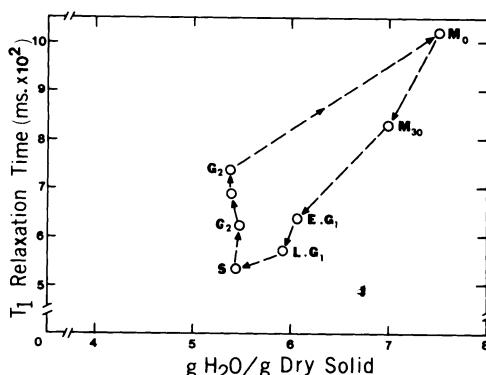


FIGURE 2. HeLa Cell Cycle: Hydration of the cells plotted as a function of the T₁ relaxation time of water protons.

Intact S-phase HeLa cells with a T_1 of 534 ms were incubated with spermine, a polyamine known to have chromatin condensing ability. (Spermine has no significant effect on the T_1 of pure water). Visible chromatin condensation was accompanied by a time-dependent elevation of T_1 to 701 ms without a significant change in percent water content of the cells (Table I). This implied that water freed from the condensing chromatin raised the mean T_1 value, but it was also possible that spermine had some effect on the cytoplasm as well.

It was, therefore, necessary to design a method for the isotonic isolation of nuclei. Most present methods for nuclear isolation use hyper- or hypo-tonic shock to rupture cell membranes. This visibly alters the state of the chromatin and produces shrunken deformed nuclei. Since this would alter the water-chromatin interactions, such methods were not acceptable. A modification of the cytochalasin-B enucleation method of Prescott (25) was used to isotonically prepare double-membraned nuclei, called karyoplasts. Nuclei isolated in this manner showed (Table II) that S-phase karyoplasts had the lowest T_1 (458 ms), while G₂ karyoplasts were higher (690 ms). When S-phase karyoplasts were treated with spermine, chromatin condensation resulted in an elevated T_1 with no significant change in percent water content (Table III).

Therefore, we propose that part of the changes in the state of water can be directly tied to the natural chromatin condensation cycle of HeLa cells. Other dynamic water-macromolecular systems in cells that could effect water

TABLE I. Effect of Spermine on the T_1 of Synchronized S-Phase HeLa Cells

Sample	Incubation	T_1 (ms)	% H ₂ O
S Control	1 hr	538	84.4
S Control	2 hr	546	84.6
Spermine (0.02M)	1 hr	638	84.4
Spermine (0.02M)	2 hr	701	85.0

TABLE II. Relaxation Times and Water Content of S and G₂ HeLa Cells and Karyoplasts

S-Phase (12 Hours)						
	Control Cells			Cytochalasin-B Treated Cells		Karyoplasts
T _{1ms}	534	±	43	681	±	14
T _{2ms}	100	±	9			53
% H ₂ O	84.42	±	.75	82.90	±	2.00
						84.01 ± .99

G ₂ Phase (19 Hours)						
	Control Cells			Cytochalasin-B Treated Cells		Karyoplasts
T _{1ms}	690	±	4	795	±	2
T _{2ms}		-		99		
% H ₂ O	84.28	±	.63	84.00	±	.51
						80.02 ± 1.14

All T₁, T₂, and % H₂O values expressed as the mean ± standard deviation

TABLE III. Effect of Chromatin Condensation on T₁ of S-Phase HeLa Nucleoplasts

Sample		T ₁ (ms)	% H ₂ O
S-Phase Nucleoplasts	Mean	457	84.0
	Std. Dev.	29	.9
S-Phase Nucleoplasts with Spermine	Mean	617	84.0
	Std. Dev.	43	.5

structure might be various membrane systems, actin-myosin fibers, and microtubules, all of which have their own cycles in the cell.

IV. RELATIONSHIP OF WATER TO DYNAMIC CELLULAR PROCESSES

The evidence just discussed pointed to a dynamic relationship between the state of water and macromolecular events in cellular life. The constantly changing biochemical processes of rapid division were further explored in our laboratory as they related to cellular water.

Over the last four years, twelve cell lines have been established from metastatic human breast cancer cells obtained by pleural effusions (26,27). The cell lines have been carefully characterized according to chromosome pattern, growth in nude mice, estrogen binding capacity, enzyme types, and growth and morphology patterns which show them to be characteristic of the primary tumor in many ways. Four of these human cancer cell lines (MB-231, MB-157, MB-330, and MB-331) were chosen for this preliminary investigation (see Note Added in Proof). The cells were grown as monolayers in Leibovitz medium (Gibco L-15) supplemented with 15% fetal calf serum, glutathione, insulin, and antibiotics at 37°C in equilibrium with air. The doubling time of cells in each culture was estimated over a period of time. The cells were then gently scraped from the flasks and centrifuged at 1000g for 20 minutes in NMR sample tubes. After removal of excess medium, the pellets were measured by a 180°-τ-90° pulse sequence on a Bruker-SXP nuclear magnetic resonance spectrometer to determine spin-lattice relaxation time (T_1) of water hydrogens at 30 MHz and 25°C. Medical records available in summarized form were studied for information that could lead to a definition of the time course and pattern of the disease in each patient.

The results of doubling time, spin-lattice relaxation times (T_1) and the medical record examinations are presented in Table IV.

The range of division times in culture was found to be 18 h for MB-231, 36 h for MB-157, 75 h for MB-330 and 7 days for MB-331. The ability of these cells to survive and multiply in culture may be indicative of their hardiness and persistent growth properties.

TABLE IV. *The Correlation of Human Breast Cancer Metastasis with the Physical State of Cellular Water and Division Rate in Culture*

Cell Line	Approximate Division Time in Culture ^a	Diagnosis of Primary Tumor	Months Survival After 1st Recurrence ^b	Spin-Lattice Relaxation Time	% H ₂ O
T ₁ msec ^c					
MDA-MB-231	18 hrs	Poorly differentiated; primary and intraductal carcinoma	7 months	1030	87.5
MDA-MB-157	36 hrs	Medullary carcinoma with lymphoid stroma and metastasis	12 months	907	87.4
MDA-MB-330	72 hrs	Infiltrating carcinoma	Advanced metastases present 10 months prior to death	725	87.1
MDA-MB-331	1 week	Unclassified with progression to chest wall, nodes and skin	27 months	581	88.5

^aDetermined from dilution and growth of culture

^bTaken from medical record summary of patient

^cRelaxation times of water hydrogen protons at 30 MHz and 25°C.

Among these four lines, no apparent correlation was observed between the type of primary malignant tumor and the ability of the cells to grow in culture. Nor was there any apparent correlation between the histological classification of the primary tumor and the survival time of the patient. Survival statistics collected for groups of patients in a specific histological classification may have a wide range whose mean survival rate is of little meaning with regard to any one patient. The data on the four cell lines, however, do show that progress of the disease in the patient after the first recurrence is related to rate of growth of the metastatic cells in culture. Therefore, it may be that the very same properties of the cancer cells that enable them to survive and grow rapidly in culture are related to these same traits in the human body.

The model system defined by the four cell lines in this study shows a direct correlation between the division time of the cells in culture and the T_1 relaxation time of the cellular water protons. The MB-231 line is the most rapidly dividing at 18 h and has the highest T_1 (1030 ms), a value near that found in undeveloped newborn rat muscle (28), as well as in mitotic HeLa cells during the cell cycle (16).

This may indicate that the macromolecular lattice in the MB-231 cell is less complex than in the MB-331 cell, which divides only once a week and has a T_1 of 581 ms, nearer that determined for molecularly complex striated muscle (28). (See Note Added in Proof).

Since the actual percentage of water in the cell lines is very similar, the differences in T_1 values are not a simple function of total water content. Thus, some fundamental difference between the macromolecular interactions with water among these four cell lines may be the key to an understanding of their differential growth properties. It is important to remember that all four cell lines are metastatic carcinomas that were eventually fatal to the patients.

Do the investigated physical properties of the system-doubling time and T_1 contain information relevant to the course of the original disease in the patients? From the available medical records, the factor in the disease that showed the greatest correlation with both doubling time and state of water was the survival time of the patient after the first post-operative recurrence of the cancer (Table IV). (Line MB-330 does not fit exactly; however, the patient did not return to the hospital until whole body metastasis had

occurred). Since these cell lines were grown from cells that had metastasized from the primary breast tumor to the lungs, their origin was metastatic and therefore, they may be truly representative of the metastatic potential of the patient's disease. The fact remains that the range of patient survival was 7-27 months, which can be correlated with the rapidity of division of these cells in culture and the degree of motional freedom of their water molecules. Thus, it is important to study further these four cell lines, as well as the eight additional ones now available, for determination of those physical properties that provide quantitative information concerning the malignant course of cancer. (See Note Added in Proof).

V. SPECULATION INTO THE FUTURE USES OF CELL BIOLOGY IN THE STUDY OF WATER

The two previous studies represent only the beginnings of what can be accomplished by the use of cultured cells in understanding the dynamic behavior of cell-associated water. It may be appropriate, therefore, to speculate on the future course of research in this area.

In the immediate future, I believe we will see increased attention in the participation of water in many areas of cell biology. The changes in state of water in the HeLa cell cycle should stir curiosity about other cell cycles and the relationship of conformational changes in various macromolecules to water structure. Coupled with this should occur the investigation of what these changes in water properties may mean in terms of electrolyte solvation. Will it be found that, especially in the nucleus, Na^+ will move across the nuclear membrane in response to a change in water structure? The rapid uptake of water just prior to mitosis implies that water is involved directly or indirectly in the triggering of division. Also, the relationship of T_1 to division rate in culture holds possibilities for further exploration.

Areas thus far unexplored should yield much information about cellular water. For example, evidence exists in whole tissues (30) and chicken embryos (31) that a very early and perhaps initial reaction to viral infection is the uptake of water into cells. A comprehensive study of this phenomena could yield new insight into the mechanism of viral infection. Drug and viral induced transformation of cells to new morphological states may be followed and better understood by

examining the properties of water. A good example of this ability of water to reflect the overall state of the cell has been under preliminary study. Chinese hamster ovary cells undergo a classical change to long spindle type cells when exposed to dibutyryl-cAMP (32). In our laboratory, these treated cells showed no significant differences in water T_1 values from normal CHO cells and they continued to divide at their regular rate (unpublished results). Such a large visual transformation without a change in water was puzzling. Recent results (33) have shown, however, that the mechanism of cAMP-transformation on these cells involves only a "sticky" surface protein and not the total cell biochemistry. This agrees with the expected sensitivity of water to the internal cellular environment. Transformation to a cancerous state, however - where total cellular biochemistry is involved and division rate increases - should be reflected in elevated T_1 values. It is likely that other areas of study of cellular water by NMR in cultured cells will be revealed, and I suggest that after this descriptive phase, a further step will be taken as discussed in the following section.

VI. MANIPULATION OF CELLULAR WATER

In general, after a period of observation in an area of science, a time usually arrives for investigation of the mechanism for a new phenomenon (this is the current stage of cellular water research). But, at some point in time, a transition from observation to application occurs. In the case of cellular water, there has, to my knowledge, not been an attempt yet to consciously manipulate the structure of water in living cells to produce a desired effect. If cellular water structure is basic to life, then the changes that appear in cancer and development may hold clues to the eventual manipulation of cellular function.

The most obvious application of water manipulation would be in the restoration of diseased cells to a more normal state where they might revive proper function. Some speculative ideas about future experiments to manipulate the state of water in normal and diseased cells are outlined below. It is assumed that the time course of changes in water structure can be followed by NMR and hydration studies. These are only a few ideas and hopefully the reader will add others. Because of the control available in cultured cells, these may provide the best system for such studies.

One physical variable that is known to have an effect on water structure is *temperature*. Usually one thinks of ice, liquid, and water vapor, but in the complex intra-cellular environment, very little is known about the details of the temperature dependence of water structure. Changes in NMR properties of water with temperature in biological tissues are just beginning to be studied. The properties of vicinal water display unusual temperature dependence according to Drost-Hansen (24) and these may play some role in biological temperature phenomena. There is a large literature on the temperature dependence of biological phenomena, and certain basic biological processes show minima and maxima at or near the temperatures where vicinal water structure also changes. In cultured cells, temperature dependence of growth and division have been studied for many different cell lines within narrow ranges of 31-42°C (35,19). The true mechanism of temperature-dependent growth is not known. It is often noted that enzymes are temperature-dependent in test tubes and that these enzymes stop functioning at temperature extremes. Actually, the mechanism cannot be that simple. Around each macromolecule are layers of water whose temperature-dependent behavior may affect the conformation and activity of enzymes. The whole field of enzyme kinetics in living protoplasm remains to be studied in this regard.

Can temperature be used to manipulate water structure in living cells? I should like to suggest that it already has been. The production of synchronous populations of cells by temperature shock (19) is a good example of a possible whole cell reaction to a change in state of water. Treatment of cancer by localized increases in body temperature, called hyperthermy (36,37) shows not only a decrease in cancer growth, but a greater sensitivity of cancer cells to radiation therapy when the cellular temperature is raised only a few degrees (38). Differential germination rates of seeds (39) at temperatures near the phasic properties of vicinal water are also interesting. Careful observation will show groups of plants and animals concentrated at certain temperatures and scarce at others (see chapters by Fisher in this volume). I have vivid memories of a particular rainbow geyser in Yellowstone Park where various colors of algae grew in distinct rings at temperatures varying from the hottest water at the bottom of the pool to the cooler water at the top. There was no mixing of plant types and each color of algae was separated from the next by a clear non-growth ring. Why there were certain temperatures where species could adapt and others where nothing grew has always been a mystery to

me. The study of multiple growth optima by Drost-Hansen and co-workers may provide insight in such phenomena.

One question that could be asked is why do mammals and birds have evolutionary selected mechanisms for temperature control while the majority of animals on this planet are functionally dependent on the temperature of their environment? Is it possible that mammalian and avian cells are so macromolecularly sophisticated that only a few degrees in temperature causes structural changes in water sufficient to shut down the giant molecules by conformational changes? Perhaps other species have more stable enzymatic configurations. These types of changes with small temperature differences may involve only a small fraction of water molecules very close to enzymes and other cellular structures. Therefore, they may never be detected by biophysical techniques.

However, the effects of large temperature changes, such as freezing, can readily be studied. A classical example is found in the NMR properties of muscle water during the freezing process. Muscle water does not freeze at 0°C, reflecting its protein and electrolyte composition. But even at -5°, -10°, and -15°C, muscle water may resist freezing. After nucleation of ice crystals, a portion of cell water freezes rapidly; however, a non-freezable fraction of mobile water remains, often as much as 20% (40). It has been postulated, but not conclusively proven, that this non-freezable fraction is closely associated with cellular macromolecules. This phenomenon is one of the most conclusive proofs of the non-ideal behavior of biological water. Upon thawing, muscle water follows a hysteresis pattern of return to normal relaxation behavior. The non-freezable fraction of water has been studied only recently in cultured cells. In our laboratory, a human breast cancer cell line (613SW) showed the same type of behavior as muscle water (unpublished data), revealing ~ 12% non-freezable (NF) water. We intend to study the NF fraction of water in cell lines in relation to their division rate.

The preceding paragraphs have been written from a different viewpoint regarding temperature dependency of growth and division than is normally expounded. One finds that most explanations of temperature phenomena are based on enzyme kinetics. However, it is interesting how enzymes that perform the same function in different species, and differ by only a few amino acids in their peptide sequence, could be the great temperature controllers. It seems that perhaps a much more general cellular phenomenon, such as a change in

the physical structure of water around macromolecules, might shut off certain functions of the cell (see chapter by Clegg in this volume).

Another area that has been explored to some extent in relation to involvement of cell water is extent of *hydration*. Some literature does exist on total tissue or total cell hydration and some investigators believe NMR relaxation time changes in water are due to an increase or decrease in total hydration (41). Certainly, the amount of water present per gram of other components is important in determining T_1 values; however, what the other components are, and their nature, is also important to the state of the surrounding water. In the HeLa cell cycle, we have seen how the T_1 values of cellular water can change in response to changes in chromatin conformation without changes in total cellular hydration (16). In breast cancer cells, increased T_1 values seem to be more related to division rate (42) than hydration (see Note Added in Proof). In breast cancer biopsies from humans, there was absolutely no correlation between tissue T_1 and T_2 values and hydration (unpublished data from this laboratory). In the spleens of rats fed carcinogens, T_1 values increased while hydration decreased (43), just the opposite of prediction. I think these examples are sufficient to show that the current mechanism for changes in T_1 and T_2 values is not completely understood.

Regardless of actual mechanism, we can ask whether increases or decreases in hydration could be useful tools in water manipulation. Current evidence indicates that sometimes highly hydrated tissues or cells have higher T_1 values. Cancer cells, especially rapidly dividing ones, seem to exhibit very high hydration and also high T_1 and T_2 values. What if cancer cells could be gradually and continually subjected to high external osmolarity? HeLa cells grown in hypertonic media do show a slower division rate (19).

Another way of changing hydration might be to chemically stimulate excessive protein production. If this protein accumulated in cytoplasm, it might slow cell growth by restricting water around it. A possible example of this technique has been accomplished. When excess thymidine is added to culture medium, the DNA replication system of HeLa cells is blocked. If the effects last only a few hours, no noticeable change in progression to mitosis is noted when the thymidine is removed. However, since the other enzyme systems of the cell are not blocked by thymidine, the cell will continue to synthesize protein and grow. In the case of an ex-

tended thymidine block of > 12 hours, an increased ratio of protein to DNA is seen and, after removal of thymidine, a lag is found in continued cell division, even though all components for division are present in excess. A simple experiment would be to study excess thymidine blocked cells with time to see if a change in the structure of cellular water could be detected. The reversal of this effect might correspond to a return to normal cellular division.

For non-dividing cells, certain chemicals might be found which would stimulate protein synthesis as a protection against dehydration and loss of electrolytes in such diseases as cholera. In this laboratory, we have followed Chinese hamster ovary (CHO) cells treated with cholera toxin. A time-dependent increase in T_1 values was seen (45). It would be interesting to see if this effect could be halted by increased protein synthesis. Although this is a case of two effects fighting one another, it certainly would have application in the treatment of diarrheal disease.

After investigating the correlation between cellular division rate and the physical state of water, I have also become curious to know if regulators of cell growth alter the physical state of water as well.

The normal growth regulating mechanisms are not well understood; however, a few clues are available. Polypeptide growth regulators isolated from human urine have been shown to inhibit the growth of human leukemia, osteosarcoma, and HeLa cells (44). Tests in breast cancer cell lines, for which we already have NMR data, are underway to determine if peptides that slow or inhibit cell growth can cause changes in water structure. As we begin to understand the regulation of cellular growth and division, it may become apparent that water can play important roles in these processes.

Speculations can also be made about artificial means of manipulating cell water. One such method might be the inclusion of inert particles in cytoplasm to increase the surface area available to influence water structure. For a number of years, tiny polystyrene latex beads have been used by electron microscopists as highly accurate size scales. These beads are incorporated by phagocytosis into many kinds of cells in large numbers. Since the beads are of highly uniform diameter, the increase in surface area can be calculated. Bruun, Sørensen and Drost-Hansen (unpublished data) have observed that polystyrene beads can affect water structure near their surfaces. Therefore, large numbers of

tiny beads in cells should change the water structure. If a slowly biodegradable bead could be invented, then it would be feasible in the future to treat diarrhea disease with this method. Intestinal cells commonly take in nutrients by phagocytosis and pinocytosis. If diarrhea is caused by a change in water structure in intestinal cells, then incorporation of beads from medicines into the intestinal epithelial cells might slow down loss of fluids in diarrhea.

The previous sections in this chapter have all been written from a specific point of view: that water plays a critical role in cellular structure and function. Classically, water has mostly been considered an inert solvent for the various components found in cells. It may be a question of whether macromolecules affect water structure which, in turn, affect macromolecules, etc., but not enough data are available to rule out at least a fifty-fifty partnership between water and other molecules. This point of view may well prove of value and be worthy of considerable study.

The manipulation presented here concerning the active manipulation of water structure offer only hints at the scope of application. By controlling the structure of cellular water, it may be possible to return cells to normal behavior more quickly during infection, to compensate for genetic abnormalities, to manage water and electrolyte balance, and to slow cellular division in cancer. It may turn out that the next generation of scientists will have reason to thank the pioneers in this field for their current observational research and explorations into the mechanisms of water structure; one day they may even praise them for efforts at practical applications in medicine.

NOTE ADDED IN PROOF

Water-Cytoskeletal Interactions in Cultured Cells. In addition to earlier work published on the NMR relaxation times of human breast cancer (HBC) cells, a comprehensive study on eleven HBC lines has now been completed.

A number of cultured cell systems have been characterized in our laboratory by the nuclear magnetic resonance relaxation times of the hydrogen protons of their intracellular water and the antibody immunofluorescence patterns of cytoplasmic actin fibers and microtubules. Human breast cancer cells have been examined for a relationship between

TABLE V. Human Breast Cancer Cell Lines: Water Relaxation Times, Doubling Times, and Cytoskeleton

Cell Line MDA-MB*	NMR Relaxation Times		% H ₂ O	Doubling Time in Culture (Days)	Microtubule Complex	
	T ₁ ms ± SD	T ₂ ms ± SD			% Full	% Diminished
231	934 ± 78	123 ± 31	86.5	1	11	89
468	---	---	---	1-1½	0	100
157	907 ± 10	135 ± 4	87.4	1-1½	7	93
461	---	---	---	1-1½	14	86
361	849	---	88.4	1½	---	---
134	717 ± 64	145 ± 39	88.9	1½-2	0	100
453	770 ± 15	113 ± 18	87.4	1½-2	1.5	98.5
330	752 ± 39	---	88.5	1½-3	55	45
435	607 ± 9	112 ± 13	87.3	6-7	34	66
331	549 ± 136	75 ± 20	88.5	5-7	48	52
431	521	126	---	12-14	83	17
436	499 ± 49	100 ± 20	84.3	16-18	79	21
415	260 ± 50	69 ± 5	85.4	24-28	84	16
213 (slow)	662 ± 70	104 ± 18	---	2		
157	669 ± 52	102 ± 18	85.2	2½		

water mobility and cytoplasmic structure. Among 11 lines of human breast cancer cells, there was a strong correlation between doubling time in culture, spin-lattice relaxation times (T_1) of water, and the amount of fibrous microtubule complex (see Table V). Fast growing cells have scarce diffuse tubulin fluorescence by antibody immunofluorescence and long T_1 's (1000-600 ms) while slow growing cells have dense fibrous cytoplasmic microtubule complexes and short T_1 's (250-500 ms). Differences between these cells may lie on the level of the "microtraebeculi" of the cytoplasm. Studies with the high voltage electron microscope may confirm this.

The relaxation times of water in Balb 3T3 and SV3T3 show differences that may be correlated with the change in amount and conformation of cytoplasmic actin and tubulin seen during the process of transformation. Studies on changes in water relaxation times as a function of the manipulation of cytoplasmic macromolecular structures by temperature and drugs are underway. Current evidence points to a strong correlation between the cytoplasmic macromolecular matrix and the behavior of water in cells.

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AQUEOUS PHASE STRUCTURE IN CELLS AND ORGANELLES¹

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I. INTRODUCTION

A. *Fundamental Questions*

The assumption that intracellular water constitutes a single aqueous phase is so commonly made in biology that it is rarely stated. Yet, there is a compelling rationale for investigating this assumption, for its validity has profound consequences for the interpretation of biological measurements. Examples abound in biology: in every instance where a relationship requires that an intensive variable (e.g., concentration) be assigned a value for the intracellular space, an *a priori* assumption of internal phase structure is made. This assumption, in turn, frequently leads to markedly different interpretations of data. Prolonged controversy ensues, most commonly in the absence of a clear recognition of the fundamental basis for the disagreement.

It may appear at first glance that the aqueous milieu of cells is so complex as to prohibit going beyond the single phase model. Whether or not it is possible to gain meaningful insight into this problem must not be decided in advance, but rather must rest on experiment. Furthermore, we should be encouraged by the very nature of the controversies that arise from uncertainties about cell water - for the most part, their resolution requires a rather minimal description

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O

- Obstetrics and gynecology 103, 104, 212, 325,
334, 348, 362-372,
538-544, 598
Osteomyelitis 267, 382, 384
Otitis 128, 216, 217, 291

P

- Pancreatic infection 298
Parasitology 340-345, 613-619
Parvovirus 603
Pelvic inflammatory disease 159, 329, 348-361
Penicillin 135, 263, 264, 422,
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Perinatal infection 374, 391-395, 408, 409,
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559, 564, 592, 598,
604-608
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Phagocytosis 19, 27, 39, 124, 400,
406, 418, 464, 481
Pharyngitis 220, 417-428
Picornavirus 390
Piperacillin 70, 161-163, 168,
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Pittsburgh pneumonia agent 246-248
Plasmids 130-132, 458
Platelets 17, 78, 163
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Pneumonia 10, 19, 25, 30, 37,
204, 221-248, 340-343,
392, 399, 488-493,
517, 536, 539B, 565,
566, 605-607

mately one-half that of dilute electrolyte solution. Taking advantage of the natural anisotropy of muscle fibers, Hazlewood and co-workers (2) have convincingly demonstrated that the obstruction effect (3) can only account for a fraction of the observed reduction in D_s . If this important conclusion is correct, then it must be agreed that the reduction in D_s reflects altered properties of cell water. Nevertheless, there remain two distinct ways of interpreting this finding.

First, consider the cytoplasm to be a single aqueous phase. Then the short NMR relaxation times necessarily indicate that all of intracellular water exists in a different state from that of bulk water (2).

Second, consider the cytoplasm to consist of multiple aqueous phases, one of which is similar to bulk solution. Even if bulk water predominates within the cell, the overall signal and hence, D_s , will be dominated by contributions from phases with short relaxation times (1).

This example provides a striking demonstration that the new techniques cannot, by themselves, resolve the issue of water structure in cells. More importantly, it suggests why they fail - the data are impartial with respect to macroscopic models of cell water; nevertheless, such a model is required *a priori* for the interpretation of the data.

C. Definitions

I have defined the following terms in an effort to avoid the unwanted inferences that may be drawn from such terms as "bound water", "structured water", etc.:

A *normal* aqueous solution is one which is indistinguishable in its properties from a bulk aqueous solution of similar composition. A normal aqueous phase will be miscible with any other dilute aqueous solution. Solute activity coefficients and the osmotic coefficient in this phase will be the same as those of a bulk solution of similar composition.

An *abnormal* aqueous solution contains water with solvent properties different from those of bulk solution. If solutes dissolve in this abnormal phase, their activity coefficients will differ, in general, from those in bulk aqueous solutions. The osmotic coefficient and freezing point will also differ from values in the normal phase.

- Rocky Mountain Spotted Fever 109
Rotavirus 303
Rubella 540-544

S

- Salmonella 483, 484
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Sarcoid 624
Schistosomiasis 615
Sepsis 39, 50, 91, 271-289,
 393, 394, 485
Septicemia 39, 50, 91, 271-289
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Sporotrichosis 519
Staphylococcus aureus 7, 8, 18, 19, 25, 28,
 114, 132, 160, 184,
 254, 260, 362-372,
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Staphylococcus, coagulase-negative
 (epidermidis) 132, 257, 286
Streptococcus, general 417-428
Strep. faecalis 179, 264
Strep., Group A 7, 124
Strep., Group B 425, 426
Strep., Group C 231, 427
Strep. milleri 290, 428

the presence of surfaces may lead to an underlying simplicity of phase structure. The induction of long-lived, long-range "structures" in biological water may permit distinctions to be made between macroscopic *classes* of aqueous environments within the cell. Such a distinction is broad indeed, and does not address the specifics of local water structure. Nevertheless, it is precisely the kind of information that is needed for further progress in understanding the nature of cell water.

II. PHASE STRUCTURE AND THEORIES OF HOMEOSTASIS

One of the liveliest controversies in biology is centered on the mechanism of homeostasis -- how do cells maintain constant volume and relatively invariant ionic contents? I shall examine this issue in some detail, because it is another instance in which the basis for the dispute can be traced directly to the *a priori* assumption of cytoplasmic phase homogeneity. Furthermore, because homeostasis is a fundamental attribute of cells and organelles, it provides an appropriate and useful background for the further development of concepts relating to intracellular water.

A. *The Membrane Theory*

Cell water is "normal". That this view of cell water is so generally accepted today is due in large part to extensive studies on the red blood cell by Dick (8,9,10), Gary-Bobo (11) and Gary-Bobo and Solomon (12).

The osmotic behavior of the erythrocyte deviates in two respects from the idea that it is a bag containing a solution of hemoglobin and salts: (1) only 50-80% of red cell volume responds to changes in medium water activity. (2) This osmotically active fraction is a function of cell volume (8). Dick and Lowenstein (13) propose that these deviations are due primarily to concentration-dependent changes in the osmotic coefficient of hemoglobin. This interpretation is supported by studies on the behavior of the osmotic coefficient of highly concentrated hemoglobin solutions. When these results are applied to studies on intact erythrocytes, considering the cytoplasm to be a bulk hemoglobin solution, many of the osmotic discrepancies disappear. Furthermore, the remaining deviations can be attributed to volume-dependent solute shifts (12).

U

- Ulcerative colitis 111, 157, 158, 630
Ultrasound 295-297
Ureaplasma 327, 350, 392, 395
Urethritis 330-340
Urinary tract infection 5, 6, 97, 98, 309-329

V

- Vaginitis 4, 5, 361
Vancomycin 146, 201, 202, 256,
 380, 415
Varicella 558-561
Vibrios 389, 477-481
Viral chemotherapy 207A, 207B, 523, 528-
 530, 548-556
Viral disease, general 7, 8, 38, 59, 107-
 109, 302, 633

W

- Whipples Disease 626
Wound infections 9, 80-104, 359

Y

- Yersinia 488, 489

Z

- Zinc 42

C. Characteristics of a Homeostatic System

If, for simplicity, we limit consideration to systems which do not maintain temperature or pressure gradients at the cellular level, we may note two salient characteristics of homeostasis: (1) cells and organelles exhibit constancy of volume in their normal environment. Nevertheless, they are freely permeable to water and they contain large amounts of protein and other poorly exchangeable solutes; (2) cells and organelles maintain relatively constant levels of ions and other small solutes, and these levels may differ greatly from those of the surrounding medium. Nevertheless, there is no absolute permeability barrier to these small solutes, and radioisotope studies show that they exchange many times over during the life of the cell.

Recognition of these essential characteristics is common to both theories of homeostasis. In addition, both schools recognize that cells are in a state of partial equilibrium with respect to the processes of solution and of water transport. In effect, this constraint provides a bridge between the two theories, for it permits us to treat each individual phase as an equilibrium phase. The Gibbs equation is therefore valid in both models:

$$\begin{aligned}\Delta\mu = \Delta\mu^\theta &+ RT \ln (\gamma_i / \gamma_o) \\ &+ RT \ln (m_i / m_o)\end{aligned}\quad (1)$$

where $\Delta\mu$ is the partial molal free energy of solute transfer between two homogeneous phases at constant temperature and pressure; R is the gas constant; T is the temperature; $\Delta\mu^\theta$ is the difference in standard state free energies; γ is the molal activity coefficient; and m is the molal concentration of solute. Subscripts i and o refer to intracellular and extracellular phase, respectively. The sign of $\Delta\mu$ indicates the vectorial direction of irreversible flow of solute toward equilibrium and not whether the process is exergonic or endergonic. If $\Delta\mu \neq 0$, the disequilibrium steady state can only be maintained by being coupled to some other exergonic process at the phase boundary. If $\Delta\mu = 0$, the system is in a state of partial equilibrium with respect to the solute in question.

D. An Apparent Distinction Between the Two Models

A major property of homeostasis is the selective accumulation (or exclusion) of certain solutes by the cell. For

example, the cell:medium concentration ratio for glycine in ascites cells has been estimated to be 25:1 (23). Let us consider the origin of this ratio from the point of view of both theories of homeostasis.

The *membrane theory* considers the cytosol to be an aqueous solution similar in its solvent properties to the medium bathing the cells; therefore, the choice of standard states for glycine will be the same for the two phases, and $\Delta\mu^\theta = 0$. For the same reason the ratio, γ_i/γ_o , will be close to 1.0. Finally, if glycine is predominantly in solution within the cell, m_i is approximately equal to total cell glycine divided by cell water. Therefore, $\Delta\mu \approx RT \ln (m_i/m_o)$, and a driving force exists for glycine efflux of about 2.0 kcal/mole. In order to maintain high levels of internal glycine, active transport is required to balance the irreversible diffusion of glycine out of the cell.

The *cytoplasmic model* considers glycine to be at equilibrium. The observed concentration ratio may be explained in two ways: (1) in the absence of adsorption, the last term in equation (1) is 2.0 kcal/mole. We should now choose to be the free energy of transfer of a mole of solute from the bulk aqueous solution to the abnormal cytosol. Suppose, $\Delta\mu^\theta = -2.0$ kcal/mole, reflecting the preferential solubility³ of glycine in the abnormal phase. Then $\Delta\mu = 0$, and active transport is not required; (2) Neville (24) proposes that glycine is adsorbed to intracellular macromolecules. Internal concentrations, therefore, cannot be equated to total solute divided by cell water, and the last term in equation (1) does not necessarily differ from zero.

³"Solubility" is a loose term in this context, but the repetitive use of more precise terminology is cumbersome. The law of distribution of a solute between two phases states that the concentration ratio will be constant at a given temperature, irrespective of the amount of the solute. If this law were valid over the entire concentration range, including saturated solutions, then the distribution ratio would equal the ratio of solubilities of the solute in the two phases. The distribution law is only valid for dilute solutions, so this quantitative relationship rarely holds. Qualitatively, however, it is generally true that a solute will have a higher saturation solubility in the phase in which it normally has the higher concentration in the dilute range. It is in this sense that "preferential solubility" is used.

In the cytoplasmic theories, solute-water and solute-protein interactions are such that active transport at the phase boundary is not required. This is an important advantage, in the view of this school, for if all concentration gradients require energy for their maintenance, there may not be sufficient energy stores to supply this need. This is the "caloric catastrophe" (25; see also chapter by Ling in this volume; for rebuttal), which cytoplasmic theories avoid by holding intracellular solutes at or near equilibrium.

These considerations emphasize an important point: the theories of homeostasis are not merely associated with distinct views of cell water; rather they are logically connected to them. If cell water is abnormal, we are not justified in setting $\Delta\mu^\theta = 0$, and cannot rule out equilibrium distributions of solute. If cell water is normal, $\Delta\mu^\theta = 0$, and concentration gradients require energy input at the phase boundary.

This strongly suggests that the properties of cell water can be used to distinguish between the two theories. What properties distinguish between a normal and an abnormal aqueous solution in which the processes of water transport and solution have come to equilibrium with the environment? Osmotic response and the behavior of solute activity coefficients are two such properties which have been examined extensively in biological systems. The results form a very consistent pattern: cells never behave as perfect osmometers (9); and nonelectrolyte distribution coefficients differ from 1.0 and show marked solute-specific differences from each other (11,15,18).

Ling and Troshin have properly emphasized the fact that these results appear inconsistent with the idea that the cytosol is a normal aqueous solution. Are we to conclude, then, that the membrane theory is invalid? Not necessarily. The view that *all* of cell water (less a small amount of water of hydration) must be either normal or abnormal is unnecessarily restrictive, as will be demonstrated in the next section.

E. *The Conditions of Validity for the Two Theories of Homeostasis*

The unnecessary restriction is the unwarranted assumption, made by both schools of homeostasis, that the in-

ternal milieu is homogeneous. When this restriction is removed, we may state the following:

It is sufficient, for the membrane theory, to demonstrate that some fraction of the cell is a normal aqueous phase different in composition from that of the extracellular phase. This follows immediately from the application of equation (1) to a normal internal phase in which $m_i \neq m_o$ for any exchangeable solute.

It is necessary, for the cytoplasmic theory, to demonstrate that no fraction of the cell is a normal aqueous phase different in composition from the extracellular phase. In addition, it remains necessary to demonstrate that all solutes are at equilibrium. That is, the finding that all cell water is abnormal does not by itself preclude the non-equilibrium distribution of solutes in the homeostatic state.

These conditions are rigorously correct, but essentially useless, since they contain specifications that are experimentally inaccessible. We must transform the specification of phase composition into one of phase extent, which may be experimentally accessible. To accomplish this, we consider the necessary condition of the cytoplasmic theory: what composition of a *normal* phase is consistent with an *equilibrium* distribution of solutes?

Macromolecules must be excluded, else there would be a gradient of water activity and swelling, violating homeostasis. In the absence of macromolecules and other poorly permeable solutes, there can be no Donnan potential. Therefore, a normal internal phase at equilibrium with the extracellular fluid must be approximately identical to the extracellular fluid. This imposes severe limits to the size of the hypothetical normal phase, since the volume of distribution of sodium, for example, is as low as 10-20% of cell water in most mammalian cells (27). Therefore, the maximum normal phase consistent with an equilibrium distribution of ions and solutes is 10-20% of cell water.

We have now established experimentally useful criteria for distinguishing between the two theories of homeostasis:

To establish the validity of the membrane theory, it is sufficient to demonstrate the existence of a normal internal aqueous phase comprising a significant (> 20%) portion of the cytosol.

To establish the validity of the cytoplasmic theory, it is necessary, but not sufficient, to demonstrate that a major portion ($> 80\%$) of the cytoplasm is an abnormal aqueous phase.

The problem has now been reduced to one of examining cells for evidence of bulk solution behavior with the important stipulation that we do not assume cytoplasmic homogeneity in advance. Once again, a major biological controversy can be resolved into the fundamental questions set forth at the beginning of this paper: (1) is the aqueous milieu homogeneous or heterogeneous; and (2) how much normal water exists within a given membrane-bounded compartment? I shall now turn to an experimental evaluation of these questions.

III. EXPERIMENTAL APPROACH

A. *The Mitochondrion as an Experimental Model*

It is extremely difficult, even with a concerted experimental approach, to prove the existence of a normal aqueous phase in whole cells. Mitochondria offer many advantages for such studies: (1) they are structurally simple, with a single osmotically active compartment, referred to as the *matrix*. This is in contrast to nucleated cells, which contain numerous intracellular compartments which are themselves surrounded by membranes. The mitochondrial matrix is enclosed by an extensively folded membrane, the *inner membrane*. This, in turn, is enclosed by an *outer membrane*, which is permeable to all but very large molecules (28); (2) the inner membrane is poorly permeable to all hydrophilic solutes, including ions and polar molecules such as sucrose. It is highly permeable to hydrophobic solutes and to small polar solutes (29); (3) the endogeneous ionic contents of mitochondria are independent of volume, under controlled experimental conditions. In contrast, ion movements are frequent concomitants of volume change in cells; (4) mitochondria can undergo very large amplitude swelling without rupture of the inner membrane. This has been demonstrated by electron microscopy and appears to be a consequence of the extensive in-folding of the membrane (30); (5) mitochondria, like cells, behave as imperfect osmometers (31) and exhibit a marked, energy-independent selectivity for nonelectrolytes (32); (6) mitochondria provide an appropriate model for considering the dispute over mechanisms of homeostasis; some 20 membrane carriers have been postulated for mitochondria, and the process of energy coupling itself is considered to be a manifestation of specialized ion transport mechanisms (33).

B. Experimental Methods

Rat liver mitochondria were isolated by standard procedures (34). They were added to 5 to 10 mls of incubation media containing sucrose, nonelectrolyte and an impermeant buffer (pH 7), giving a final concentration of 10-20 mg protein/ml. Parallel tubes contained the isotope pairs $^3\text{H}_2\text{O}/^{14}\text{C}$ -sucrose and ^3H -sucrose/ ^{14}C -nonelectrolyte. These non-electrolytes were studied: ethanol, glycerol, urea, antipyrine and dimethylsulfoxide (DMSO). Incubations were carried out at 0° to 2°C for sufficient time to assure an equilibrium distribution of the nonelectrolyte probe, usually 10 to 15 minutes. Pellets were separated by centrifugation; pellet wet weights were obtained; and pellet extracts and media were analyzed for K, Mg and radioactivity. Media densities and freezing point depressions were measured. Pellet wet weights were 0.2 to 0.4 gm, and dry weights were 50 to 100 mg. Pellets therefore contained on the order of 10¹² mitochondria (35). Highly reproducible results were obtained by means of rigorous sample handling methods, which will be presented in detail in other reports.

C. Mitochondrial Compartmentation

We define the total "volume" of distribution, W_{xp} , of solute X as the ratio of amount in the pellet, X_p (moles/kg dry wt), to molal concentration in the medium, m_{xo} (moles/kg water):

$$W_{xp} = X_p / m_{xo} \quad (2)$$

It is found, experimentally, that the volume of distribution of tritiated water is equal to the difference between wet and dry weights. Therefore, total pellet water may be estimated by either method. In addition, there is substantial evidence that sucrose penetrates the matrix space very slowly under the conditions of these experiments (36). Hence, the volumes of distribution of radioactivity labeled sucrose and water may be used to determine the amount of matrix water, W_m :

$$W_m = W_{\text{H}_2\text{O}} - W_{\text{suc}} \quad (3)$$

Under the assumption that the composition of the sucrose space is identical to that of the medium, we may correct for solute trapped in the sucrose space:

$$X_m = X_p - W_{suc} m_{xo} \quad (4)$$

where X_m is the net amount of X in the matrix (moles/kg dry wt.).

IV. NON-ELECTROLYTE DISTRIBUTIONS IN MITOCHONDRIA: PRELIMINARY INTERPRETATION

A. Evidence for Aqueous Phase Distribution

In the 1930's there was considerable controversy over the extent of normal water in cells. In a review, Blanchard (37) 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 volume of distribution and, if all water is normal, their concentration ratios should be close to 1.0.

These expectations are strikingly unfulfilled in mitochondria (Fig. 1), where volumes of distribution range from 67% of matrix water for DMSO to 150% for antipyrine. Before we attribute these findings to properties of the aqueous phase of mitochondria, it is necessary to rule out other explanations.

1. *Radioactive Contaminants.* This technical source of error deserves special mention, because it can lead to large and *reproducible* errors. Furthermore, while most workers use radioactive probes as received from the supplier, it is essential to recognize that commercial purity (95-99%) is inadequate for this type of experiment. Contaminants will be added in vanishingly small amounts, and their tendency to adsorb to biological tissue will lead to erroneously high volumes of distribution which are unaffected by varying the concentration of the putative solute. This phenomenon has been exhibited by most of the probes used in these experiments. For this reason, all radioactive probes except ethanol were purified by chromatography prior to use. Contamination was considered to be absent when the volumes of distribution in mitochondria of different isotopes of the same compound agreed to within 0.5%. These test procedures were carried out using compounds labelled with different isotopes (^3H vs. ^{14}C); labelled at different positions; obtained at different specific activities; and obtained from different manufacturers. This laborious, but essential, procedure

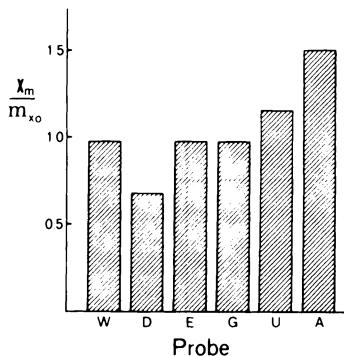


FIGURE 1. Variation of equilibrium distributions of nonelectrolytes. Mitochondrial suspensions were incubated for 15 min at 0°C in the presence of radioactively labelled probes. W = THO; D = dimethylsulfoxide; E = ethanol; G = glycerol; U = urea; A = antipyrine.

permits us to rule out artifacts due to radioactive contamination.

2. *Metabolism.* Mitochondria respire very slowly at 0°C, and this respiration (using trapped endogenous substrates) is fully blocked by the inhibitor, rotenone. Rotenone has no effect on the volumes of distributions of these probes. Furthermore, distributions do not exhibit saturation (next section) as would be expected of metabolically dependent phenomena.

3. *Adsorption.* If surface and/or site specific adsorption were responsible for elevated distributions, then these distributions should be concentration dependent, due to progressive saturation of adsorption sites. Adsorption can be eliminated as a cause for these findings, since the volumes of distribution are independent of solute concentrations from 10^{-4} M to 0.5 M (see Figures 2 and 3).

4. *The Membrane Phase.* These probes are polar non-electrolytes with low oil:water partition coefficients. It

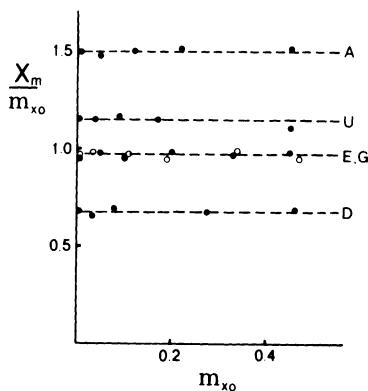


FIGURE 2. Effect of solute concentration on equilibrium distribution of nonelectrolytes. Conditions and symbols are as described in the legend to Figure 1.

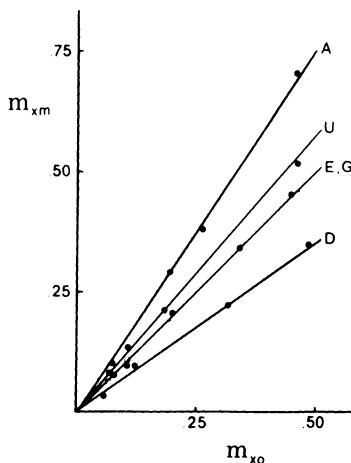


FIGURE 3. Total matrix concentration *vs.* medium concentration for various nonelectrolytes. Conditions and symbols are as described in the legend to Figure 1. Matrix water was held constant at 1.0 ± 0.05 g/g dry wt.

is highly unlikely that urea and antipyrine are selectively dissolved in the membrane, while glycerol and ethanol are excluded. Furthermore, this explanation cannot account for the DMSO results.

It is concluded, therefore, that the distributions of these probes refer to the aqueous phases of mitochondria.

B. Evidence for Phase Heterogeneity in Mitochondria

In Figure 3 are presented the results of experiments in which medium concentration was varied, while matrix volume was held constant. The distribution coefficient (slope) of each solute is independent of concentration up to 0.5 M, the highest level measured. However, the coefficients are solute-specific. The expression for the distribution of a non-adsorbing solute is simply

$$m_{xm} = Qm_{xo} \quad (5)$$

This is the relationship used by Ling (18) and Troshin (15) and the results in Figure 3 are qualitatively similar to their studies on various gels, tissues and cells.

What do these results tell us about biological water? If W_m is a single phase, then Q is a true partition coefficient and W_m evidently has solvent properties very different from those of the aqueous medium. This is, in fact, the conclusion drawn by Ling and Troshin. Before accepting this conclusion, however, note that m_{xm} (matrix "concentration" of X) is a derived, rather than measured, quantity:

$$m_{xm} = X_m/W_m \quad (6)$$

There is no question but that m_{xm} , thus defined, is a "concentration" in the general sense of the term. However, it is a concentration in the thermodynamic sense if, and only if, W_m constitutes a single phase. It is more desirable to study the relationships between measured quantities, such as can be obtained by combining equations (5) and (6):

$$W_{xm} \equiv X_m/m_{xo} = QW_m \quad (7)$$

Equation (7) relates the volume of distribution to matrix water content, which may be independently varied. This relationship has the advantage over equation (5) that it does not depend on the assumption of matrix homogeneity. In fact,

equation (7) appears to provide a direct test of that assumption. If there is a single phase, the slopes of curves plotted according to equation (7) should be identical to those plotted according to equation (5).

The results plotted in Figure 4 demonstrate conclusively that this is not the case. In contrast to the expectations of the Ling-Troshin model, the slopes of Figure 4 differ from those of Figure 3. Furthermore, the various solute distributions fall on parallel curves, and their common slope is approximately 1.0.

The simplest conclusion to draw from this result 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 Figure 3 vs. Figure 4.

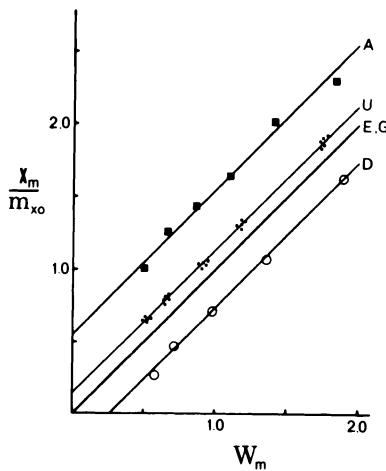


FIGURE 4. Nonelectrolyte distributions as a function of matrix water content. The symbols and conditions are as described in the legend to Figure 1. Data points for ethanol and glycerol have been omitted for clarity. Multiple points for urea represent data obtained at different urea concentrations ranging from 10^{-4} to 0.5 M. All other probe concentrations were .05 M. Matrix water (W_m) 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.

C. Tautology in the Ling-Troshin Analysis

How do Ling and Troshin reach the conclusion that non-electrolyte distributions such as those illustrated in Figure 3 are a consequence of the abnormality of intracellular water? Let us grant that it is adequate for this purpose to show that the thermodynamic partition coefficient, f , is significantly different from 1.0. At equilibrium, the partition coefficient between internal matrix and the medium may be defined as follows:

$$f \equiv \gamma_{xo}/\gamma_{xm} = m_{xm}/m_{xo} \quad (8)$$

where the symbols are as previously defined (see also Appendix A). The first equality is an identity, while the second is a consequence of the equilibrium of solute between phase o and m (equation [1]).

By experiment, it is found that a plot of the ratio X_m/W_m vs. m_{xo} has a slope different from 1.0. It is evident from equation (8) that if it could be shown that m is a single phase, then $m_{xm} = X_m/W_m$ and $f \neq 1.0$. But Ling and Troshin make the prior assumption that there is only one phase (equation [6]). This unwarranted (and unstated) assumption of phase homogeneity leads inescapably to the conclusion that f is different from 1.0; i.e., their argument is tautologous.

Let us examine a more complex phase structure to see why equation (5) is insensitive to heterogeneous phase structure. Suppose that there are two internal phases, W_1 and W_2 , and that solutes partition into both phases with phase-specific partition coefficients. For total matrix solute,

$$X_m = W_1 m_{x1} + W_2 m_{x2} \quad (9)$$

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

$$X_m = (f_1 W_1 + f_2 W_2)m_{xo} \quad (10)$$

By comparison with equation (5), we observe that Q is, in general, a weighted sum of partition coefficients:

$$Q = (f_1 W_1 + f_2 W_2)/W_m \quad (11)$$

Since W_m is held constant in the experiments of Figure 3, the compartment size parameters (W_1 , W_2 , W_m) will remain

constant. Furthermore, the partition coefficients, f , will remain constant within the range of validity of Henry's Law. Under these conditions, a constant slope will be obtained from Figure 3, even if Q is not a true partition coefficient. That is, Q is insensitive to phase heterogeneity when determined at constant volume.

D. Normal and Abnormal Water in Mitochondria

The general expression for solute distribution is

$$X_m/m_{x_0} = \sum f_j W_j \quad (12)$$

where

$$\sum W_j = W_m \quad (13)$$

At first glance this appears to be intractable due to the multitude of possible phases in a general system. The best we can do is to assign each phase to one of two hypothetical classes: phases of variable water content (designated by subscript 1) and phases of constant water content (designated by subscript 2).

$$W_2 \equiv \sum W_{2j} = \text{constant} \quad (14)$$

and

$$W_1 \equiv \sum W_{1j} = W_m - W_2 \quad (15)$$

Note that W_2 represents water in a collection of osmotically inactive phases. This is true by definition, since W_2 has been defined as constant; i.e., independent of media water activity. Such a compartment may represent, for example, multi-layered adsorption into membrane surfaces (38) which is already saturated at low vapor pressures and hence is unaffected by further increases of water activity. If such a water compartment does not exist, then experiments must be consistent with $W_2 = 0$.

The possible existence of such "bound" water has frequently been admitted and discussed (5,9,10,37). It has been almost universally assumed, however, that such "bound" water must simultaneously be non-solvent. In contrast, multilayered adsorption admits the possibility of phase properties -- that is, the water layers may be sufficiently numerous (> 5 , for example) to permit such a phase to have

solvent properties. Thus, two weighted partition coefficients may be defined:

$$f_1 \equiv \sum f_{1j} W_{1j} / W_1 \quad (16)$$

and

$$f_2 \equiv \sum f_{2j} W_{2j} / W_2 \quad (17)$$

The degree of complexity may or may not permit us to attribute significance to these weighted terms in f and W . These definitions represent what we are *capable* of knowing about the compartmentation of water and solute. For example, consider a system consisting entirely of two "normal" aqueous phases (separated, perhaps by an internal membrane) with their associated parameters, f_{11} , f_{12} , W_{11} , W_{12} . Both phases are osmotically active; however, we can only sample from the total compartment, $W_1 \equiv W_{11} + W_{12}$. It is a simple matter to show that observations relating to equilibrium distributions of solutes and water cannot distinguish this two-phase system from a single phase exhibiting the same properties. The thermodynamic coefficients will simply be assigned different values. It is in this sense that the separation into two phases, W_1 and W_2 , as defined in equations (14) and (15) accounts for all phase types which can be distinguished experimentally.

Inserting these definitions into equation (12),

$$X_m/m_{x0} = f_1 W_m + (f_2 - f_1) W_2 \quad (18)$$

E. Two-Phase Interpretation of Nonelectrolyte Distributions

In Figure 4 are plotted data for several solutes, using the parameters of equation (18).

1. We note that equations (14) and (15) do not preclude the possibility of water shifts between the two classes, W_1 and W_2 , over the range of W_m studied. However, the fact that the data conform to the linear relationship of equation (18) suggests that such shifts do not occur.

2. The validity of the linear relationship is further strengthened by the fact that the results are independent of the means used to vary W_m . W_m may be changed by experimental alterations in any of three independent parameters: internal

solute content, medium water activity and medium concentration of impermeant solute. Solute distributions are dependent on W_m and independent of the parameter or parameters used to achieve a given matrix volume.

3. $f_1 = 1.00 \pm 0.02$ for DMSO, urea, glycerol, ethanol and antipyrine. f_1 remains constant over a 5-fold range of matrix water content and is independent of probe concentrations; i.e., Henry's Law is obeyed. Phase 1 may therefore be considered to be a normal phase indistinguishable in its solvent properties from the medium. There may indeed be variations of f_{1j} 's in regions of this phase, but such variations may also occur in bulk solutions. Therefore, we are led to conclude that W_1 represents a single normal aqueous phase indistinguishable from a bulk solution and that f_1 represents a true partition coefficient between this phase and the medium.

4. DMSO, antipyrine and urea have non-zero intercepts. From equation (18) it may be concluded that W_2 is different from zero. That is, mitochondria contain a fraction of osmotically inactive water. It is noteworthy that this finding is deduced without consideration of medium osmolality or osmotic swelling curves.

5. From the non-zero intercepts, we may also conclude that $f_2 \neq f_1$ for urea, DMSO and antipyrine. Like f_1 , the f_2 's are independent of concentration, so solutes in phase 2 also obey Henry's Law.

6. Unlike f_1 , the f_2 's are solute specific. This behavior, together with osmotic inactivity, characterizes phase 2 as an abnormal aqueous phase. The question whether W_2 represents a single homogeneous phase or a collection of abnormal phases cannot be resolved by these results. In the case of W_1 , we had a model (bulk solution) and a comparative measure (partition coefficients close to 1.0) which led us to conclude that W_1 is homogeneous in the sense that bulk solution is homogeneous. In the case of W_2 , it may also be possible to find an analogous model (e.g., certain gels) which exhibit similar solute specificities.

7. The quantitative distribution of water between these normal and abnormal phases cannot be resolved by non-electrolyte probes; however, limits can be deduced from Figure 4. DMSO is evidently excluded to a considerable extent from W_2 . If it were completely excluded ($f_2 = 0$) W_2 would equal the negative intercept of the DMSO curve, since

$f_1 = 1$. This value is 0.27 g H₂O/g dry wt., representing the *minimum* value of W_2 . This is very close indeed to the estimate of W_2 from osmotic studies, using the two-phase model (next section). This agreement suggests that DMSO may represent, in mitochondria at least, the ideal probe of "normal" water proposed by Blanchard nearly 40 years ago.

V. OSMOTIC SWELLING IN MITOCHONDRIA: PRELIMINARY INTERPRETATION

A. Osmotic Swelling Curves

The relationship between the water content of an osmotically active phase and the inverse medium osmolality is derived in Appendix B, equation (B13):

$$W_1 = \frac{\phi_s S_1}{\phi_{emo}} \quad (19)$$

This relationship will be linear, provided $\phi_s S_1$ is independent of volume.

Curve 1 of Figure 5 is a plot of matrix water content, W_m , vs. inverse osmolality. The relationship is linear, with a positive intercept. This finding is characteristic of most cells and organelles.

Since total internal solutes remained constant in these experiments, the interpretation of these results must rest entirely on the value of ϕ_s , the osmotic coefficient of the internal phase. Thus, it may be inferred either (1) ϕ_s is approximately constant, and the intercept measures an osmotically inactive phase within the mitochondrial matrix, or (2) ϕ_s is highly volume-dependent in a single aqueous phase, leading artifactually to a non-zero intercept. These possibilities will be considered more rigorously in subsequent sections. In this section, I will continue to use the two-phase model as a framework within which to present the data.

In the two-phase model, the intercept is considered to reflect osmotically inactive water. The constant slope, together with the constancy of S_1 , implies that ϕ_s is relatively constant. Equation (19) may be rewritten,

$$W_m = W_2 + \frac{\phi_s S_1}{\phi_{emo}} \quad (20)$$

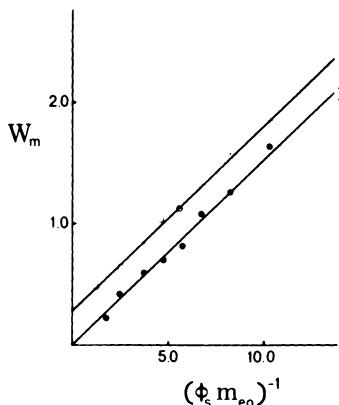


FIGURE 5. Matrix water content vs. inverse osmolality. Osmotic swelling of 0°C was effected by varying medium sucrose plus buffer concentration. Curve I represents total matrix water as described by Equation (3). The intercept of curve I is 0.28 g H₂O/g dry wt; the slope is 0.15 osmoles/kg dry wt. Curve II represents DMSO-accessible matrix water. The slope is the same as curve I, and the intercept is 0.01 g H₂O/g dry wt.

This equation is in accord with the previous interpretation of nonelectrolyte distribution studies (Section IV-E); that is, there exists an osmotically inactive phase, W_2 .

Furthermore, if DMSO is excluded from phase 2, then W_1 should approximately equal W_{DMSO} (see equation [18]). Curve II of Figure 5 is a plot of W_{DMSO} versus $1/\phi_e m_{eo}$. Trace amounts of DMSO were used to assure negligible effects on internal solute content. Curve II very nearly obeys equation (19) in that the intercept is 0.01 ± 0.02 g H₂O/g dry wt. Furthermore, the slope is identical to curve I, as expected if both ϕ_s and S_1 remained constant over the volume range.

The implication of these results is that matrix non-electrolyte, 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.

B. The Effect of Nonelectrolytes on Matrix Volume

Until now, we have looked at volume response and solute distribution independently. What does the two-phase model predict relative to the effect of added internal solute on volume?

We begin with the familiar observation that the equilibrium volume of cells and organelles is relatively unaffected by the presence of permeant solutes. Consider, for example, the transfer of mitochondria from isotonic sucrose to an identical medium to which has been added 0.5 M urea. There will be a transient shrinking on exposure to the hypertonic solution, followed by swelling as urea and water enter the matrix. After some minutes, an equilibrium water content is achieved which is very close to the initial value. This phenomenon is observed with all the nonelectrolytes studied in mitochondria.

At equilibrium, X_1 moles of nonelectrolyte will have entered the matrix, and equation (20) may be written:

$$W_m = W_2 + \frac{\phi_{sx}(S_1 + X_1)}{\phi_{ex}(m_{eo} + m_{xo})} \quad (21)$$

It is necessary to expand the osmotic coefficients. The ternary system, urea-sucrose-water has been studied (40). From their data, it is possible to estimate that interaction terms in the dilute solutions used in the present study are small, amounting to a 2% error in osmolality in 0.5 M urea and 0.3 M sucrose (see Appendix B). Neglecting this interaction term (it will be considered in a later section), an additivity approximation may be used for the term in the denominator:

$$\phi_{ex}(m_{eo} + m_{xo}) \approx \phi_e m_{eo} + \phi_x m_{xo} \quad (22)$$

The partition coefficient, f_1 , is taken equal to 1.0 in the two-phase model. From this, it follows that the interaction term between matrix solutes, S_1 , and nonelectrolyte, X_1 , must also be small, leading to

$$\phi_{xs}(S_1 + X_1) = \phi_s S_1 + \phi_x X_1 \quad (23)$$

If $f_1 = 1.0$, $m_{x1} = m_{xo}$, and equation (21) becomes

$$W_m = W_2 + \frac{\phi_s S_1}{\phi_e m_{eo}} \quad (24)$$

Thus, matrix water in the presence of nonelectrolyte depends solely on the concentration of impermeable solute in the medium, m_{eo} . Water content is unaffected by permeant non-electrolyte in the two-phase model, in accordance with the experimental findings.

C. Other Modifiers of Matrix Volume

The major endogenous solutes of freshly isolated mitochondria are potassium salts of phosphate, citrate and other organic anions (41). Experimentally, it is a simple matter to alter the amount of K^+ and the amount and type of anion in respiring mitochondria. However, it is very difficult to maintain constant amounts of endogenous solutes through subsequent volume changes. We have recently presented evidence for a Mg^{++} controlled K^+/H^+ exchange carrier in the inner membrane (42,43). The role of this carrier is to prevent massive swelling associated with diffusive K^+ uptake, caused by the respiration-driven negative membrane potential. The role of the Mg^{++} inhibition of the carrier is to prevent the loss of excessive K^+ and consequent collapse of the pH gradient. By manipulation of this mechanism, we have succeeded in preparing mitochondria containing widely varying amounts of K^+ salts which do not lose these solutes on swelling. Preliminary swelling data are presented schematically in Figure 6.

Curves I and II represent high and low K^+ mitochondria, respectively. The difference in slopes is found to correspond to the difference in solute content. Note that the intercepts of the two curves are identical.

Curves II and III represent pellets with similar potassium contents; however, the mitochondria represented in curve III were treated with 2×10^{-5} M CCP (carbonylcyanide phenylhydrazone). Note that the slopes are now similar, while CCP has caused the intercept to drop from 0.28 to 0.19. CCP is a very hydrophobic weak acid which localizes in the mitochondrial membrane. It uncouples oxidative phosphorylation by virtue of its ability to act as a hydrogen ion carrier (44).

The interpretation of these findings is as follows: added salts are localized primarily in the normal aqueous phase. Increasing the salt content of the normal phase, therefore, has no effect on the size of the abnormal phase (no change in intercept), but rather is reflected in an increase in S_1 (increased slope). Conversely, CCP is localized in the membrane and, therefore, does not affect the normal phase. We postulate that the character of abnormal water is determined largely by the membrane surface in mitochondria (32). In the presence of CCP, the extent of the abnormal phase is reduced due to the presence of the drug in the membrane. The mechanism by which this effect is mediated is not known.

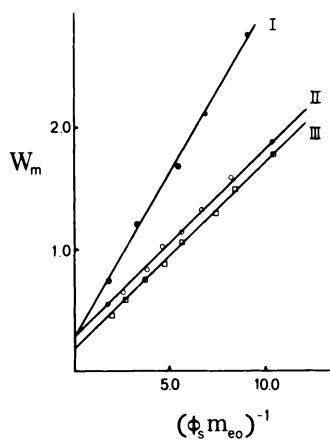


FIGURE 6. Modulators of osmotic swelling curves in mitochondria. Experiments were carried out under the same conditions as those of Figure 5. Curve I represents mitochondria which were pre-incubated at 25°C in the presence of substrate, O₂ and K⁺. Prior to swelling exposure, this preparation was treated with rotenone and subjected to a 5 min 0°C wash in K⁺-malate to block the K/H carrier (43). Following a large volume wash in isotonic sucrose, they were subjected to media of varying osmolality for 3 min. The final K⁺ content was 165 μmoles/g dry wt. Curve II represents mitochondria that lost K⁺ during a pre-incubation in hypotonic sucrose (0.08 M). Following this procedure, the K/H carrier is blocked, and no further K⁺-loss occurs on subsequent swelling (42). The final K⁺ content of this preparation was 80 μmoles/g dry wt. The mitochondria in curve III were prepared identically to those of curve II. Immediately after aliquots of stock suspension were added to swelling tubes, 2 × 10⁻⁵ M CCP was added to each tube. The final K⁺ content of this preparation was 77 μmoles/g dry wt.

If this interpretation is correct, then the distributions of urea and DMSO should be unaffected by changes in K⁺ content, at a given matrix volume. Conversely, the volume of distribution of urea should decrease, and that of DMSO should increase, in the presence of CCP due to the decrease in W₂. All of these expectations have been confirmed experimentally, in support of the two-phase model.

We have also found conditions in which mitochondrial K⁺ can be entirely replaced by tetraethylammonium cation. Since

the latter cannot exit on the cation carriers, we expect to be able to study the properties of these mitochondria without volume-dependent solute shifts.

These findings represent experiments in progress. It is hoped that they will contribute to a further understanding of the solution structure of mitochondria.

D. Hydration of Proteins and Membranes

It is self-evident that a perfectly dehydrated system must have a water content of zero. Therefore, the osmotic swelling curve (Figure 5) must break down toward the origin as external osmolality approaches infinity (dehydration). The point at which the curve deviates from apparent linearity is of considerable importance. If this deviation were shown to occur in the dilute solution range, one could argue that the linear osmotic curve is an artifact of the varying osmotic coefficient. On the other hand, if the osmotic swelling curve could be shown to remain linear down to very high osmolalities, this would support the two-phase concept that macromolecules become saturated at water activities much lower than those of dilute solutions.

In support of the two-phase model, it is the latter finding that dominates hydration studies of proteins and membranes. Ingenious studies on hemoglobin crystals suggest the presence of "bound" water at low water activities (45). The quantitative relationships of this fraction to the osmotic intercept in red cells has also been demonstrated (46). Thus, the value for "bound" water in hemoglobin suggests that the linear part of the osmotic curve extends very close to the y-axis ($1/\phi_{\text{emo}} \rightarrow 0$).

This attribute is also seen in hydration studies of red cell membrane preparations (38). These authors fit isopiestic adsorption data to the Brunauer-Emmett-Teller isotherm and, by extrapolation, estimated the saturation value of hydration to be about 0.70 g H₂O/g dry membrane. Using their values for membrane surface area, and taking the density of this water to be 1.0, this works out to a 50 Å saturation layer of water. This estimate is of the same order as that of the thickness of abnormal water associated with the mitochondrial membrane (32). Of equal interest is the fact that the membrane is already 30% hydrated when in isopiestic equilibrium with saturated BaCl₂. The equivalent inverse

osmolality of such a system is about 0.16, a very low value on the x-axis of plots such as those of Figure 5.

In summary, it appears from these types of studies that water associates with surfaces of proteins and membranes, leading to rapid saturation at low vapor pressures (high osmolalities). Further increase in water activity, in the dilute solution ranges used in whole cell/organelle studies, have little further effect on this surface phase. These findings support the two-phase model of biological water.

VI. APPLICATION OF THERMODYNAMICS TO BIOLOGICAL WATER STRUCTURE

Any model of phase structure in any biological system must be consistent with thermodynamics. It does not follow, however, that thermodynamic measurements can distinguish between these models. This derives from the fact that thermodynamic parameters (activity coefficients, osmotic coefficients, Setchenow coefficients) are *assigned* on the basis of the model. That is, in our ignorance of the phase structure, these coefficients must remain essentially phenomenological -- derived from experimental data treated according to a particular model. (One of the difficulties in using thermodynamics to establish the validity of a particular phase model was illustrated in Section IV-C).

Despite these difficulties, a thermodynamic treatment of aqueous solution behavior in biological systems is of considerable value. Inconsistencies of a particular model may show up. Certain measurements may be suggested which are capable of distinguishing between models. Features which previously appeared to satisfy only one model may prove to be compatible with several models. Finally, such an evaluation may prove useful in bringing out the essentially extra-thermodynamic assumptions that are used in *all* treatments of biological water.

Three models of intramitochondrial phase structure may now be distinguished: *normal* (9,12), *abnormal* (15,16) and *heterogeneous* - 70% *normal* and 30% *abnormal*, as described in this chapter.

The data on mitochondrial solution behavior which have been presented in the preceding sections will now be evaluated according to the thermodynamics of multi-component solutions. The basic expressions are derived in Appendix B.

VII. THE INTERNAL OSMOTIC COEFFICIENT: THERMODYNAMIC TREATMENT

A. Normal Phase Model

In this model, the osmotic coefficient is postulated to be dominated by the osmotic coefficients of internal macromolecules, which, in turn, are highly concentration dependent. Thus, the internal osmotic coefficient, ϕ_s , will increase as mitochondria are contracted. Dick (10) has argued strenuously in favor of this interpretation. The expression for matrix water in a single phase model is, once again,

$$W_m \equiv W_1 = \frac{\phi_s S_1}{\phi_e m_{eo}} \quad (25)$$

Over the entire experimental range, W_m obeys the following empirical equation, as previously noted (Figure 5):

$$W_m = a + \frac{b}{\phi_e m_{eo}} \quad (26)$$

Combining these two expressions gives the equation for ϕ_s :

$$\phi_s = \frac{b W_m}{S_1 (W_m - a)} \quad (27)$$

It is evident from Figure 5 that ϕ_s must, in the one-phase model, deviate from this expression as W_m approaches a . At higher dilutions, however, there is no reason to expect equation (27) to take a different form. Therefore, b must equal S_1 , since

$$\lim_{(W_m \rightarrow \infty)} \phi_s = 1 = \frac{b}{S_1} \quad (28)$$

Therefore,

$$\phi_s = \frac{W_m}{W_m - a} \quad (29)$$

Osmotic coefficients are normally expressed as functions of concentration, and this is readily achieved by expanding equation (29):

$$\phi_s = 1 + \kappa m_{s1} + \kappa^2 m_{s1}^2 + \kappa^3 m_{s1}^3 + \dots \quad (30)$$

where

$$\kappa \equiv \frac{a}{S_1} \quad (31)$$

The value of κ in the experiment of Figure 5 is 1.7. The value of the osmotic coefficient, calculated according to equation (29), ranges from 2.0 (0.6 osmolal) to 1.1 (0.08 osmolal). In high K^+ experiments (curve I of Figure 6), the corresponding values for ϕ_s range from 1.76 to 1.1.

This analysis has several consequences: (a) it permits the identification of the experimental slope, b , with internal solute content, S_1 ; (b) it provides a useful expression for ϕ_s in terms of experimental quantities; (c) it illustrates, via equation (30), the extremely non-linear behavior of the osmotic coefficient in the single phase model, even in dilute solutions; (d) it illustrates the purely phenomenological nature of ϕ_s : its value is determined by experiment assuming the internal phase to be a homogeneous solution.

B. Heterogeneous Phase Model

Following an analogous treatment for a two-phase system in which $W_m = W_1 + W_2$,

$$W_1 = \frac{\phi_s S_1}{\phi_{eo}} \quad (32)$$

$$\phi_s = \frac{b W_1}{S_1 (W_1 - a')} \quad (33)$$

where

$$a' \equiv a - W_2 \quad (34)$$

Note that it was not assumed here that $W_2 = a$. There are three possibilities for a' : (1) $W_2 < a$, so that $a' > 0$. In this case, $b = S_1$, using the same arguments as before. (2) $W_2 > a$, so that $a' < 0$. In this case, the requirement that $W_2 < W_m$ is sufficient to assure that $-a'/W_1 < 1$, and once again, $b = S_1$. These two possibilities permit a simple expression for ϕ_s , entirely analogous to equation (29):

$$\phi_s = \frac{W_1}{W_m - a} \quad (35)$$

(3) If $W_2 = a$, then $b = \phi_s S_1$ and ϕ_s must be approximately constant, as if, for example, phase 1 were a salt solution. We may assume for most purposes that a constant $\phi_s \approx 1.0$ and equation (35) is adequate to cover this special case as well.

C. Abnormal Phase Model

The current status of this school's treatment of water equilibrium in cells, together with an excellent historical review of hydration theories and experiments, is given by Ling (5). He emphasizes the finding that cells and non-biological polymers exhibit similar adsorption isotherms. Whatever adsorption theory is used to obtain parameters from these results, the parameters will necessarily be phenomenological.

It is interesting that there is a very steep rise in "adsorbed water" above relative vapor pressures of 0.8, and Ling suggests that this represents cooperative adsorption as polarized multilayers. An alternative explanation is that this region represents formation of "normal" bulk phase water after the surfaces have been saturated with several layers of water; i.e., that the steep rise is equivalent to the linear portion of the osmotic swelling curve.

D. Interpretation of Osmotic Swelling Data

In effect, equation (35) is a general expression for the osmotic coefficient in all three models. The quantitative value of ϕ_s depends on the extent of phase 1, the phase in osmotic equilibrium with the medium. Thus, ϕ_s is seen as a phenomenological quantity dependent on the model chosen to represent the data. Evidently, therefore, such data cannot determine which model is valid. Data supporting, but not establishing, the heterogeneous phase model have already been discussed in previous sections.

VIII. NONELECTROLYTE DISTRIBUTION COEFFICIENTS: THERMODYNAMIC TREATMENT

A. Normal Phase Model

1. *Solute Partition Coefficient.* Consider phase 1 to

be a normal aqueous phase containing m_{s1} moles/kg of total solute and separated by a semipermeable membrane from aqueous phase 0 containing m_{eo} moles/kg of total solute. Solutes s and e cannot cross the membrane; however the system is in osmotic equilibrium. Now add a permeant non-electrolyte, X, to the system and allow it to come to equilibrium across the membrane. To complete the analogy with mitochondria, the number of moles of S_1 remain constant, and osmotic equilibrium is maintained by means of shifts of water and nonelectrolyte. The expressions for the partition coefficient, f_1 , and the activity coefficients of X in the two phases may be obtained from equations (B5), (B10) and (B15):

$$f_1 = \frac{m_{x1}}{m_{xo}} = \frac{\gamma_{xo}}{\gamma_{x1}} \quad (36)$$

$$\ln \gamma_{x1} \approx 2\alpha m_{x1} + k_{xs} m_{s1} \quad (37)$$

$$\ln \gamma_{xo} \approx 2\alpha m_{xo} + k_{xe} m_{eo} \quad (38)$$

Combining equations (36-38),

$$\ln f_1 = k_{xe} m_{eo} - k_{xs} m_{s1} - 2\alpha m_{xo} (f_1 - 1) \quad (39)$$

This expression should hold for any normal internal phase, within the range of validity of the simplifying approximations (Appendix B). Note that the partition coefficient is a function of the concentration of X in both phases and of the concentrations of all other solutes in those phases, via the weighted interaction terms, k_{xe} and k_{xs} .

2. Comparison with Data. In the single phase model, $Q = f_1$; that is, the distribution coefficient is the true partition coefficient given by equation (39). Note that all of the terms in equation (39) are experimentally accessible, with the exception of k_{xs} , the internal Satchenow coefficient. Equation (39) thus permits the calculation of k_{xs} for the uniphase model.

In the discussion of Figure 4, it was noted that non-electrolyte distributions obeyed the expression

$$W_x \equiv QW_m \equiv W_m + C_x \quad (40)$$

where W_x is the "water" of distribution of X, W_m is the matrix volume and the constant, C_x , is the experimentally determined, solute-specific intercept of each curve in Figure 4. Our interest is to compare these experimental findings with the

theoretical expression, and this will require the linearization of $\ln f_1$. Although the distribution coefficient differs considerably from 1.0 for some of the solutes studied, it is adequate for present purposes to let $\ln f_1 \approx f_1 - 1$. Since the distribution coefficient was shown to vary little with nonelectrolyte concentration, we may neglect the terms in m_{x0} , leading to the following expression for W_x ($m_{x0} \rightarrow 0$):

$$W_x \approx W_m - S_1(k_{xs} - \frac{\phi_s k_{xe}}{\phi_e}) \quad (41)$$

When expressed in this form, it is evident that the non-electrolyte distributions of Figure 4 are generally consistent with the uniphase model. Furthermore, we may calculate the difference in Setchenow coefficients from the intercepts, using $S_1 \approx .17$ moles/kg dry wt. They are -3.2 (antipyrine), -0.9 (urea), 0 (ethanol, glycerol), and +1.6 (DMSO). If the uniphase model is correct, these large and divergent values must presumably result from prominent salting-in and salting-out effects of macromolecules.

If γ_{x0} were known for each of these solutes in the sucrose media employed, then γ_{x1} could be determined from $f_1 = Q$ and equation (36). Equation (37) could then be used to determine k_{xs} as a function of m_{s1} and m_{x1} . Once again, the desired coefficient is determined phenomenologically and depends on the model, since m_{s1} and m_{x1} are determined by the choice of $W_1 = W_m$.

B. Abnormal Phase Model

1. *Solute Partition Coefficient.* According to the Ling-Troshin Model, all internal water is abnormal; that is, $W_2 = W_m$. The chemical potential of nonelectrolyte, X, in this abnormal phase may be written

$$\mu_{x2} = \mu_{x2}^\theta + RT \ln \gamma_{x2} m_{x2} \quad (42)$$

where μ_{x2}^θ is the solute chemical potential in a hypothetical ideal 1.0 molal solution with solvent W_2 (i.e., not in "normal" bulk solution). This solute standard state is chosen in the usual manner, so that $\gamma_{x2} \rightarrow 1.0$ as $m_{x2} \rightarrow 0$ in a binary solution.

Data do not exist for the behavior of solute activities in "abnormal" water; hence, there is no basis for the expan-

sion of the activity coefficient that was carried out in Appendix B. However, we have minimized variations in γ_{x2} by defining a new solute standard state for phase 2. At equilibrium, the solute *activities* in the two phases must always differ by a constant proportionality factor (see discussion on p. 248 in [47]). It is convenient to define a new standard state quantity, f_2^0 , representing the ideal partition coefficient between phases 1 and 2 when the solutes are in their standard states. Equation 42 then becomes

$$\mu_{x2} = \mu_x^\theta + RT \ln \frac{\gamma_{x2} m_{x2}}{f_2^0} \quad (43)$$

where

$$f_2^0 \equiv \exp \left(\frac{\mu_x^\theta - \mu_{x2}^\theta}{RT} \right) \quad (44)$$

At equilibrium, the general partition coefficient, f_2 , is given by

$$f_2 = \frac{\gamma_{x0} f_2^0}{\gamma_{x2}} \quad (45)$$

where

$$f_2 \equiv Q \equiv \frac{m_{x2}}{m_{x0}} \quad (46)$$

Recall that the purpose of this analysis is to determine whether the large differences in slopes between Figure 3 and Figure 4 are consistent with the abnormal phase model. For this purpose, it is adequate to ignore variations in γ_{x0} ; that is, to let $\gamma_{x0} \approx 1.0$. Furthermore, the results portrayed in Figure 3 tell us that γ_{x2} is independent of the concentration of X when m_m is held constant. It follows, therefore, that γ_{x2} is, at most, a function of endogenous solute concentration, m_{s2} . This dependence may be expressed in a manner analogous to equation (37):

$$\ln \gamma_{x2} = 2k_{xs} m_{s2} \quad (47)$$

where $2k_{xs}$ is the interaction coefficient between non-electrolyte and endogenous solutes. This is merely a convenient expression; $2k_{xs}$ is not considered to be constant, but rather a function of m_{s2} . Under these conditions, the partition coefficient may be written

$$\ln \frac{f_2}{f_2^0} = -2k_{xs}m_s^2 \quad (48)$$

In particular, equation (48) should be valid in the limit, $m_{x0} \rightarrow 0$.

We may draw an interesting comparison between solute distributions in an abnormal phase (equation 48) and those in a normal phase (equation 39). In both cases, solutes are at equilibrium. Their distribution coefficients will be determined by interactions with internal solutes and macromolecules and by interactions with the solvent. In the Normal Phase Model, the former interaction predominates, leading to salting-in or salting-out of nonelectrolyte. In the Ling-Troshin (Abnormal Phase) Model, solute-solvent interactions predominate, due to the "structuring" of intracellular water. This effect of abnormal solvent properties is expressed in f_2^0 , reflecting the free energy of transfer of nonelectrolytes from bulk solution to the abnormal phase.

2. Comparison with Data. In the Abnormal Phase Model, $f_2 = Q$ by hypothesis, so that $W_x = f_2 W_m$. f_2^0 (via μ_{x2}^0) may be defined at any convenient value of W_m . For example, f_2^0 may be defined as the standard partition coefficient associated with the conditions of Figure 3 ($W_m \approx 1.0$ g/g dry wt., in equilibrium with 0.27 osmolal sucrose). In the neighborhood of this region we may use the approximation

$$\ln f_2/f_2^0 \approx f_2/f_2^0 - 1 \quad (49)$$

Using the definition, $W_x \equiv x_m/m_{x0}$,

$$W_x = f_2^0(W_m - 2k_{xs}S_2) \quad (50)$$

In the Ling-Troshin Model, abnormal water characteristics determine the partition coefficients of non-adsorbed nonelectrolytes. This dependence is explicitly included in the f_2^0 term, which should, therefore, dominate the partition coefficient. That is, $f_2 \approx f_2^0$. This case has already been considered (Section IV). Plots of W_x vs. W_m , as in Figure 4, should yield a family of curves with zero intercepts and solute-specific slopes. These slopes must equal those found in plots of m_{xm} vs. m_{x0} (Figure 3). In contradiction to this expectation, the experimental results (Figure 4) yield a family of curves with identical slopes. Furthermore, $f_2^0 \neq 1.0$ by hypothesis, while the observed slopes are equal to 1.0.

Therefore, the Ling-Troshin model is not in accord with experimental results.

The inconsistency of this model with thermodynamics is not removed by considering the second term in equation (50). Since S_2 is found to be constant by experiment, equation (50) predicts a family of curves with different intercepts, as observed experimentally. However, the problems with the slopes, f_2^0 , are unchanged, and the model remains inconsistent with results.

Equation (48) can only agree with experiment if f_2^0 varies with water content. This requires that, in the limit, $\mu_{x2}^0 \rightarrow \mu_x^0$, rendering meaningless the definition of the standard state quantity, f_2^0 . That is, this type of variation of chemical potential is already encompassed within the usual definition of activity coefficient. Thus, the equation fits the data provided $f_2 \approx 1.0 + 2k_{xs}m_s$, and $f_2^0 \approx 1.0$. But this is equivalent to the definitions of the Normal Phase Model, and therefore this possibility is inconsistent with the basic postulates of the Abnormal Phase Model.

Finally, we note that both the Normal Phase Model and the Abnormal Phase Model treat the internal solution as a single phase, via the implicit assumption made in the definition of internal concentration: $m_{xm} \equiv X_m/W_m$. The hypothetical solution properties attributed to this phase by the two models are mutually exclusive. Since these experiments examine those properties directly, and since the data are consistent with the Normal Phase Model, the Abnormal Phase Model must be rejected on the grounds that the two models are mutually exclusive.

The Abnormal Phase Model may be modified by removing the restriction that the internal milieu is a single phase. This modification makes it equivalent to the Heterogeneous Phase Model. The consequences of this model contradict the conclusions of Ling and Troshin, as has already been noted in the discussion of active transport (Section II).

Since equation (48) is either inconsistent with the data or inconsistent with the postulates of the model, it must be concluded that this model is incorrect.

C. Heterogeneous Phase Model

1. Solute Partition Coefficients. The internal milieu

is treated as two phases. The partition coefficient in the normal phase (1) is given by equation (39), and that for the abnormal phase (2) by equation (47). All the considerations used in obtaining these expressions apply to this model.

2. Comparison with Data. The approximation that $f_1 \approx 1$ at low values of m_{X_0} is given by hypothesis. We may also take $f_2 \approx f_2^0$, considering the major component of f_2 to be the free energy of transfer of X between phase 2 and normal aqueous solution. Then

$$W_X \approx f_1 W_m + (f_2^0 - f_1) W_2 \quad (51)$$

which is identical to equation (18) and agrees with the observed relationship, equation (40). It must be noted that the parameters of equations (39) and (47) cannot be obtained directly for the two-phase model without prior knowledge of the extent of W_2 . If we take $W_2 = a$, on the basis of arguments presented in Section V, the essential parameters of the two-phase model can be calculated (Table I).

TABLE I. Properties of Mitochondrial Water

Parameter	Abnormal Phase	Normal Phase
Extent (g H ₂ O/g dry wt)	0.28 (30%)	0.67 (70%) ^a
Osmotic Coefficient	inactive	0.94 \pm .05 ^b
<i>Phase:medium partition coefficients:</i>		
antipyrine	3.0	1.0
urea	1.5	1.0
glycerol	1.0	1.0
ethanol	1.0	1.0
dimethylsulfoxide	0.04	1.0

^aValue given is for mitochondria in equilibrium with 0.272 osmolal sucrose.

^bObtained from combined urea distribution - osmotic swelling studies.

Note that solutes which have excess hydrogen bond donors are concentrated in the abnormal phase, while the aprotic solute is excluded.

D. Interpretation

Ling (16) and Troshin (15) place considerable emphasis on the divergent Q values found in biological water, using data exemplified by those in Figure 3. Both authors consider these findings to be inconsistent with the concept that cell water is normal. The foregoing analysis has demonstrated that this is decidedly not the case. Furthermore, the Ling-Troshin model as presently stated is shown to be inconsistent with experiment. If the model is modified, to fit observation, it becomes indistinguishable from the Normal Phase Model or from the Heterogeneous Phase Model. Therefore, the Abnormal Phase Model of cell water can be rejected. The importance of this conclusion for cell biology cannot be overstated, for it rules invalid the arguments of the Ling school relating to active transport (see Section II).

Distinguishing between the remaining two models is more difficult. Both are consistent with the data by virtue of the phenomenological nature of the coefficients; however, the Setchenow coefficients calculated from the Normal Phase Model are an order of magnitude larger than those normally seen in aqueous solution. It is of interest that Gary-Bobo (11) considered, and rejected, salting-in and out interactions to explain the solute specificity of alcohols in hemoglobin solutions.

IX. EFFECTS OF NONELECTROLYTES ON MATRIX WATER CONTENT: THERMODYNAMIC TREATMENT

It is well to restate, at this point, two features of this work which represent departures from most studies of this kind: (a) experimentally, solute distribution studies are carried out together with, rather than separate from, osmotic swelling studies; (b) theoretically, abnormal water is treated as a (possible) solvent, rather than as "bound" water without solvent properties. These two features permit a detailed analysis of the solute-water interactions, leading to a possible means of distinguishing between the Normal and Heterogeneous Phase Models.

A. Volume Consequence of Large Satchenow Coefficients

This development will consider matrix water in the presence (W_m^+) or absence (W_m^-) of nonelectrolyte, but at the same concentration, m_{eo} , of external impermeant solutes. Osmotic equilibrium in the presence of nonelectrolyte is given by

$$\phi_{sx}(m_{x1} + m_{s1}^+) = \phi_{ex}(m_{eo} + m_{xo}) \quad (52)$$

where m_{s1}^+ denotes the concentration of endogenous solutes in the presence of nonelectrolyte. Both sides may be expanded, using equation (B18):

$$\begin{aligned} & \phi_s^+ m_s^+ + \phi_{x1} m_{x1} + k_{xs} m_{s1}^+ m_{x1} \\ &= \phi_e m_{eo} + \phi_x m_{xo} + k_{xe} m_{xo} m_{eo} \end{aligned} \quad (53)$$

Note that $\phi_e m_{eo} = \phi_s^- m_s^-$, the internal osmolality in the absence of nonelectrolyte at the same external sucrose concentration, m_{eo} . Using this relationship together with equation (39) to eliminate $k_{xs} m_{s1}^+$, equation (36) to eliminate m_{x1} , and equation (B11) to eliminate the ϕ_x (which are osmotic coefficients of a solution of pure x at the given concentration), we obtain

$$\begin{aligned} & \phi_s^+ m_s^+ - \phi_s^- m_s^- = m_{xo}(f_1 - 1) \\ & \frac{\{f_1 \ln f_1\}}{f_1 - 1} - 1 + \alpha m_{xo}(f_1 - 1) - k_{xe} m_{eo} \end{aligned} \quad (54)$$

The left side of the equation may be transformed by means of equation (35) and $m_{s1} = s_1/W_1$.

$$\phi_x^+ m_s^+ - \phi_s^- m_s^- = \frac{s_1(W_m^- - W_m^+)}{(W_m^+ - a)(W_m^- - a)} \quad (55)$$

Note that equation (55) is independent of the size of W_2 ; that is, it is a measurable quantity which is independent of the model chosen to represent the phase structure of the matrix. Furthermore, the right side of equation (54) is completely determined by f_1 and properties of the medium. f_1 , however, does depend on the size and properties of $W_1 = W_m^- - W_2$; i.e., the osmotically active phase. Therefore, equation (54) provides a potential test of whether the non-electrolyte is distributed in all of matrix water or in some

fraction of matrix water.

To illustrate this, we use a more accurate approximation for $\ln f_1$; thus, $\ln f_1 \approx (f_1 - 1)(f_1 + 1)/2f_1$, leading to

$$\frac{S_1(W_m^- - W_m^+)}{W_m^+ - a} = \frac{m_{xo}(f_1 - 1)^2(1 + 2am_{xo})}{2}$$

$$- (f_1 = 1)k_{xe}m_{eo}m_{xo} \quad (56)$$

If the single phase model is correct, $f_1 = Q$, and any solute with $Q \neq 1$ will cause shrinkage of the matrix, when compared at the same value of m_{eo} , assuming the last term may be neglected. Urea is one of the few solutes whose activity coefficient in sucrose has been studied (40), permitting evaluation of media quantities. However, $(Q-1)^2$ for urea is too small to permit detection of discrepancies in equation (56). DMSO is a much better candidate; however, evaluation of equations (39) and (54) for DMSO and other solutes must await analysis of additional ternary systems, to obtain values for α and k_{xe} .

When such information is available, it should be possible to test experimentally the hypothesis that the matrix is a single phase. Consider an excluded solute, such as DMSO, whose distribution coefficient, $Q = 0.6$ in 0.3 M sucrose. If it can be shown that equation (54) requires $f_1 \approx 1.0$, then it follows that phase 1 comprises less than total water; i.e., that there is an osmotically inactive phase. Similarly, if equation (54) requires $f_1 \approx 1.0$ for a solute with $Q = 2$, then it follows that this solute is distributing into phase 1 and into the osmotically inactive phase 2.

This analysis may also be appropriate for protein solutions, where similar behavior of nonelectrolyte distributions and osmotic coefficients are observed. For example, Gary-Bobo (11) has found that certain alcohols are extensively "salted out" of hemoglobin solutions and that the excluded volume depends solely on the amount of protein, as in mitochondria. It may turn out that an analysis of the type proposed here could be used to examine the possibility of water compartments in the neighborhood of protein molecules.

B. Higher Order Effects

The most important assumption made in the above analysis

is that the "Setchenow" coefficients, k_{xs} , are constant. In effect, this permits the simple substitution of equation (39) into equation (53) and leads to the tentative conclusion that phase structure must necessarily be reflected in the effect of permeant solutes on volume. This conclusion is of sufficient importance to warrant further examination of the approximation involved. For this purpose, we return to the work of Ellerton and Dunlop (40) on the system water-urea-sucrose.

In fitting the data up to 7.0 M urea and 4.3 M sucrose at 25°C, they found that a second order polynomial best fit the data. They make the statement, however, that a first order equation "was also found to fit the data quite well". I have plotted their data up to 3.6 M (total) and find that k_{us} is linearly related to $m_u + m_s$ over this range. Furthermore, the scatter at a given total molality appears not to be related to the relative proportions of urea and sucrose. Thus, linear regression of 55 values gives

$$k_{us} = -0.12381 + 0.01335 (m_u + m_s) \quad (57)$$

(for $m_u + m_s < 3.6$ M). Note that equation (B16) requires that the coefficients of m_u and m_s must be equal in a first-order expression of k_{us} .

In general, a non-constant Setchenow coefficient of first order leads to the following relationships for the mitochondrial system:

$$k_{xj} = \alpha_{xj} + \beta_{xj} (m_j + m_x) \quad (58)$$

$$\ln \gamma_x = \ln \gamma_x^o + \alpha_{xj} m_j + \beta_{xj} m_j m_x + \frac{\beta_{xj}}{2} m_j^2 \quad (59)$$

$$\begin{aligned} \ln \gamma_j &= \ln \gamma_j (m_x = 0) + \alpha_{xj} m_x + \beta_{xj} m_x m_j \\ &\quad + \frac{\beta_{xj}}{2} m_x^2 \end{aligned} \quad (60)$$

$$\phi_{xs} (m_{x1} + m_{s1}) = \phi_x m_{x1} + \phi_s m_{s1} + k_{xs} m_{x1} m_{s1} \quad (61)$$

$$k_{xs} = A + B m_{x1} + C m_{s1} \quad (62)$$

$$\ln \gamma_{x1} = \ln \gamma_{x1}^o + k_{xs} m_{s1} - \frac{C m_{s1}^2}{2} \quad (63)$$

$$A = \sum \alpha_{xj} f_j \quad (64)$$

$$B = \sum \beta_{xj} f_j \quad (65)$$

$$C = \sum \beta_{xj} f_j^2 \quad (66)$$

$$m_{1j} = f_j m_{s1} \quad (67)$$

Note that $B \neq C$, in general, because of the squared term in m_j that appears in the integral. As before, equations (61) and (63) can once again be combined to eliminate k_{xs} . In this case, however, there is an extra term in $C_{ms}^{2/2}$ in equation (54). Experiments on the variation of Q with m_{xo} and m_{s1} are, therefore, required to obtain B and C . The combined equation may then be evaluated to test the single phase hypothesis, $f_1 = Q$.

From Figure 4 and the large apparent k_{xs} values derived therefrom, it appears that the constant term in k_{xs} predominates and that the presence of higher order terms will not have a major quantitative effect.

C. Can Equilibrium Thermodynamics Distinguish Aqueous Phase Heterogeneity? The answer is yes, provided we limit the scope of the question to the two alternatives previously defined: (1) in a single phase system all water is osmotically active; (2) a two-phase system includes, as well, an osmotically inactive fraction of water which can dissolve solutes. The question can be resolved with a purely hypothetical model.

Consider a model "mitochondrion" containing a mixture of solutes. We suppose that these solutes are all small ions which have no capacity to induce abnormal water. These mitochondria, therefore, either will have no abnormal water at all (single phase) or this abnormal water will be associated exclusively with the membrane (two-phase), since there are no dissolved proteins. We now suspend these mitochondria in a solution of identical proportions of ions as found in the matrix. The membrane is impermeable, over the time range of our experiments, to all of these substances, but rapidly permeable to nonelectrolyte and water. The internal osmotically active solution is now *identical* to the external solution, and it will remain identical if we add water and/or nonelectrolyte, by virtue of the given properties of the model.

Under these hypothetical conditions, the left side of equation (54) is necessarily zero for all solutes and for

both phase models. In contrast, the right side (using $f_1 = Q$) will be zero for all solutes only if the single phase model is correct; i.e., only if $Q = 1$ for all solutes. In this hypothetical experiment, the finding that $Q \neq 1$ requires a two-phase model. Thus, thermodynamic measurements are capable, in principle, of distinguishing phase heterogeneity from homogeneity.⁴

X. RESOLUTION OF THE QUESTION OF PHASE STRUCTURE

The "Abnormal Phase Model" of Ling (7) and Troshin (15) may be excluded on the basis of the analysis given in Section VIII. What remains is to decide between a Heterogeneous Phase Model and a Normal Phase Model of intracellular (and intramitochondrial) water. Deciding this issue, as noted in the Introduction, will determine how we interpret all data relating to the intracellular solution. Even a quantity as fundamental as "intracellular concentration" requires knowledge of the extent of normal aqueous solution within the cell.

The thermodynamic analysis of these two surviving models has shown that intracellular solution behavior may be interpreted in two distinct ways.

⁴For completeness, another potential effect of non-electrolyte on water content must be mentioned. If the depth of abnormal water over a surface is related, ultimately, to surface forces, then the depth should remain relatively constant. If this is true, the volume of the abnormal phase, and not water content, should remain fixed. The presence of space-occupying nonelectrolyte should, therefore, displace some of the water from phase 2, and this effect (which will be related to the solute molar volume and its concentration in phase 2) may be significant at high concentrations of solutes whose $f_2 > 1.0$. This effect does not, however, destroy the usefulness of equation (54). That is, it cannot lead erroneously to the conclusion that phase heterogeneity obtains, since the effect is in the same direction (shrinkage) as that predicted by the normal phase model. Furthermore, an excluded solute, such as DMSO, cannot affect the volume of abnormal water by this mechanism.

Heterogeneous Phase Model: The extrathermodynamic assumptions are: (a) the osmotic coefficient is relatively constant over the range studied; therefore, the osmotic intercept, a , represents an abnormal phase, $W_2 = a$; or (b) there is an osmotically inactive phase whose extent is approximately equal to the osmotic intercept. These assumptions are equivalent and lead to the same conclusions: the normal phase has the characteristics of a dilute solution. Solute activity coefficients and osmotic coefficients are close to 1.0 and vary only slightly with concentration. Water in the abnormal phase is invariant with changes in water activity or solute content of the normal phase, but sensitive to membrane-localized drugs. Solute activity coefficients in this phase vary widely with the net hydrogen bond donor capacity of the solute. Accordingly, the treatment of solute distribution data is that $m_{x1} \approx m_{x0}$ and $W_2 \approx a$, the osmotic intercept. The extrathermodynamic assumption thus permits the calculation of the thermodynamic partition coefficient in phase 2 (f_2).

Normal Phase Model: The extrathermodynamic assumption is made that $W_1 = W_m$. Thus, the entire cytoplasm is a bulk aqueous solution whose properties are profoundly influenced by macromolecules. The osmotic coefficient is a highly non-linear function of macromolecule concentration. Solutes are strongly salted-in or out by macromolecules, so that non-electrolyte concentration ratios are also highly dependent on macromolecule concentrations. Accordingly, the treatment of solute distribution data is that $m_{x1} = X_m/W_m$, and $f_1 = 1 + C_x/W_m$; where C_x is a term containing the Setchenow coefficient. The extrathermodynamic assumption of this model permits the calculation of C_x for each non-electrolyte studied.

We note that each concept of phase structure determines its own model-specific thermodynamic parameter: one relating to the free energy of solute transfer to an abnormal phase and the other relating to a weighted Setchenow coefficient. Which approach is correct?

A. Fence Sitting

Neither model is preferable, since each appears to explain the data equally well. It is merely a choice of the most convenient parameters to use in expressing the data.

This alternative is unacceptable, since the two models lead to very different interpretations of fundamental experi-

mental data ... for example, of the diffusion coefficient of water in the cell; of the energy required for active transport, etc. These two models lead, therefore, to different quantitative results. A choice *is* made every time a biologist calculates an "intracellular concentration" or any other fundamental solution property. Thus, fence-sitting is not an acceptable approach for quantitative biologists.

B. The Normal Phase Model

The assumption of a single phase is more conservative, and therefore, to be preferred for the present, until convincing evidence to the contrary is available.

I object to this reasoning, first, on the grounds that the extrathermodynamic assumptions of the two models are equivalent in scope. Thus, neither model is more "conservative" than the other. In this respect, it is the extra-thermodynamic assumptions themselves which must be critically evaluated. On this basis, the Normal Phase Model is found wanting (Section V).

My second objection is that this model is an *inherently* incomplete description of biological solution structure. That is, the findings of abnormally large Setchenow coefficients and an abnormally nonlinear osmotic coefficient, being purely phenomenological, tell us nothing. They beg for interpretation. A parallel situation exists in solution chemistry, where the approach is not to *describe* the concentration-dependence of overall solution properties, but rather to *explain* it in terms of fundamental interaction models. For example, Scatchard (48) very early took this approach to explain the variation of the osmotic coefficient of sucrose with concentration.

Another interesting example of model building in a ternary (urea-sucrose-H₂O) system is found in the paper of Ellerton and Dunlop (40). The phenomenological Setchenow coefficients describe the effects of salt concentration on non-electrolyte activity coefficients. Solution chemists are actively engaged in searching for models that *account* for the sign and magnitude of these coefficients (see references in Appendix B). All of these attempts to explain the behavior of the phenomenological coefficients begin with the setting up of a model describing the interactions that are thought to occur within the solution. The data are then fit to this model, resulting in specific interaction parameters

rather than broad solution parameters. The validity of the model necessarily rests on its ability to predict experimental results. The Debye-Hückle limiting law for the activity coefficients of salts is an example of such an extrathermodynamic approach. It is important that biologists similarly move beyond the purely phenomenological approach toward a physical description of biological solutions.

At best, therefore, the Normal Phase Model is incomplete; truly, it is not a model at all. It remains to develop a satisfactory explanation for the abnormal osmotic and Setchenow coefficients observed in all biological solutions.

C. *The Heterogeneous Phase Model*

There is every reason to expect multilayered adsorption of water onto macromolecules, forming an abnormal phase. This concept has been extensively discussed by Ling (18). In contrast to Ling, I consider multilayered adsorption to be very limited in extent, on the order of 5 to 10 water molecules deep. Raising water activity further leads to accumulation of bulk solution. This interpretation is in accord with the experimental findings.

This model provides an explanation for the behavior of the (apparent) osmotic coefficient and the large (apparent) Setchenow coefficients. It permits calculation of the energy required for active transport, provided the extent of abnormal water and the solute partition coefficient into the abnormal phase are known. On the basis of data presently available, I consider this to be the model of choice in treating biological solution structure.

D. *Experimental Resolution*

The analysis of Section IX has shown that these two models can, in principle, be differentiated experimentally. Such an achievement would represent a major breakthrough in our understanding of biological solution structure. The data required for such a test is well within the limits of accuracy obtainable on mitochondrial preparations (and probably on red cells and hemoglobin solutions as well). The major stumbling block at present is the lack of information on solute activity coefficients in the external solutions required for these studies. Such data can be obtained by

investigators experienced in isopiestic methods. Alternatively, external activity coefficients can be estimated by freezing point determinations. The labors of such an investigation must not be underestimated, but the potential rewards are high, and these experiments should be pursued.

E. Conclusion

On the basis of these considerations, the Heterogeneous Phase Model appears to be the best available description of biological solutions at the present time. In the final sections, I will touch briefly on some consequences of this conclusion.

XI. THE NATURE OF THE ABNORMAL PHASE

It is impossible to forego some speculation on the properties of the abnormal phase; however, the brief description presented here must be recognized as conjectural.

A. The Location of the Abnormal Phase

It seems reasonable to postulate that the abnormal phase is induced by macromolecular surfaces. This is in general agreement with the starting point of Ling's model of cell water (5). Drost-Hansen (6) has paid particular attention to the water structuring properties of surfaces, and considers these findings to have great significance for biology.

The reader will recall that the term "abnormal water" was defined phenomenologically in accordance with the techniques used to study it. Thus, a phase is "abnormal" with respect to its solution properties. Drost-Hansen has introduced the morphological term "vicinal water" to describe water near surfaces. I consider it very likely that the abnormal phase observed in the present study is a phase consisting of vicinal water.

B. Membrane Surfaces vs. Protein Surfaces

Taking a value of $1.5 \times 10^6 \text{ cm}^2/\text{g}$ dry wt. for the inner membrane area of mitochondria (28), the thickness of a uniformly distributed abnormal water layer is about 25 Å. This

amounts to 5-6 water molecules, and allows a very rough idea of the extent of an abnormal phase associated with the membrane. 25 Å represents an underestimate of thickness if macromolecules are concentrated in this phase (see Section XII), or if there is localization of abnormal phases to particular regions of the membrane. An overestimate will result if abnormal phases are found on both sides of the inner membrane. It appears that vicinal water, while sufficiently deep to confer phase properties, is rather limited in radial extent.

Just as mitochondria are remarkable for their high surface-to-volume ratio, red blood cells are remarkable for their low value of this ratio. Furthermore, red cells are uncomplicated by internal membranous structures. As a consequence, the amount of *membrane* localized abnormal water in red cells should be on the order of 0.1% of total water, using 25 Å as the thickness of this phase. This is to be compared with 30% for mitochondria, and one surely would not expect to be able to detect such a small fraction. Nevertheless, the osmotic intercept is similar in red cells and mitochondria. The explanation for these findings is that water associated with hemoglobin surfaces predominates in red cells. This interpretation is borne out by the finding that the apparent osmotic coefficient of cell-free hemoglobin can largely account for the osmotic behavior of red cells (13). If the abnormal water is indeed associated with hemoglobin, then one would expect abnormal nonelectrolyte distributions in red cells to be duplicated in the cell-free hemoglobin solutions. This is precisely the finding of Gary-Bobo (11). I conclude, therefore, that the majority of abnormal water in red cells is associated with intracellular hemoglobin.

The cytoplasmic structure of the red cell is virtually unique. I consider it most likely that abnormal water in nucleated cells is associated predominantly with their extensive internal membrane surfaces and with structural protein elements. The question of "soluble" proteins is discussed in Section XIII-F.

C. Forces Acting to Maintain the Abnormal Phase

It is of interest to consider the nature of abnormal water from two related points of view: what underlying water structures confer abnormal properties to this phase? What forces maintain water in this state? The latter question must be addressed first since these forces must surely

determine, in some manner, the structure of water in the abnormal phase. We may enumerate four factors that must have a predominate influence on the maintenance of the abnormal phase:

1. The non-aqueous protein or membrane surface is the surface to which the initial water layer adsorbs. The orientation of subsequent layers must be profoundly affected by any two-dimensional order on the underlying surface, as emphasized by Ling (5).
2. Cooperativity, via hydrogen bonding, will cause additional layers of water to adsorb to the first. The pronounced cooperativity of water molecules will, therefore, confer long-range effects to the underlying surface:water interactions. The work of Peschel (this volume) and Drost-Hansen (6) suggests that vicinal water may extend many hundreds of Angstroms from the surface. Much of this work, however, was carried out in pure water or very dilute solutions.
3. The presence of salts and other solutes in biological water may be expected to reduce greatly the long-range order, by comparison with that observed in their absence. The abnormal phase undoubtedly has a different "structure" from the bulk phase, and the presence of solutes must uniformly be structure-breaking in the abnormal phase, leading to attenuation of long-range order.
4. The phase boundary between the normal and abnormal phase not only must reflect the previously mentioned factors, but also must contribute to the maintenance of the abnormal layer. Thus, a tendency to minimize this surface may influence underlying surface conformation, and vice versa.

D. *The Abnormal:Normal Phase Boundary*

Drost-Hansen (6) has illustrated several types of phase transitions that may exist between vicinal and bulk water. The most important question is whether the transition is abrupt or gradual (continuous). I believe it is most likely that the transition is abrupt, leading to a well defined phase boundary. This conjecture is not entirely without foundation: it is difficult to conceive of gradual transitions, given the extraordinary cooperativity of water molecules. On the average, therefore, a water molecule will orient itself according to the structure of the solution in which it finds

itself. The strength and orientation of hydrogen bonds in a given phase may not permit the molecule to take an intermediate position.

E. The Structure of Abnormal Water

"Bound" water evokes the image of an ice-like or caged structure, extensively hydrogen bonded. The ability of hydrogen-donor solutes to penetrate this phase may be due to their ability to enter clathrate cavities and, perhaps, to form H-bonds with solvent walls. I do not share this view of abnormal water, since polar solutes are already extensively H-bonded in the normal phase. It is difficult to imagine how a transfer to a more structured region would be energetically favorable. Thus, the possibility that abnormal water is ice-like (more H-bonded than normal water) appears unlikely, considering the finding that this water is an avid solvent for certain polar solutes.

It seems equally plausible that abnormal water may be less H-bonded than normal water. The phospholipid head groups on the membrane may induce a region of solvent disorder, for example. Under the constraint of constant volume (that is, assuming a constant range of disordering influence), transfer of a solute molecule into the "abnormal" phase would be accompanied by the transfer of water to the more ordered bulk phase. The negative free energy change of water displacement would be counterbalanced by the energetically unfavorable transfer of hydrogen bonded polar solutes from bulk water to abnormal water.

In a review of the effects of drugs on cell membranes, Allison (49) points out that hydrogen bonds are undoubtedly of great importance in maintaining membrane structure, and further, that hydrogen bonds are good candidates for the action of reversible drugs. The hydrogen bond acceptor nature of the membrane leads to another conjecture as to the structure of vicinal water: suppose the first monolayer to be adsorbed to H-bond acceptor groups. 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. Such a phase may tend to exclude aprotic solutes, such as DMSO, while concentrating solutes with excess H-bond donor groups, such as urea.

My own prejudice, at present, is that abnormal water is not ice-like, but rather, has less order than bulk water. The experimental approach taken in the present work offers some promise of finding clues to this question. In particular, it is planned to study the effects of temperature on the distributions of nonelectrolytes.

XII. BIOLOGICAL CONSEQUENCES OF PHASE HETEROGENEITY IN CELLS AND ORGANELLES

A. *Validity of the Membrane Theory*

The presence of bulk solution to the extent of 70% of matrix water has been shown in mitochondria. This has consequences for the interpretation of mitochondrial ionic balance: is it maintained by membrane processes (permeability and/or carriers) or does it represent a state of equilibrium due to the abnormality of the matrix water? It is a straightforward matter to show that membrane processes are required to explain the observations of K^+ and anion movements, given the finding that 70% of matrix water behaves as a bulk aqueous solution.

All evidence points to the same conclusion for whole cells. For example, the giant barnacle muscle has been studied with exceptional thoroughness by Hinke and co-workers, with the objective of defining the state of muscle water and solutes (50-52). A variety of techniques have been applied, including osmotic swelling, ion activity measurements with intracellular electrodes, and diffusion kinetics of ions, water and nonelectrolytes. The results consistently indicate that 70-80% of fiber water is in a normal aqueous solution. As shown in Section II, this result establishes the necessity for membrane localized active transport processes to maintain homeostasis.

B. *NMR Studies on Biological Solutions*

The overall reduced spin-spin diffusion coefficient for water in cells (2) must be considered to reflect weighted signals from different phases, rather than a true diffusion coefficient. Thus, the present findings support multiphase interpretations of NMR and EPR measurements on biological systems (for instance, 53-55).

As stated in the Introduction, physical studies on biological water cannot, by themselves, resolve the issue of phase heterogeneity, since a phase model is required for the interpretation of the data. This fact has been explicitly noted by some workers in this field; for example: "It is not possible with our present state of knowledge of macromolecular systems to use NMR relaxation times as direct evidence for altered solvent properties of the water of biological systems" (56).

C. Intracellular pH

In a heterogeneous phase system internal concentration is not, in general, equal to the observed solute-to-water ratio of the internal phase. Nevertheless, such an assumption is normally made, and is equivalent to the adoption of a single phase model of cell water. Conclusions drawn from internal concentration data are exquisitely sensitive to assumptions about phase structure. A notable example is found in the controversy over the value of intracellular pH.

Attempts have been made to estimate intracellular pH directly, using microelectrodes, leading, in one case, to a value of about 5.99 (57). This value is of particular significance, since the H^+ ion equilibrium potential associated with it is very near the membrane potential, suggesting that H^+ ions are in equilibrium across the cell membrane. In contrast, numerous studies of the distribution of weak acids, such as DMO, yield values in the neighborhood of 7.0 to 7.1 for intracellular pH (58). This value indicates that H^+ ions are distributed far from equilibrium in the cell. Wiggins (59) has reviewed this controversy and has concluded that the discrepancy is due to a reduced ionization constant of water in cells which, in turn, results from enhanced water structure.

The heterogeneous phase model suggests another solution; namely, that the true concentration of DMO in the "normal" cytosol phase is not necessarily equal to the apparent (total) concentration. Furthermore, one must not dismiss lightly the suggestion by Waddell and Bates (60) that pH heterogeneity is the result of varying pH within intracellular anatomic compartments. For example, if 90% of the cell solution has a pH of 5.99, the observed results are consistent with a second compartment, comprising 10% of cell water, having a pH of 8.00. This is well within the limits of mitochondrial matrix pH and volume, and it must be concluded that compartmentation can readily account for the discrepancy between the two

measurements. This explanation points up the problem of studying whole cells with such techniques; membrane-bounded compartments have vastly different properties from the cytosol, heavily weighting the results. In this respect, it is premature to invoke alterations in water structure, since there is already sufficient anatomical heterogeneity in whole cells to account for such observations.

D. Intramitochondrial pH and the Driving Force for Oxidative Phosphorylation

The problem of determining the transmembrane pH gradient in mitochondria is particularly important because of its central role in the chemiosmotic theory of energy transduction (61). Nicholls (62) has recently carried out acetate distribution studies in mitochondria to estimate the pH gradient in various energetic states. Simultaneously, he has estimated the transmembrane electrical potential from the concentration gradient of rubidium. The protonmotive force of Mitchell is proportional to the free energy of transfer of hydrogen ions across the membrane and equal to the sum of the electrical potential term and the concentration term:

$$\Delta p \equiv \Delta \mu_H / F = -\Delta E + 59 \Delta pH \quad (68)$$

where Δp is the protonmotive force, F is the Faraday, ΔE the membrane potential and ΔpH the pH gradient across the membrane. Nicholls calculates the protonmotive force from concentration gradients of acetate (a) and rubidium (r) determined in the presence of valinomycin. These solutes should remain close to their equilibrium values because of the high permeability of the membrane to acetic acid and Rb^+ -valinomycin complex.

The heterogeneous phase model requires (1) that there is solute equilibrium between the two contiguous aqueous phases in the matrix, and (2) that solute activity coefficients in the normal phase are approximately equal to those in the medium. It follows that

$$\Delta p = 59 \log \frac{m_{lr}^{m_1 a}}{m_{or}^{m_0 a}} \quad (69)$$

where 1 and 0 refer to concentrations in the normal internal phase and in the external phase, respectively. The major approximation enters when Nicholls assumes that m_{lr} and $m_1 a$

are equal to their respective *total* solute:volume ratios. Equation (69) may be reorganized to express this assumption explicitly:

$$\Delta p = 59 \log \frac{R_m A_m}{W_m^2 m_{or} m_{oa}} + 59 \log \frac{f_r f_a}{f_w^2} \quad (70)$$

where the first term on the right is the protonmotive force as calculated by Nicholls, and where $f_a = A_1/A_m$; $f_r = R_1/R_m$, representing the ratios of solute in normal phase to total matrix solute (A_m and R_m). $f_w = W_1/W_m$, the fraction of normal phase water. If these ratios differ from 1.0, then the calculated Δp will be different from the true Δp by the amount given in the second term of equation (70).

The significance of this potential error is immense, because it goes to the heart of the mechanism of energy coupling. Nicholls (62) has also calculated the phosphate potential in terms of the millivolts of protonmotive force required to maintain the equilibrium on the assumption that two protons are translocated into the matrix compartment for every molecule of ATP appearing in the extra-mitochondrial compartment. He finds that while the phosphate potential and protonmotive force change in parallel, the former parameter indicates a requirement for a potential some 50 to 80 millivolts in excess of that recorded as the protonmotive force. As Nicholls points out in his conclusion:

"Unless determinations of protonmotive force are substantial underestimates, it is clear that either a stoichiometry of two protons translocated per ATP synthesized is not valid under the approach to equilibrium conditions, or that the mechanism of energy transduction is other than chemiosmotic."

We are carrying out experiments designed to provide an estimate of matrix pH using the heterogeneous phase model. The partition coefficient, f_1 , for acetate anions in phase 1 is given by

$$f_1 \equiv \frac{m_{al}}{m_{ao}} = \frac{m_{ul}}{m_{uo}} 10\Delta pH - \Delta pK \quad (71)$$

where m_u represents the concentration of undissociated acid, HAC, and m_a that of the anion, Ac^- . We have confirmed the observation (61) that acetic acid enters the matrix by non-ionic diffusion. Since phase 1 is a normal aqueous phase in this model, we may set $m_{u1} \approx m_{a1}$ and $\Delta pK = 0$. Therefore,

$$f_1 \approx 10^{(pH_1 - pH_0)} \quad (72)$$

As before, the experimentally determined volume of distribution of acetate is given by

$$W_{ac} = f_1 W_m + (f_2 - f_1) W_2 \quad (73)$$

These experiments are more complex than those with non-electrolyte because it is difficult to vary W_m without varying ΔpH . Internal buffering capacity must also be evaluated simultaneously with distribution studies. Nevertheless, families of curves derived from appropriate experiments do yield the necessary data, permitting the evaluation of f_2 in equation (73).

f_2 is formally defined by an expression similar to equation (71). Needless to say, the attribution of changes in f_2 to changes in pH_2 , pK_2 , or m_{u2} is not strictly possible. Nevertheless, we are primarily concerned, not with these formal parameters, but rather with the acid-base properties of the abnormal phase with respect to particular weak acids. Note that most of the substrates and products of intramitochondrial reactions are weak acids.

The major finding such a study is hoped to yield is the "true" pH gradient between phase 1 and the medium; this is the appropriate variable to use in the work function defined by Mitchell.

E. The Membrane Potential and the Distribution of Ions

An analogous treatment of ion distribution data can be used for those ions which are in diffusion equilibrium across the mitochondrial membrane. This condition very nearly obtains for K^+ and Rb^+ in the presence of valinomycin. Once again, the ions are in equilibrium in all phases, and a well defined electrical potential difference must obtain between the normal phase and the medium. The magnitude of this potential can, in principle, be determined from f_1 , as before.

It is of interest to note that a potential difference should also exist between the normal and abnormal phases within the matrix.

F. The Distribution of Macromolecules and Metabolic Consequences of Phase Heterogeneity

1. *Macromolecular Localization.* Macromolecules will not, in general, be distributed uniformly throughout cell water. More specifically, soluble enzymes may be preferentially localized in or excluded from abnormal water, as is the case with other solutes. In contrast to small solutes, proteins are very likely associated with abnormal water layers due to their extensive surfaces. If these soluble enzymes were localized in the normal phase, the total interfacial surface within a given compartment would be magnified many-fold. The tendency to minimize this collection of interfacial surfaces may lead to association of proteins in the bulk phase (as occurs with hemoglobin in the red cell); however, an even greater reduction of interfacial surface will be achieved if the "soluble" enzymes are localized within the abnormal phase itself. The metabolic consequences of such localization are extensively discussed by Clegg (this volume) and the present analysis supports his model of enzyme segregation.

2. *Substrate Localization.* It also follows from this model that substrate concentrations in the abnormal phase will differ, in general, from those of the normal phase. Furthermore, the partition coefficients of substrates, products and modulators of enzymes will, in general, differ from each other. This generality may have profound consequences for the qualitative and quantitative interpretation of kinetic studies. It is true, of course, that thermodynamic activities must be the same in the normal and abnormal phases. It would be accurate, therefore, to measure concentrations in the normal phase (activity coefficients ≈ 1.0) as a reasonable estimate of activities in the abnormal phase. The consequence of the present model for kinetics arises from the fact that we normally use *total* concentrations as estimates of activity, and these may be very different from actual concentrations in the normal phase.

One of the most important modulators of enzyme activity is pH, and H^+ ions are substrates and products of many biological reactions. It has been noted by Wiggins (59) that

the dissociation constant of "structured" water may differ from that of bulk water.

3. *Reaction Kinetics.* Finally, the kinetics of a reaction are expected to be affected by the "structure" of the solution in which the reaction takes place. Once again, it is primarily our *perception* of this environment which may affect the interpretation of results. The concept that many reactions may take place in a milieu very different from a simple salt solution may provide a useful expansion of mechanistic possibilities.

G. Release from the Tyranny of "Bound" Water

Finally, the proposal that abnormal water constitutes a phase has an important liberating consequence for biologists. It frees us from the limiting view that osmotically inactive water, or "bound" water, must simultaneously be non-solvent. At this state of development, it cannot be said with absolute certainty that abnormal, vicinal water exists. What can be stated with confidence is that, if it exists, this water has solvent properties, and the abnormal phase is a solution.

NOTE ADDED IN PROOF

The heterogeneous phase model of cell water raises several questions about the aqueous milieu of the cell. As already stated, one such question is whether the phase boundary is continuous or abrupt. Can there be, as I have proposed, sharp phase boundaries between regions of abnormal water and normal (bulk) water? I will take this opportunity to call the reader's attention to additional results which bear on this question.

A review of water in biological systems by W. A. P. Luck (*Topics in Current Chemistry*, 64, 113-136, 1976) focuses on the microscopic structure of water. A logical development, beginning with the structure of pure water and

aqueous solutions, provides new insights into water structure with emphasis on the quantitative limits imposed by physical measurements. In pure liquid water at 25°C, only 10% of the OH groups are free; therefore, the number of water monomers must be vanishingly small. Furthermore, Professor Luck suggests that the free OH groups are not randomly distributed, but rather occur only at the surfaces of ice-like water aggregates. With this assumption, one can calculate the idealized cubic aggregate to contain about 300 water molecules at 25°C. It is of interest that the mean thickness of such an aggregate is about 7 water molecules, similar to that deduced for the abnormal phase in the present paper. Furthermore, Luck suggests that certain hydrophilic gel-formers interact with the free OH groups in the fissures, immobilizing the large aggregates. Dr. Luck's review lends support to the concept that there is an abrupt, rather than gradual, phase transition between the abnormal phase and the neighboring bulk aqueous phase.

The lifetime of these large aggregates is on the order of 10^{-12} sec, as deduced from dielectric or NMR measurements. That is, the fissures containing the free OH groups are moving rapidly through the isotropic liquid. The "flickering" of these clusters must, however, be somewhat static in regions near a surface, somewhat like a standing wave. This leads once again to a picture in which phase separation of the heterogeneous phase model is a sharp boundary at which the "orientation defects" of the bulk solution face those of the surface solution.

I should have noted in the manuscript that aqueous phase separations can readily be created in the test tube. For example, a mixture containing 1.1 g% of dextran and 0.36 g% methylcellulose in water will separate into two phases with a sharp phase boundary. Nevertheless, both phases contain more than 98% water. This type of aqueous phase separation is used in cell separation techniques and has been extensively discussed by Albertsson (P. Å. Albertsson, Partition of Cell Particles and Macromolecules, Wiley-Interscience, New York, 1971).

Finally, I wish to call attention to subscript errors in Figures 5 and 6 of this manuscript. In both figures, ϕ_s should read ϕ_e , referring to the osmotic coefficient of the external solution.

APPENDIX A: SYMBOLS USED IN PAPER

This appendix is a collection of the symbols, and their definitions, which are used throughout the paper.

1. Subscripts

Subscripts p, m, 0, 1, 2 designate the compartment or compartments to which these concentrations refer.

<u>'phase' subscript</u>	<u>designates</u>
0	external phase; the medium in which mitochondria are suspended.
p	mitochondrial pellet, including trapped media.
m	mitochondrial matrix.
1	hypothetical 'normal' phase.
2	hypothetical 'abnormal' phase.

Three solute classes are distinguished by subscripts e, x and s.

e = media solutes (sucrose plus buffer) which do not penetrate the matrix.

s = endogenous matrix solutes (primarily potassium salts and proteins) which are retained by the inner membrane.

x = nonelectrolyte probe which comes to equilibrium in all phases.

2. Internal Contents (moles/kg dry wt)

X₁, X₂ = nonelectrolyte in phases 1 and 2.

S₁, S₂ = endogenous solutes in phases 1 and 2.

3. Internal Concentrations (molal)

m_{x1} , m_{x2} = nonelectrolyte concentrations in phases 1 and 2.

m_{s1} , m_{s2} = endogenous solutes in phases 1 and 2: note that $m_{s1} \equiv \sum v_j m_{j1}$, $j \neq x$.

4. External Concentrations (molal)

m_{xo} = nonelectrolyte.

m_{eo} = impermeant solute, where

$$m_{eo} = \sum v_j m_{jo}, j \neq x.$$

5. Mitochondrial Water Spaces (kg H₂O/gm dry wt)

W_m = total matrix water content, determined experimentally.

W_2 = water in (hypothetical) osmotically inactive matrix phase 2.

W_1 = water is osmotically active matrix phase 1.
(Note $W_m = W_1 + W_2$).

W_{xm} = the 'water' of distribution of solute X in the matrix, experimentally defined by $W_{xm} \equiv X_m/m_{xo}$.

a = the experimentally determined intercept of osmotic swelling curves.

6. Solute Activity Coefficients

γ_{xo} , γ_{x1} , γ_{x2} = molal activity coefficient of solute X in phases 0, 1, 2.

γ_{xo}^o , etc. = molal activity coefficient at the designated concentration, m_{xo} , in a pure binary mixture of X and H₂O. Super-script o denotes the binary solution.

7. Osmotic Coefficients

ϕ_x , ϕ_s , ϕ_e = osmotic coefficients of pure solutions of x, s, e, resp.

ϕ_{xs} , ϕ_{xe} = osmotic coefficients of mixed solutions.

8. Solute Interaction Coefficients (molal⁻¹)

k_{ex} = interaction coefficient between e and x (external medium).

k_{sx} = interaction coefficient between s and x (internal phase 1).

${}^2k_{sx}$ = interaction coefficient between s and x in phase 2.

APPENDIX B: MULTICOMPONENT SOLUTION THERMODYNAMICS

A. *The Osmotic Coefficient and Solute Activity Coefficients*

The free energy of a system at constant temperature and pressure is a linear homogeneous function of the number of moles, N_j , of the system components. Therefore, using Euler's Theorem (63),

$$G(T, P, N_1, \dots, N_n) = \sum N_j \mu_j \quad (B1)$$

where

$$\mu_j \equiv \left(\frac{\partial G}{\partial N_j} \right)_{T, P, N_i} \quad (B2)$$

Differentiating both sides of equation (B1) leads, at constant temperature and pressure, to the Gibbs-Duhem equation:

$$\sum N_j d\mu_j = 0 \quad (B3)$$

The chemical potential of the solvent (water), μ_w , may be written

$$\mu_w = \mu_w^0 + RT \ln a_w , \quad (B4)$$

where μ_w^0 is the chemical potential of pure water. The chemical potential of the solute, μ_j , is

$$\mu_j = \mu_j^\theta + RT \ln \gamma_j m_j \quad (B5)$$

where μ_j^θ is the solute chemical potential in a hypothetical ideal 1.0 molal solution, and γ_j is the molal activity coefficient. Since m_j is the number of moles per kg of solvent, equation (B3) may be written, for the case of a single, non-dissociating solute:

$$\frac{1000}{M_w} d(\ln a_w) + m d \ln \gamma_m = 0 \quad (B6)$$

where M_w is the molecular weight of water. The osmotic coefficient, ϕ , is defined by

$$\frac{1000}{M_w} \ln a_w \equiv -\phi m \quad (B7)$$

Differentiating equation (B7), substituting equation (B6) and integrating over the composition variables leads to the relationship between the osmotic coefficient and activity coefficient of a nonelectrolyte:

$$(\phi - 1)m = \int_m d(\ln \gamma) \quad (B8)$$

Equation (B8) may be generalized as follows:

$$(\phi - 1) \sum v_j m_j = \int \sum v_j m_j d(\ln \gamma_j) \quad (B9)$$

where v_j is the number of species into which solute j dissociates ($v = 1$ for nonelectrolytes), and γ_j for a salt is the mean ionic molal activity coefficient.

In the case of a binary solution of nonelectrolyte and water, equation (B8) may be integrated if the dependence of the activity coefficient on concentration is known. Since $\gamma \rightarrow 1.0$ as $m \rightarrow 0$, the solute activity coefficient of a binary mixture of nonelectrolyte and water may be expanded in a Taylor's series:

$$\ln \gamma \approx 2\alpha m + \dots \quad (B10)$$

The osmotic coefficient of such a binary solution is readily obtained by integrating equation (B8):

$$\phi \approx 1 + \alpha m \quad (B11)$$

Since ϕ is experimentally accessible, equation (B11) provides a means to test the linear approximation of equation (B10). Lewis and Randall (47, p. 264) have plotted data for sucrose; at concentrations less than 1.0 molal, the deviation from equation (B11) is very small indeed. For the present purpose, therefore, it will be adequate to consider only the linear term in equation (B10).

B. Osmotic Equilibrium Between Normal Aqueous Phases

Osmotic equilibrium is the state in which, in the absence of pressure gradients, water activity, a_w , is equal in the two phases. Using equation (B7), the expression for osmotic equilibrium between an internal phase, 1, and the medium, 0, is therefore

$$\phi_s m_{s1} = \phi_e m_{eo} \quad (B12)$$

where $m_{s1} = \sum v_k m_{k1}$, representing the total ideal osmolal concentration of all solutes in phase 1, and $m_{eo} = \sum v_j m_{j0}$, representing the osmolal concentration of medium solutes. Solutes designated by subscripts s and e are incapable of crossing the membrane over the time course of these experiments (App. A). m_{s1} is not susceptible to direct measurement in mitochondria; however it may be written $m_{s1} = S_1/W_1$, where S_1 is the amount (moles) of osmotically active solute in phase 1, and W_1 is the water content. Rearranging, we achieve the expression for osmotic equilibrium,

$$W_1 = \frac{\phi_s S_1}{\phi_e m_{eo}} \quad (B13)$$

This analysis demonstrates that water content and molality are proper co-variables for osmotic equilibrium studies.

In the present experiments, the osmolality, $\phi_e m_{eo}$, was estimated by freezing point depression. There is a fundamental error in this estimation by virtue of the fact that the osmolalities refer to the temperature at which the solution freezes, rather than the temperature of the experiment. This error should be small in these studies, which were carried out at 0°C and in which solute concentrations were less than 1.0 molal.

C. The Setchenow Coefficient

The Setchenow coefficient refers to the effect of salts on the activity coefficient of a nonelectrolyte in aqueous solution. Nonelectrolytes may be "salted in" or "salted out". This is evidently applicable to the case at hand, where we could say that matrix urea is salted in by matrix solutes, while DMSO is salted out. The theory of salt effects on nonelectrolytes has received considerable attention since the review of Long and McDevit (64). In particular, the reader is referred to Krishnan and Friedman (65), Conway, *et al.* (66), Desnoyers, *et al.* (67), Aveyard and Heselden (68), Cross (69) and articles referred to therein.

Our interest is to focus on the thermodynamic relations between activity coefficients in multicomponent solutions. These relationships are developed below for a solution containing two nonelectrolytes and water. The thermodynamic theory for a ternary system was derived by Robinson and Stokes (70), to which reference may be made for a more rigorous derivation.

The activity coefficient, γ_1 , of solute 1 may be expressed in a Taylor's series

$$\ln \gamma_1 = \sum_i \sum_j A_{ij} m_1^i m_2^j \quad (B14)$$

All terms in $j = 0$ are functions solely of m_1 , and hence represent $\ln \gamma_1^0$ for a binary solution of solute 1 and water. Retaining only the linear term in m_2 , equation (B14) may be approximated by

$$\ln \gamma_1 \approx \ln \gamma_1^0 + k_{12} m_2 \quad (B15)$$

where k_{12} represents the influence of m_2 on γ_1 and is similar in definition to the Setchenow coefficient. From equation (B3) and equation (B5), it follows that

$$\frac{\partial \ln \gamma_1}{\partial m_2} = \frac{\partial \ln \gamma_2}{\partial m_1} \quad (B16)$$

therefore

$$\ln \gamma_2 \approx \ln \gamma_2^0 + k_{12} m_1 \quad (B17)$$

These approximations permit equation (B8) to be integrated, resulting in an additivity rule of improved accuracy:

$$\phi_{12}(m_1 + m_2) = \phi_1 m_1 + \phi_2 m_2 + k_{12} m_1 m_2 \quad (B18)$$

In equation (B18), ϕ_{12} is the osmotic coefficient of the solution containing both solutes at concentrations m_1 and m_2 . ϕ_1 is the osmotic coefficient of a solution containing only 1 solute at concentration m_1 , and ϕ_2 has the equivalent definition. To gain some idea of the effect of the approximations made in equations (B10) and (B15), we may refer to Ellerton and Dunlop (40), who carried out a careful isopiestic study of the ternary system, urea - sucrose - water. They obtained expressions for the terms in equation (B18) up to 7.0 M in urea and 4.3 M in sucrose at 25°C. The osmolality of a solution 0.5 M in both urea and sucrose (comparable to the highest concentrations used in the mitochondrial experiments) is 0.98193 OsM, retaining all terms in their equations. Dropping the higher-order terms (i.e., using equation (B8) with $k_{12} = -0.12597$) gives an osmolality of 0.97515 OsM, an "error" of -0.7%. Dropping the last term in equation (B18) entirely leads, with the same concentrations, to a total osmolality of 1.00664 OsM, an error of +2.5%.

For a nonelectrolyte and a salt dissociating into v ions, equation (B18) becomes

$$\phi_{12}(m_1 + vm_2) = \phi_1 m_1 + \phi_2 vm_2 + k_{12} m_1 m_2 \quad (B19)$$

D. Effective Satchenow Coefficients

The interior phase of mitochondria contains a variety of unspecified solutes, and it may appear impossible to apply the above relations to such a system. However, the experimental condition that it is possible to hold internal solutes constant with swelling permits such an application. The expansion for the nonelectrolyte, X, may be written

$$\ln \gamma_X = \ln \gamma_X^0 + \sum k_{Xj} m_j \quad (B20)$$

where the summation in this and subsequent equations are taken over all solutes except $j = X$. Similarly, by virtue of equation (B16),

$$\ln \gamma_k = \ln \gamma_k(m_X = 0) + k_{Xk} m_X \quad (B21)$$

where $\ln \gamma_k(m_X = 0)$ refers to the activity coefficient of solute k in the absence of nonelectrolyte. (All endogenous

solutes are treated as nonelectrolytes for simplicity. The final result will not be affected). Equation (B9) becomes

$$\begin{aligned} (\phi_{sx} - 1)(m_x + m_s) &= \int m_x d \ln \gamma_x^0 \\ &+ \int \sum m_k d \ln \gamma_k (m_x = 0) + \int \sum k_{xj} d(m_x m_j) \end{aligned} \quad (B22)$$

where $m_s \equiv \sum m_k$, the total molal concentration of endogenous solutes (excluding nonelectrolyte). By virtue of previous definitions, the first two integrals are

$$(\phi_x - 1)m_x + (\phi_s - 1)m_s \quad (B23)$$

where ϕ_s is the osmotic coefficient of the internal phase in the absence of nonelectrolyte. The last integration of equation (B22) is achieved as follows: if total solute content is maintained constant during swelling, each solute remains in a constant ratio to total solute content. That is, $m_k = \lambda_k m_s$, and each λ_k is constant. The final integration then becomes

$$\int \sum k_{xj} f_j d(m_x m_s) = k_{xs} m_x m_s \quad (B24)$$

where $k_{xs} \equiv \sum k_{xj} \lambda_j$, the "effective" Satchenow coefficient for the internal phase. The additivity rule for the osmolality of phase 1 in mitochondria containing nonelectrolyte, X_1 , and endogenous solutes, S_1 , is therefore analogous to that of a ternary system:

$$\phi_{xs}(m_{x1} + m_{s1}) = \phi_s m_{s1} + \phi_x m_{x1} + k_{xs} m_{x1} m_{s1} \quad (B25)$$

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METABOLISM AND THE INTRACELLULAR
ENVIRONMENT: THE VICINAL-WATER
NETWORK MODEL¹

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I. INTRODUCTION

It seems to be widely believed that much of the metabolic activity of eucaryotic cells occurs in what amounts to an aqueous solution, containing about 20% protein and variable concentrations of metabolites and ions. I refer here to the "soluble phases" of cells; namely, the cytosol, nucleoplasm, and the interior compartments of various membrane-bounded cytoplasmic organelles. Although a significant fraction of the enzymatic complement of cells is known to be "particulate" in the sense of being an integral part of various cellular structures, it would appear that a large proportion of metabolic reactions, perhaps the majority, are catalyzed by enzymes believed to be located exclusively, or in large part, in these "soluble phases" (1). Furthermore, the enzymes, and other components of the soluble pathways they make up, are believed by many to function and be regulated by what amounts to ordinary statistical mass-action mechanisms, operating by essentially random 3-dimensional diffusion of the molecular participants.

The belief outlined very briefly above seems to me to be the prevailing or "consensus view", as I will refer to it

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here. In many ways, this view is really not greatly different from the old "bag-of-enzymes" notion so popular in the early days of biochemistry. The consensus view modifies this only in terms of certain enzymes that are embedded in, or tightly held by various membranes and other structures such as ribosomes, and the occurrence of soluble multi-enzyme complexes. I am confident that few, if any, would actually refer to cells as "bags of enzymes" these days. Nevertheless, it seems to me that this conception remains firmly entrenched with regard to metabolic activity occurring in the cytosol, nucleoplasm, and interior compartments of cytoplasmic organelles. While it is certainly true that not everyone agrees with this view of the intracellular environment, the extent of disagreement in my opinion has been rather minor; in any event, it has had relatively little impact on contemporary thought, judging from the scientific literature and the coverage given this topic in current textbooks (1). The latter consideration is not trivial since it can be assumed to not only represent the prevailing view, but to provide the basis upon which future scientists are trained. A major purpose of this paper is to challenge certain features of the consensus view, and present an alternative.

II. EVIDENCE FOR THE CONSENSUS VIEW

I think it is instructive to briefly consider how the consensus view arose: what is the experimental basis for this picture of the intracellular environment? I believe most of it comes from three general sources: 1) *Cell Fractionation Studies*. The cytosol is generally defined as that fraction of cells and tissues that remains soluble after all particulates have been sedimented by centrifugation of homogenates usually prepared with dilute buffer solutions. Likewise, membrane-bounded organelles are first isolated, and their "soluble" contents released by various treatments. Thus, on this basis, the majority of the Krebs cycle enzymes appear to be dissolved in the mitochondrial matrix, the Calvin cycle enzymes in the chloroplast stroma, and so forth. This approach has clearly provided the bulk of the evidence. 2) *Reconstitution Experiments*. Here the velocity and regulation of a pathway believed to exist in one of the soluble phases of cells can be approximated by reconstruction of the components of the pathway *in vitro*. The implication of this is that one can account for intracellular events by dilute solution studies *in vitro*.

3) *Intracellular Water.* The widely held belief that almost all of the intracellular water is no different than ordinary bulk water permits the reality of a conventional cytosol, nucleoplasm, etc. Indeed, such a notion is mandatory. This consideration is also closely related to the "bag of enzymes" idea referred to earlier, and its origin can be traced back to the early days of modern biochemistry (2).

The consensus view, generated by this and other evidence, has apparently worked very well in explaining what is known about the nature of metabolism and its regulation, and it is understandable that most have been willing to go along with something that appears to work so well. However, this does not guarantee that such a view is entirely correct. Later in this chapter I will take up some of the evidence, from a diverse array of experiments, that is hard to reconcile with the consensus view; indeed, some of the evidence is directly contradictory. First, however, a few remarks on the evidence for the consensus view outlined above.

It should be appreciated that not all fractionation studies have produced the same results. For example, Green, et al. (3) concluded from their work that the enzymes of the glycolytic pathway are "associated" with the membrane fraction in yeast and red blood cells, Mowbray and Moses (4) presented evidence that these enzymes exist in *Escherichia coli* as a multi-enzyme complex particle, while de Duve (5) concluded that the enzymes of the glycolytic pathway are freely dissolved in the cytosol of liver cells, and he somewhat dogmatically implies this to be the case for most cell types. The well known micro-localization of glycolytic enzymes in skeletal muscle has been considered in an excellent recent review by Clarke & Masters (6). Those authors also consider the consequences of "enzyme binding" versus "soluble pathways".

One must read these papers very carefully to decide who makes the stronger argument since the particular experimental techniques employed and the cell type under study are crucial to the interpretation. The analysis by Clarke & Masters (6) emphasizes this point. It is my opinion that all research carried out on the micro-localization of enzymes using disrupted cells (and a great deal could be cited) must carry with it a significant measure of uncertainty inherent in this kind of study, a view that I think many would agree with. While it is true that the bulk of the evidence may be consistent with the consensus view, such experiments in my opinion cannot be decisive in settling the issue either way.

As to the second category of evidence presented in support of the consensus view, I can only say that I agree: reconstitution experiments can work. I would add, however, that this has only been examined in any detail for the glycolytic pathway, as far as I have been able to ascertain. In any case, one is not compelled to believe that the outcome of such studies provides conclusive evidence for the consensus view of the intracellular environment, only that the results can be taken as consistent with this interpretation.

Finally, the long held view that the physical structure of almost all intracellular water is not significantly different from that in a relatively dilute aqueous protein-electrolyte solution has been challenged by a large amount of data generated in several different laboratories. I will consider this topic in more detail later. For now, however, I would like to emphasize that if the structure of even a significant fraction of the total intracellular water is proven to differ appreciably from "bulk water", then this should be considered in studies dealing with the location and operation of "soluble" pathways within the cells. In my opinion, such proof is available.

While much debate on the issue of the physical state of intracellular water has centered around solute distribution and active transport, it seems that the overall *metabolic* consequences have largely been ignored. I will also consider this at some length. First, let us consider some examples that, to greater or lesser degree, suggest that revision of the consensus view may be required.

III. STUDIES ON STRATIFIED CELLS

In two very carefully executed studies on *Euglena*, Kempner and Miller (7) presented evidence that no macromolecules, examined by a variety of sensitive cytochemical methods employing 31 different stains, could be detected in the soluble zone of intact cells stratified by centrifugation. The cells, incidentally, remain viable after centrifugation. A total of 19 enzymes was also searched for, but none were detected in the "soluble phase" of the stratified cells. However, when these authors fractionated the cells by conventional procedures, several selected "cytosol enzymes" were easily demonstrated in the "soluble fraction" or cytosol. In a more limited study, Zalokar (8) arrived at a similar conclusion using stratified *Neurospora*.

These results are, of course, contrary to expectation in terms of the consensus view of the intracellular environment. Although not completely free from interpretive difficulty, the conclusion of such studies seems evident: the cytosol has a much different meaning in intact cells compared with disrupted ones. Kempner and Miller (7) suggested that the macromolecules were somehow "associated" with the particulate components of the cells, but did not further consider the nature of this association. However, one thing seems clear: such "associations" are readily disrupted by most cell fractionation procedures, an important point I would ask the reader to remember.

IV. WATER-REPLACEMENT AND CELL DEHYDRATION

Extensive work by Webb and associates (9) on aerosols of vegetative bacterial cells that are not inherently resistant to desiccation has consistently shown that significant dehydration damage does not occur until about 90% of the total water has been removed, only about 0.3g water/g dried cell solids remaining. Although the metabolism of the cells was not monitored in their studies, the results must mean that all ongoing metabolic activity during the course of, and after dehydration to various water contents was not significantly upset, in spite of the fact that the cells had lost variable amounts of water and, in the extreme, virtually all of their "cytosol" water. The point should be made that the amounts of "non-freezable" water in a variety of cell types, ranges over 0.25-0.8gH₂O/g cell solids (10). This fraction of intracellular water is apparently not bulk water in any sense of the term. When considered in the context of Webb's studies such findings suggest the possibility that these cells can metabolize without the presence of a bulk aqueous phase.

The results of Morowitz (11) are similar to those of Webb, although based on a different experimental approach. He showed that about 30% of the total water of two bacterial species, also not particularly resistant to water loss, could be replaced by glycerol without stopping the growth and normal division of the cells. His conclusion is provocative: "*The high degree of specificity necessary for bacterial replication is not connected with rate processes taking place in the aqueous phase, but must be sought elsewhere.*" This conclusion should also be evaluated in terms of the consensus view of "soluble" metabolic pathways.

A variety of mammalian cells, including those from humans, can tolerate the loss of about 30% of their total water and survive (for review see 12). A specific example of much interest in the context of the present paper is the observation that glucose metabolism in rat liver cells is not significantly altered, in rate and direction, by a loss of about 25% of the intracellular water (12), a rather surprising observation, in my judgment, if one considers it within the context of the consensus view of soluble metabolic pathways. Other examples of a similar nature can be cited.

The area of cryobiology also provides information of interest, since it seems clear that cellular dehydration, due to removal of liquid water as crystals or by osmosis, is responsible for much of the damage associated with exposure to low temperatures. Meryman, a leading investigator in this area, has advanced a notion called the "minimum cell volume model" to explain the damage inflicted upon cells by exposure to low temperatures (13). In the latter paper (p. 262) the statement is made that "*In all the animal cells we have studied, no adverse effects are seen until a volume is reached that corresponds to the loss of approximately 65 percent of the cell liquid.*" Nei (14) concludes from his extensive research that most microbial cells exhibit similar properties and Billingham and Medawar (15) found that even mammalian skin could lose as much as 75% of the total water without killing all the cells as judged from grafting experiments. Of course, the low temperature conditions prevailing in some of these studies limit, to some extent, their metabolic significance. On the other hand, it is well known that a wide variety of microbial, plant, and animal cells can lose very large percentages of their total water without loss of viability at ordinary temperatures (see 16 for references to the vast literature on this subject).

Taken together, I believe that all of the observations noted in this section strongly suggest that marked fluctuations in the total amount of water in cells may have remarkably little effect with regard to the qualitative, and in some cases quantitative, nature of their metabolism. I hasten to add that this alone certainly does not disprove the consensus view of the occurrence of "soluble" metabolic pathways. However, it is worth considering such experimental findings within this context. We should ask how these cells can maintain metabolic balance and be able to regulate their metabolism properly in the face of such wide fluctuations in cellular water content. According to the consensus view, such fluctuations can be expected to result in constantly changing concentrations not only of enzymes,

but of all the other components of the soluble pathways which, if operating in free solution as this view requires, must nevertheless be regulated in rate and direction by simple mass action mechanisms. This would seem to pose insuperable problems as different cell parts lose water differentially. On the other hand, if these pathways are not really taking place in free solution, but are instead due to enzymatic reactions occurring somewhere other than the "bulk aqueous phase", then the consequences of removing variable amounts of the total intracellular water might be far less serious. The question then arises as to the intracellular location of such metabolic pathways, if we assume for the moment that they are not occurring in the bulk aqueous phases of cells. This question is considered next.

V. INTRACELLULAR INTERFACES AND VICINAL WATER

The relationships existing between intracellular interfaces, water adjacent to those surfaces (vicinal water), and ongoing intermediary metabolism are, in my opinion, of great potential importance to any assessment of the intracellular environment, but have not, of late, been given the attention they deserve. A great deal of evidence obtained from the study of various systems, biological and otherwise, has shown that water near interfaces (vicinal water) exhibits properties that differ appreciably from bulk water (see Drost-Hansen, 17, and the present volume). The evidence, in my opinion, is overwhelming that this is so. However, much uncertainty exists regarding the detailed structure, properties, and distance from the surface over which the water is so influenced. In fact, at present it isn't even clear whether such vicinal water exhibits more, or less, order than bulk water. Although investigators believe that this influence can extend only over a few molecular diameters of water from the surface, perhaps no more than 3-5, there is a body of evidence, obtained chiefly from inanimate systems, that indicates the water is perturbed for distances on the order of 300-1,000 Å (17). Water vicinal to various membranes, biological and otherwise, appears to be perturbed over about 25 Å, at the very least (17,18). That this is an extremely important consideration is evident if one takes into account the enormous interfacial surface area in the ordinary eucaryotic cell. One of my colleagues at the University, Dr. Peter Luykx, called to my attention the careful work of A. V. Loud (19) in which quantitative surface area estimates of certain membrane systems of normal rat

liver cells were calculated from electron microscopy. I have summarized some of Loud's estimates in Table I.

TABLE I

Estimates of Some Membrane Surface Areas and Hydration of Rat Liver Cells

A. Hydration and cell numbers^a

Number cells/g fresh weight	1.28 x 10 ⁸
Number water molecules/cell	1.81 x 10 ¹⁴

B. Membrane surface areas (μm^2)^b

Smooth endoplasmic reticulum	16,900
Rough endoplasmic reticulum	25,400
Mitochondria (inner & outer membranes)	<u>44,220</u>
TOTAL	86,520

C. Estimates for vicinal water (per cell)

No. of Water Layers	Thickness (Å)	Cumulative Water Molecules ($\times 10^{-14}$)	% of Total Cell Water
1	2.8	0.011	0.6
3	8.4	0.033	1.8
5	14.0	0.055	3.0
10	28.0	0.111	6.1
25	70.0	0.277	15.3
50	140.0	0.555	30.7

^aThese calculations use the data of Seglen, P. O. (1973), *Exp. Cell Res.*, 82, 391, obtained for isolated rat liver cells, the water content of which was 69% of the wet weight after correcting for glycogen loss.

^bData from Loud, A. V. (1968), *J. Cell Biol.*, 37, 27.

These data apply only to the endoplasmic reticulum and mitochondria of mid-zonal cells, the surface areas for these structures being less than those of central cells, but greater than for peripheral cells. Obviously, such estimates involve appreciable uncertainty, but even allowing for this, the amount of membrane surface area is very large indeed. It is instructive to estimate the amount of water that could be influenced by these surfaces alone, using 2.8 \AA as the thickness of, and $7.8 \times 10^{-8} \mu\text{m}^2$ as the surface area covered by one water molecule (Table I). An estimate of the total amount of water in the average liver cell is also given. If we now consider the surface-perturbed water to exist as a layer 25 \AA thick (17,18) the calculations suggest that roughly 5% of the total cellular water will be so involved. This figure seems minimal because the surface area calculations (Table I) assume "molecular smoothness" for the membrane surface. Clearly, the *actual* surface area (i.e., that accessible to water) must exceed the estimates, perhaps by a factor of 2. Of course, it is also possible that more than 5-10 layers of water are perturbed by the membrane surface. In view of these considerations, and emphasizing that these membranes represent only a small fraction of the total available surface, it seems inescapable that a large fraction of the total cellular water must be strongly influenced by proximity to membranes and other ultrastructural surfaces.

At this point reference can also be made to more recent electron microscopical evidence that exceedingly large numbers of actin-containing microfilaments ramify throughout the cytoplasm of many eucaryotic cells (96) being much more abundant than earlier work had indicated (see, for example, 20). Arrays of microtubules may be extensive in some cells, and there is also evidence (20) that the cytoplasmic area of a number of cell types contain an intricate 3-dimensional lattice of filaments averaging $30-60 \text{ \AA}$ in thickness (microtrabeculae), the surface area of which must be enormous. I imagine this meshwork of dense cytoplasmic strands to be somewhat like a bath sponge in 3-dimensions, the channels within the network being of the order of $500-1000 \text{ \AA}$. I believe that this recently discovered micro-organization of the cytoplasm will prove to be of paramount metabolic importance, and I will consider it in this connection later in more detail. It may be noted now, however, that this network is dynamic in the sense that it is modified during various cellular activities (i.e., passage through the cell cycle, motility, etc.). In other words, the cell apparently can control the amount and disposition of its "cytoplasmic sponge".

Of some importance to the topic of this section is the structure of the interior compartments of membrane-bound organelles, in particular the mitochondrion and nucleus. There is considerable doubt that the mitochondrial matrix is simply a "structureless gel". In fact, there is evidence that the matrix seems to be composed, in part, of an intricate network of tortuous branching strands of protein. The excellent work of Pihl and Bahr (21) on critical-point dried rat liver mitochondria describes this "matrix-skeleton" as a branched network of strands (about 300 Å in diameter) generating a total length of 80 μm per mitochondrion and constituting roughly one-third of the total mitochondrial protein. Thus, if we take this to be a smooth solid rod, the surface area per mitochondrion would be at least $7.5 \mu\text{m}^2$. This area should be compared with the previously mentioned measurements of Loud (19) who estimated that a single rat liver mitochondrion has an outer-membrane surface area of about $7 \mu\text{m}^2$ and a total inner membrane surface area of about $27 \mu\text{m}^2$. In other words, the "matrix skeleton" makes up roughly 18% of the total ultrastructural surface area in this organelle. (In this connection, see the article by Garlid in this volume). I should point out that the "matrix-skeleton" surface area is not included in the calculations summarized in Table I.

A significant question concerns the relationships between mitochondrial surfaces and the enzymes that are housed within the mitochondrial matrix. As mentioned earlier, the consensus view of the intracellular environment holds that most of the enzymes of the Krebs cycle are freely dissolved in the mitochondrial matrix material. However, there is reason to believe that the Krebs cycle enzymes actually may be located near or on the inner mitochondrial membrane (22-25). I will show later why it is important to note that these investigators view the association between membrane and Krebs cycle enzymes as "a very loose one". Whether or not the "matrix-skeleton" of Pihl and Bahr (21) could also serve as a focus of association for these enzymes has not been evaluated, but certainly remains a candidate for such a function. I believe the foregoing casts much doubt on the view that the Krebs cycle takes place essentially in free solution within the mitochondrial matrix. I have not searched the literature for comparable studies on the location of the Calvin-Bensen cycle enzymes in the chloroplast stroma. It would be interesting and of importance to know whether a similar situation occurs in that organelle.

Recent studies on the ultrastructure of nuclei have revealed the presence of a non-membranous array consisting of rod-like and annular structures, interconnected by strands (26,27). This network, in some ways analogous to the cytoplasmic trabecular network (20), is so extensive that it would be extremely difficult even to attempt an estimate of its surface area. Suffice it to say that such a network, plus the chromosomes, other intranuclear structures, and the nuclear-envelope, provides what must be a massive total surface area per unit volume of nucleus, and that a corresponding amount of the nuclear water should be strongly influenced by these surfaces. To my knowledge, no studies have been carried out on the association of nuclear enzymes with this network.

Note should be made of extensive evidence that several enzymes of the glycolytic pathway in skeletal muscle tend to "associate" with the ultrastructural components of this tissue (6). These associations depend strongly on pH and ionic strength, are generally fully reversible, and can be influenced by certain metabolites. Current thinking seems dominated by the view that direct "high energy" interactions between groups on the ultrastructural surface with groups on the enzyme are responsible for these associations. While this may be so, the possibility that water-water interactions might also be involved has, to my knowledge, not been given much attention (69). It is clear, however, that the extensive surface area present in this tissue also allows for the potential involvement of vicinal water in such enzyme-surface interactions.

We should now consider what has been described in this section in the context of the consensus view of "soluble metabolic pathways". Apparently, we must suppose that the presence of such vast intracellular surface areas has little effect on the assumed "dilute solution" that presumably provides the matrix for so much of cellular metabolism. In my mind, this is highly questionable. The model I will present later rests heavily on the metabolic participation of vicinal water existing near intracellular surfaces. But in addition to the general issue of the potentially important role of vicinal water in enzyme micro-localization in cells, the foregoing account of intracellular interfaces raises doubt, in my opinion, as to whether very much of the total intracellular water could find itself far enough from some surface to exhibit the properties of bulk water. Yet, as we shall see in the next section of this paper, most of those engaged in research on the physical state of intracellular water

maintain that the overwhelming majority behaves like neat water. This seems to me to be a paradox of the first order!

VI. THE CURRENT CONTROVERSY SURROUNDING THE PHYSICAL STATE OF INTRACELLULAR WATER

This controversy is well known and it is not my intent to undertake a detailed and critical review of the matter. Yet, it is central to the issues under examination here, and must be briefly considered. Opinions range from the view that all the water in cells is different from bulk water (see Ling and Walton, 28, and the article by Ling in this volume for reference to evidence given over the last 15 years in support of this view, the "association-induction hypothesis") to the opposite extreme, the conventional or majority view, in which very little cell water, perhaps 5-10% of the total, is believed to exhibit properties that differ from water in dilute solutions (reviewed by Cooke and Kuntz, 10). The position of Hazlewood, Chang and their associates (see Hazlewood, this volume) at the present time seems to me to be somewhat intermediate, although clearly much more in line with the association-induction hypothesis than with the "majority view". Although the investigators interested in this topic have employed a variety of techniques, nuclear magnetic resonance spectroscopy (NMR) has provided the bulk of the most recent data. The reader will have to cope at first hand with the extensive literature on this topic to fully appreciate the complexity of the situation. My own position is that sufficient evidence has been gathered to permit the conclusion that an appreciable percentage of the total water in cells, of the order of 30% at least, has properties that differ from bulk water. With regard to the precise amount of such perturbed water, and the issue of different sub-populations of intracellular water exhibiting different structural properties in different cell types, I believe we must await the outcome of further research. However, it is not possible for me to accept the view of some (29,30) that only one or two percent of the total intracellular water differs from bulk water. I believe this doubt is fully justified by considerations already taken up in this article, and additional topics I will consider later.

Other "probes" of intracellular water have been employed in addition to NMR, and the results of these studies should also be considered. Experiments in which the behavior of

various solutes has been studied in different cell types have been interpreted to mean that roughly 75% of the total intracellular water behaves like that of an ordinary aqueous solution while the remainder exhibits significantly different osmotic and solvent properties (for examples, see 31 and 32). The elegant work of Horowitz and Paine (32) is particularly convincing in this regard. Consequently, it can be concluded that, with regard to the behavior of such "probes", about one third of the intracellular water differs in its solvent properties from ordinary liquid water. Garlid (33, and this volume) has carried out some important work on solute distribution and the state of water in isolated mitochondria. His findings, interpreted as a "2-phase" model, are that about 25% of mitochondrial water is "abnormal" with regard to its solubility properties for non-metabolizable low molecular weight solutes.

While these "solute distribution studies" have usually been interpreted in terms of essentially 2 phases of cell water, one of which is bulk water, it is not clear to me how the possibility of altered solvent properties for all cell water is ruled out.

The behavior of spin-labels in cells as measured by electron spin resonance spectroscopy (ESR) is also of much interest. Although some disagreement exists (34) the consensus view seems to be that the effective viscosity of the intracellular environment as "seen" by the diffusing probe is in the range of 1-5 cp (35,36,37), being not greatly different from that of water in bulk aqueous solutions. Furthermore, most authors state that the probe, depending on the type used, samples a very large fraction of the total cellular water. It must be kept in mind that such studies are by no means free from interpretive difficulty. The conclusions arrived at often rest very heavily on the nature of the probe used, and the assumptions made concerning binding of the probe, and the extent to which the probe measures all of the cellular water. Nevertheless, the study by Sachs and Latorre (35) is particularly interesting because they measured the probe's behavior (TEMPOL) as a function of water content in giant barnacle muscle cells. They concluded that as much as 75% of the total muscle water could be removed without producing a qualitative change in the spectrum. This seems remarkable if viewed in terms of the consensus view of the "soluble phases" of cells which suggests that the solute concentration in the soluble compartments should increase markedly as water is removed. Indeed, one should expect an increase in viscosity due only to the increasing protein con-

centrations, which are usually considered to be in the vicinity of 20% in the "soluble phase" of fully hydrated cells. Yet, this was not observed by Sachs and Latorre. In a subsequent discussion of these results Sachs (36) made note of this somewhat paradoxical observation and provided the interesting comparison that doubling the concentration of a glycerol solution of 5 cp viscosity causes a 100-fold increase in its viscosity, whereas removing half the total muscle water resulted only in a doubling of the intracellular viscosity. Sachs (36) provided an explanation of his own for how this might come about. In view of what has been written thus far in the present article, I would like to offer an alternative interpretation of these data: namely, that the cytosol, nucleoplasm, and other "soluble" compartments may actually contain only a very low concentration of proteins and other large molecules in *free* solution, such macromolecules being instead associated with various surfaces within the cell. If this were the case and the probe sampled primarily the bulk aqueous phase, as the authors maintain, its behavior might not be expected to be excessively damped by removal of quite large amounts of water, since the probe would still find itself in a highly aqueous environment containing a relatively low concentration of solutes (inorganic and metabolites) that would contribute appreciably to viscosity as dehydration occurred.

I find the recent ESR results of Henry, *et al.* (38) very important in this connection. Using a variety of spin-probes having different affinities for water, they evaluated the probes' behavior in yeast mutants lacking fatty acid synthetase activity. Such auxotrophs eventually die when deprived of saturated fatty acids in culture. These authors observed that while an increase in the restriction of molecular rotational diffusion of various spin labels dissolved in the *aqueous phase* of cells of cells occurred during fatty acid starvation, the apparent state of cellular hydrocarbons remained essentially the same under these conditions. This research group (Keith and Snipes, 39) also found that another yeast auxotroph, requiring inositol for growth and viability, exhibited essentially the same behavior. Thus, it is the *aqueous phase* in which the abnormal processes occur in both mutants, even though deficient for different components of membrane structure. The authors suggested that the death of the cells was due to an alteration in the normal diffusion of metabolites within the aqueous compartments of the cells. Naturally, I find this possibility very attractive, since it agrees nicely with the importance of membrane surface and vicinal water interactions as described earlier in this paper,

as well as the model I will describe later.

A final point regarding the physical state of intracellular water concerns the inability of a large fraction of it to fit into the crystal lattice of ice when cells and tissues are cooled to temperatures well below the freezing point of water, the so-called "non-freezable water". Based on various calorimetric studies (DSC and DTA) and low temperature NMR work, the amount of such water ranges from 0.3-0.8 g/g dry wt. of cell or tissue solids (for review see 10). In other words, an average of roughly 25% of the total water. It is, of course, tempting to relate this "repetitious figure" to the amount of water that appears abnormal in terms of the solute distribution studies since they are roughly the same. I believe this temptation should be avoided since one is not compelled at present to believe they both must represent the same fraction of cell water. Nevertheless, such findings do provide further evidence that a significant fraction of cellular water, at least of the order of 25%, does indeed behave differently from bulk water.

In conclusion, it appears to me that the only thing that can be said with any degree of agreement and confidence at the present time is that a significant fraction of intracellular water does indeed differ in its properties from "bulk water", and that the amount of such "unusual" water is a function of the probe used to measure it, a point made previously by several authors (10,40). Another important variable seems to be the particular investigator interpreting the data, particularly the model-dependent NMR results.

I would like to stress again that while the question of the physical status of intracellular water is of exceptional importance to many areas of cell biology, the roles that such abnormal or "non-bulk water" may play in the overall metabolism of cells has been given considerably less attention than it deserves. James (41), who is not particularly sympathetic to the view that the majority of cell water differs from bulk water, puts it this way:

"Whether such ordered water exists, and if it does exist, whether it differs significantly from pure liquid water, are questions still open to argument. The majority viewpoint would appear to be the classical conception (with possible modifications); the onus largely is on the minority viewpoint to show that cellular

water differs from ordinary water. If the minority viewpoint is ultimately proved, it may have tremendous impact on currently held ideas concerning bioenergetics."

I would suggest that the last word in this quote could easily be changed to "metabolism". It seems surprising to me that so very little research has been devoted specifically to an examination of the interrelationships that are likely to exist between cellular metabolism and the structure of intracellular water, other than that associated with the energetics of ion and other solute transfers across cell membranes. Although the recent work of Beall, *et al.* (42) and Beall (this volume) represents a good start in this direction, current views on the nature of cellular metabolism largely ignore the involvement of water except in its obvious roles as a substrate or product of a number of enzymatic reactions, and as a bulk solvent for metabolites and other cellular constituents. Yet, it is very well known that water plays a direct and crucial role in the structure, stability and therefore function of proteins and nucleic acids, as well as a number of other cellular components. By definition, therefore, alterations in the nature and disposition of water in cells can be expected to have profound effects on intermediary metabolism. While most would agree with this point of view, very few have actually attempted to analyze the relationships. It would seem that those interested in metabolism and its control have simply forgotten, or more likely, are not interested in the potential significance of cell water in this capacity. On the other hand, those involved in the description of the physical state of cell water have done the same for metabolic considerations. Both groups seem to neglect the recent discoveries in the area of cellular ultrastructure described in the preceding section.

Since no one seemed to be examining such interrelationships, an interdisciplinary research program was initiated in 1973, the participating laboratories being those of Dr. Carlton Hazlewood (Baylor College of Medicine), Dr. Walter Drost-Hansen (University of Miami) and my own. The overall objective was to perform a detailed analysis of the physical structure of intracellular water, and to examine a variety of selected metabolic parameters, both as a function of water concentration in living cells. Any correlations revealed by this initial phase of the program would then be further analyzed for possible causal relationships. For this work we chose a biological system (the cellular cysts

of the brine shrimp, *Artemia salina*) that is capable of undergoing repeated cycles of dehydration and re-hydration and exhibits a number of other experimentally useful characteristics (48,53). In the next section I will describe this model system in some detail, and then briefly summarize our findings.

VII. INTERRELATIONSHIPS BETWEEN WATER AND METABOLISM IN ARTEMIA CELLS

Sexual varieties of *Artemia salina* (a primitive crustacean) reproduce either viviparously or oviparously. In the latter case, encysted dormant embryos or "cysts" are released into the environment which is usually brine, as the species is found in highly saline lakes and salterns (48). Desiccation is the *normal* biological fate of these cysts, and they can undergo repeated cycles of hydration and dehydration without irreversible damage. The cysts are available commercially in kilogram quantities, and can also be produced in gram quantities in large-scale laboratory cultures. All studies we have published since 1972 have been carried out on a large standardized population of cysts stored at -20°C under nitrogen gas, and all work described here has utilized the same population, the viability of which (88%) has not changed during this time.

Figure 1 illustrates some of the structural features of these cysts. The insert at the upper left shows the histological picture obtained from light microscopy of a fully hydrated cyst in cross section. About 0.2 mm in diameter when fully hydrated, the cyst is composed of an embryo at the early gastrula stage (43,44) and an outer complex but acellular shell. Purified preparations of the shells are readily obtained by allowing the embryos to develop and leave the shell behind as they hatch into motile nauplius larvae (45). By determining the behavior and properties of isolated shells, we can evaluate contributions from the internal cellular mass by experiments performed on whole cysts under comparable conditions. There are roughly 4,000 cells in each cyst (46,47) and it appears that we are dealing here with an essentially undifferentiated mass of cells (43,48). That the internal mass of the cyst is cellular, and not syncytial as suggested earlier by Dutrieu (44) is evident from the massive study of Benesch (43) and our own unpublished results.

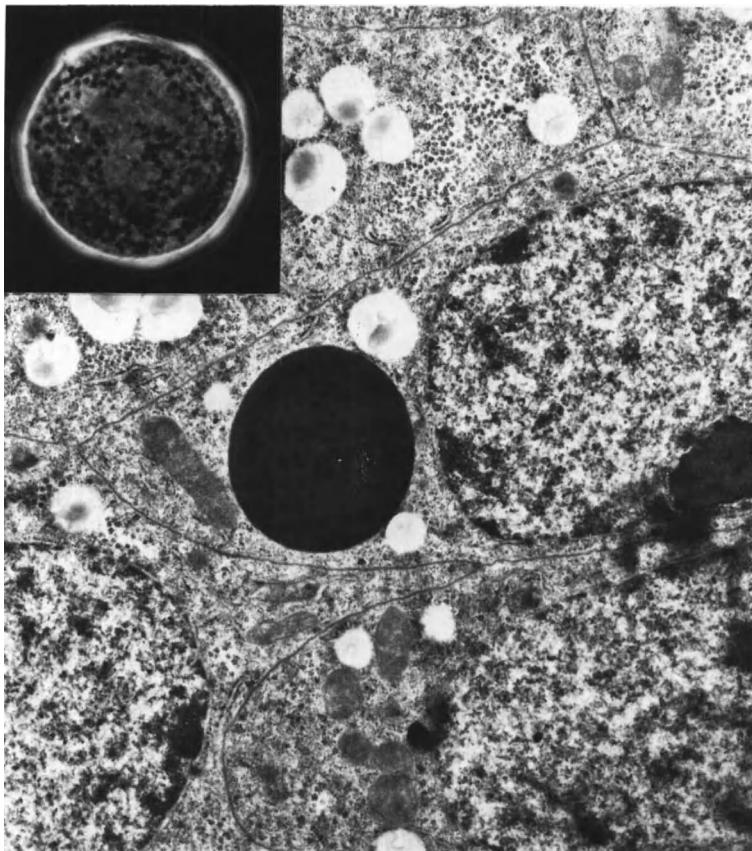


FIGURE 1. Structural features of the encysted gastrula (cyst) of *Artemia salina*. The insert at top left is a light microscope photograph of a fully hydrated cyst in cross-section. The major part of the figure shows the ultrastructural detail of a few cells. (See text for description).

The main part of the photograph shows the ultrastructural features of several cells as revealed by conventional transmission electron microscopy performed by Dr. Clark Olson in my laboratory. Except for the presence of yolk platelets (the large dark object in the center), these cells exhibit the usual features of animal cells and should not be considered "unique", in a structural sense, any more or less than liver or muscle cells are "unique". Several studies have been published on the ultrastructural details of *Artemia* cysts (49,50,51). Upon desiccation, the cellular mass becomes highly compacted (50) since the shell components do not

act as a water-retaining device. When dried over desiccants such as CaSO_4 the cysts retain only "residual" water (about 2% by weight) and are ametabolic but viable (52,53).

Upon suitable conditions of incubation (53) the dried cysts hydrate and resume metabolic activity. However, cell division and DNA synthesis do not occur until after the embryo emerges from the cyst, usually after 10 or more hours of incubation of fully hydrated cysts under optimal conditions (46,48,54). Hence, studies carried out on cysts that have not yet emerged are uncomplicated by changes associated with gene replication, growth, and changes in cell number. Therefore, the cyst can be converted from a hydrated, metabolically active mass of living cells into a desiccated, ametabolic state (and vice-versa), simply by manipulating environmental water activity in the liquid or vapor phase (45). Furthermore, no "metabolic triggers" seem to be involved in these metabolic transitions, the only requirements being adequate amounts of water and molecular oxygen (44,45, 48,52,55,56). Consequently, no complexities arise from hormonal or other growth-factor regulators (as is often the case in dried seeds and other plant propagules) or from metabolic-activation mechanisms of the kind that occur in many microbial spores.

Hydration can be carried out by immersing the cysts in solutions of NaCl and other solutes (45). All available data indicate that the cysts are effectively impermeable to non-volatile organic and inorganic solutes (48,50,57); such solutes act only by lowering water activity in the environment and do not directly interact with the cellular component. Hydration and dehydration can be achieved equally well from the vapor phase, and suitable methods have been developed and used for both procedures (45).

In summary, these cysts can be manipulated to obtain any desired degree of water content between full hydration (about 140g $\text{H}_2\text{O}/100\text{g}$ dried cysts) and extreme desiccation (about 2g/100g) by immersion in solutions, or by water sorption directly from the vapor phase. Hydration can be carried out over a wide range of temperatures, and under conditions permitting metabolism or strongly suppressing it, simply by controlling the gas phase and external water activity (58,59,60). All these conditions can be examined without significant damage to the cells. Therefore, the results of experiments on the role of cellular water can be interpreted with the confidence that induced alterations in water content have not produced significant change. This is rather important since

most previous studies on the physical state of water in cells have been carried out on excised tissues that are either dead, or in the process of dying, and it is a rare case where much attention is given to their metabolic status.

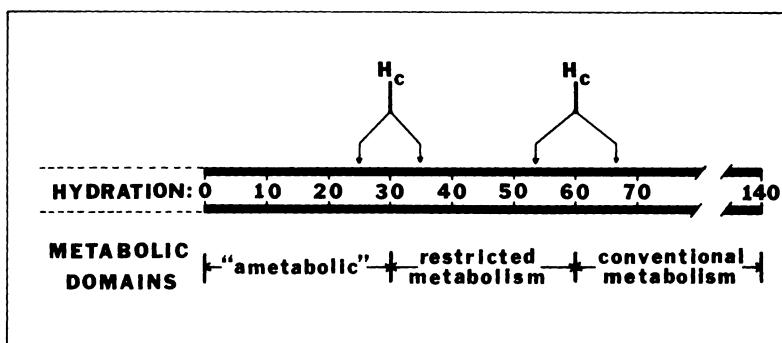
These features have been described in some detail to illustrate the utility of the *Artemia* cyst for studies on intracellular water, as well as to provide sufficient basis to understand the results of the studies to be described next.

A. Metabolic Activity and Hydration Levels

Our initial goal was to "map" the hydration dependence of selected metabolic parameters. For each metabolic activity examined, two experimental approaches were taken: one, the "Kinetic" approach, involved a determination of the initiation of a given metabolic event in cysts undergoing continuous hydration from the dried state (the standard condition for "dried" being equilibration of cysts over CaSO_4); the other, an "Equilibrium" approach, utilized cysts that had first been "pre-hydrated" (equilibrated to a given water content at 0-2°C), then transferred to temperatures permitting metabolic activity, and incubated at the same environmental water activity used for pre-hydration. In both approaches the cysts were either hydrated by immersion in solutions of known water activity, or by incubation in the vapor phase over such solutions (45).

Using these approaches we have determined the range of cyst hydration required to initiate various metabolic events, and I will refer to these as "critical hydrations". Figure 2 and Table II summarize the current status of our findings. Except for results on incorporation of ^{14}C -labelled formic, acetic and propionic acids, all of this work has either been published or is in press at the present time (45,61-66). All of the metabolic activities described here have been shown to be restricted to the cellular component of the cyst, and contributions from possible microbial contamination of our cyst preparations have been shown to be negligible. Finally, $^3\text{H}_2\text{O}$ -measurements on individual cyst hydration have shown that the water contents of large populations of cysts at a given environmental water activity do reflect the hydration levels of individual cysts within acceptable error (64).

On the basis of these results we have tentatively described three "metabolic domains" in terms of cyst hydration, being separated from each other by a critical hydration.



*FIGURE 2. Summary of results on the initiation of hydration-dependent metabolic events in *Artemia* cysts. H_c refers to "critical hydrations" that separate the metabolic domains. Hydration is in units of g H₂O/100g dried cysts. See text and Table II for further description and supporting evidence.*

Obviously this description may be subject to revision as more data become available. Nevertheless, this device is a convenient way to summarize a large amount of information. Several features are worth noting.

1. No evidence has been obtained for the occurrence of any metabolic event (i.e., enzymatic involvement) in cysts containing less than 10g H₂O/100g cysts. At this hydration, and up to about 30g/100g, the only indication of enzymatic activity observed was a slow but significant decrease in the concentration of ATP (66). However, even in this single case, the evidence for enzymatic involvement was indirect, and further study is required. Consequently, we assign cysts containing between 0-30g H₂O/100g dry weight to an "ametabolic" status, realizing, of course, that certain isolated enzymatic reactions may be taking place.

2. We have obtained a considerable amount of evidence that several pathways of intermediary metabolism are initiated at cyst hydrations in the vicinity of 30g/100g (63,65). These include the metabolism of certain amino acids, Krebs' cycle and related organic acids, and pyrimidine nucleotides, at least 15 metabolites being involved. The operation of these pathways was detected by the use of ¹⁴CO₂ as a labelled precursor as the cysts are effectively impermeable to organic compounds of low vapor pressure. In addition, incorporation of ¹⁴C-labelled formic, acetic and propionic acids from the vapor phase into a variety of low molecular weight compounds

TABLE II. Hydration Dependence of Cellular Metabolism in *Artemia* Cysts^a

Cyst Hydration (gH ₂ O/100g cysts)	Metabolic Events Initiated	Reference
0-10	none observed	45, 52, 61-66
~ 10	decrease in ATP concentration	66
10-30 ± 5	no additional events observed	see above
30 ± 5	metabolism involving several amino acids, Krebs cycle and related intermediates, short chain aliphatic acids, pyrimidine nucleotides, slight decrease in glycogen concentration	61-66
30-60 ± 7	no additional events observed	see above
60 ± 7	cellular respiration, carbohydrate synthesis, mobilization of trehalose, net increase in ATP, major changes in the free amino acid pool, hydrolysis of yolk protein, RNA and protein synthesis, resumption of embryonic development and eventual emergence from the cyst	45, 52, 61-66
60-140	no additional events observed	see above

^asee Figure 2 for a summary of these relationships.

has also been observed at about the same hydration threshold of 30g/100g (unpublished results). Thus, we consider the hydration range of 30-60g/100g as a second metabolic domain (Figure 2 and Table II) which will be referred to as "Restricted Metabolic Pathways".

3. Another critical hydration occurs in the vicinity of 60 gH₂O/100g and this appears to be particularly significant. This is the lowest cyst hydration at which all of the following are initiated: embryonic development eventually resulting in emergence of the larva from its shell (45), the utilization of trehalose (the primary source of stored energy, and carbon skeletons for synthesis) and the synthesis of glycogen and glycerol (54,83), cellular respiration (54,84), a net increase in ATP concentration (66), marked changes in the free amino acid pool (66a) and the synthesis of protein and nucleic acid (63,65). In other words, the initiation of a metabolism characteristic of cells in fully hydrated cysts. For these reasons, we assign cysts containing this amount of water, or more, to the domain of "Conventional Metabolism".

4. Above hydrations of about 60g/100g and up to a maximal hydration (about 140g/100g) no additional metabolic events have yet been observed to take place, although the rates at which metabolism proceeds appear to be related to the hydration level, in general increasing with increasing hydration. This aspect requires further study. Such results indicate that less than half of the maximum cyst hydration results in a metabolism that is *qualitatively* the same as that occurring in fully hydrated cysts. This interesting and, I believe, very important observation will be considered later in more detail.

5. Up to hydrations of about 65g/100g the water content of the cysts is a strict function of environmental water activity. However, once this hydration is reached, ongoing metabolic activity in the cells participates directly in determining the level of cyst hydration (45,54).

The general conclusion of all these studies is that two rather narrow ranges of cyst hydration appear to be of major metabolic significance: 30 + 5 and 60 + 7, the latter resulting in the initiation of a conventional metabolism characteristic of fully hydrated cysts (about 140g/100g dried wt.).

Such findings take on more significance in the context of our studies on the physical state of water in these cells.

Although this work has not been published, and space limitations preclude its complete description here, a large amount of data has been obtained. The present picture emerging from this work is that little, if any, water with properties like those of bulk water is present in the cells until they have achieved about 60% hydration. A brief account of some results obtained from Differential Scanning Calorimetry (DSC) and dielectric measurements will illustrate the approach used and the nature of this preliminary evidence.

B. DSC Measurements as a Function of Cyst Hydration

This work was done in collaboration with Dr. Walter Drost-Hansen and Mr. Chet Braun, Laboratory for Water Research, Department of Chemistry, University of Miami. The instrument used was a DuPont Model 900 differential thermal analyzer modified for DSC. The cysts were pre-hydrated to various levels at 0°C, then transferred to calibrated cups (hermetically sealed) for measurement against empty cups, also previously calibrated. Heat capacity measurements (C_p) were made over the range 15–35°C. The results in the main body of Figure 3 show the observed relationship between cyst hydration and C_p , and the insert represents the use of these data to graphically estimate the partial heat capacity of the cyst water (\bar{C}_{p1}) which are given in Table III. These data indicate that the partial values for cyst-associated water exceed those of pure water by a significant amount, at least up to cyst hydrations in the vicinity of 70g/100g. Above this hydration a large amount of scatter occurs in the data, the significance of which is not yet understood. Preliminary work has revealed that none of the water in these cysts freezes when they are placed at -12°C for 6 hours, regardless of the hydration level. We intend to examine much lower temperatures in the future.

While these findings are certainly not without interpretive difficulty, particularly in terms of estimating partial heat capacities (40), they do suggest that little, if any, of the water contained in these cysts behaves calorimetrically like that in bulk aqueous solutions up to cyst hydrations of about 70g/100g. Yet, we have shown that the cells of cysts at this hydration have already initiated conventional metabolism (Figure 2 and Table II). Consequently, such metabolism does not appear to require the presence of an appreciable bulk aqueous phase. Further interpretation of these data will be carried out after presentation of some results of dielectric measurements. I should first point out, however, that studies

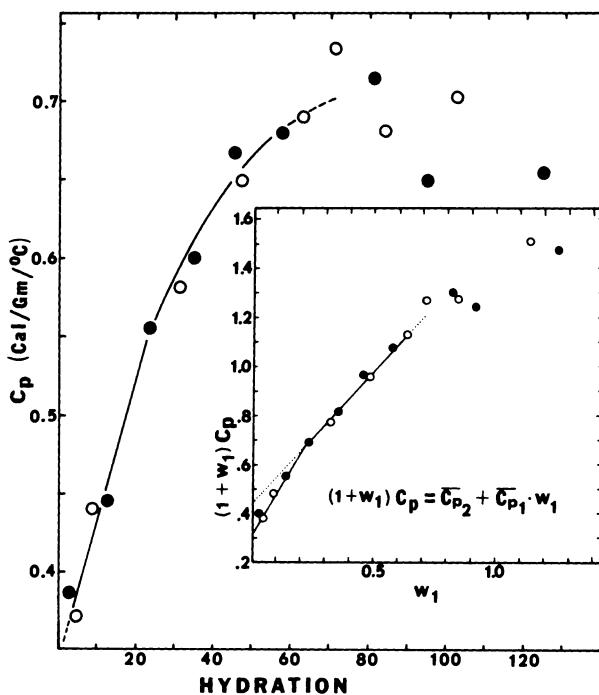


FIGURE 3. Heat capacity measurements on *Artemia* cysts as a function of hydration ($\text{gH}_2\text{O}/100\text{g}$ dry cysts). The insert is a plot of the equation shown, in which w_1 is cyst hydration ($\text{gH}_2\text{O}/\text{g}$ dried cysts), C_p is total heat capacity and \bar{C}_{p1} and \bar{C}_{p2} are partial values for the water and dry mass of the cysts, respectively. Table II gives these estimates for two ranges of w_1 .

TABLE II. Estimates of Partial Heat Capacities of Water (\bar{C}_{p1}) and the Dry Mass (\bar{C}_{p2}) of *Artemia* cysts^a

Cyst hydration range ($\text{g H}_2\text{O}/100\text{g}$ dried cysts)	\bar{C}_{p1}	\bar{C}_{p2}
0-25	1.47	0.33
25-65	1.11	0.44

^aThese data were obtained from Figure 3 using the equation given in the insert.

done on isolated shells show that the thermal behavior of the whole cyst is dominated by contributions of the cellular component. Thus, the behavior illustrated in Figure 3 can be viewed as that generated chiefly by the cells.

C. Some Dielectric Properties of Cysts as a Function of Hydration

These studies were carried out by me in the laboratory of Dr. Walter Drost-Hansen with the skilled assistance of Mr. Marcos Puga, then a graduate student in the Department of Physics, University of Miami.

The experiments were performed at 23° (+ 1°)C using a General Radio Capacitance Bridge (Model 1615A) for the frequency range 500 Hz-100kHz, and a Hewlett-Packard Vector Impedance Meter to cover the range 0.5-100 MHz. A specially designed three-terminal capacitance cell was constructed which permits control of the gas phase surrounding the sample. In effect, the cell consists of two concentric capacitor plates between which the cysts are packed. The dielectric constant (ϵ') and loss (ϵ'') were calculated from measurements on dried cysts, and cysts of various water contents. When freed from surface water (45) the cysts behave as a "free flowing powder" and are easily transferred to the cell where they are packed uniformly by gentle tapping of the cell until no further packing takes place. Measurements on cyst populations of a given hydration are highly repeatable.

1. *Dielectric Constant vs. Applied Frequency.* The data shown in Figure 4 depict the relationship between the real part of the dielectric constant (permittivity) and applied frequency for cysts containing different amounts of water. Note that ϵ' is essentially independent of frequency for CaSO₄-dried cysts (a hydration level of about 2% by weight) indicating that all components of the cyst are "frozen" over the frequency range employed. (The apparent increase in ϵ' at higher frequencies is an instrumental artifact). However, the addition of only a small amount of water results in the appearance of a significant dispersion over the low to intermediate frequency range, and a 2.5 fold increase in the dielectric constant at 1 kHz. Clearly, even a small amount of water over the "residual" level enables some component (or components) of the cysts to respond to the applied field. The nature of this component(s) has not yet been identified. However, we do know from studies on isolated shells that the response noted in Figure 4 is due chiefly to the cellular component of the cyst.

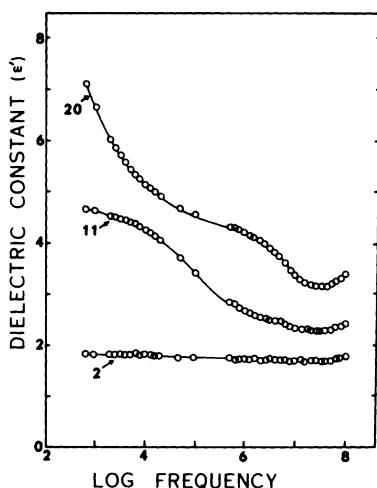


FIGURE 4. Dielectric constant (ϵ') as a function of applied frequency (Hz) for *Artemia* cysts at three different hydration levels. Hydration is in units of g $H_2O/100g$ dried cysts.

Upon further addition of water, ϵ' increases sharply at the lower frequencies. We believe that some of this increase is due to electrode polarization as well as Maxwell-Wagner processes, and are currently evaluating these contributions. As more and more water is added to the cysts, the shape of the ϵ' vs. frequency curves remains similar to that for the highest hydration (20g/100g) shown in Figure 4. Inspection of these data reveals the appearance of a higher frequency dispersion which persists in cysts at hydrations in excess of 20g/100g. It will be analyzed in more detail shortly.

2. ϵ' and ϵ'' as a Function of Cyst Hydration. We have measured the dielectric parameters over the entire range of frequencies available to us, on 26 cyst samples in various states of hydration. The results in Figure 5 show the relationship between the dielectric parameters and cyst hydration at 40 MHz. Transitions in ϵ' and ϵ'' occur in the vicinity of 25g/100g. Whether or not another transition in ϵ' takes place at 60g/100g cysts remains to be determined. However, it is important to note that above a hydration of approximately that level the real part of the dielectric constant is essentially independent of cyst hydration, even up

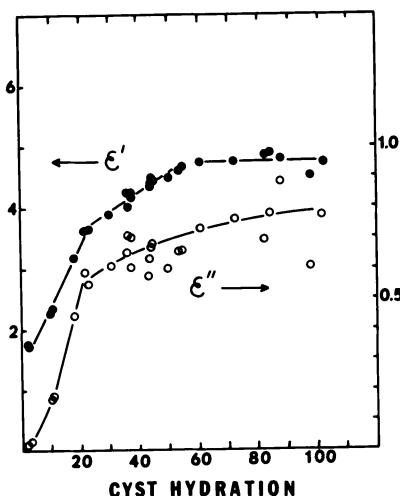


FIGURE 5. Dielectric parameters as a function of hydration of *Artemia* cysts at 40 MHz. The real (●) and imaginary (○) parts of the dielectric constant are shown. Hydration is in units of g H₂O/100g dried cysts.

to maximal water contents of about 140. This finding was not expected since, in principle, one would expect ϵ' to increase as a function of hydration. A great deal of scatter occurs in the data for the dielectric loss (ϵ'') above 60g H₂O/100g cysts, and no interpretation of this relationship will be advanced here.

These "kinetic" (relaxation) data are most interesting in comparison with our results on the hydration dependence of heat capacity which is a "thermodynamic" (though not an equilibrium) quantity: both indicate relatively abrupt transitions in physical properties at cyst hydration ranges of critical importance to the metabolic behavior of the cells (Figure 2) and an increase in scatter of the data above the hydration required for conventional metabolism (Figures 3-5).

3. Preliminary Analysis of the Two Major Dispersions. The "low" frequency dispersion is shown in Figure 6 along with parameters calculated from the data. A Cole-Cole plot of the results (insert) indicates the participation of several processes having different relaxation times ($\alpha = 0.46$). Also, the macroscopic relaxation time (τ) of about 2×10^{-6} sec. for a characteristic frequency (f_0) of 100 kHz suggests that we may be observing the α -dispersion that has been noted

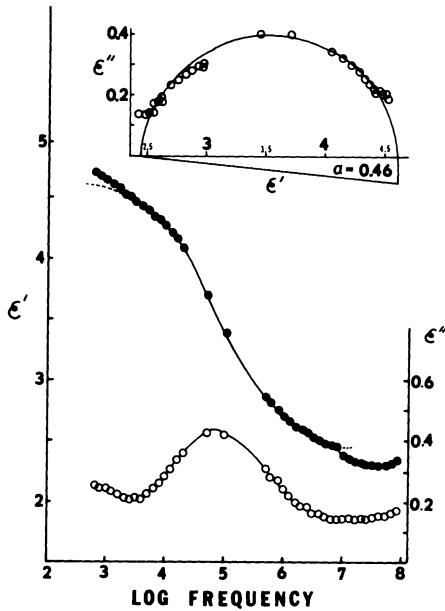


FIGURE 6. Real (●) and imaginary (○) parts of the dielectric constant as a function of frequency for *Artemia* cysts at a hydration level of 11g H₂O/100g dried cysts. The insert at the top is a Cole-Cole plot of these data.

for a variety of biological systems (see Schwan, 92, and Grant, 93).

Of some interest is the "higher frequency" dispersion. For this we obtain, by similar analysis, $\tau = 2.5 \times 10^{-8}$ sec., and $f_0 = 6.5 \times 10^6$ Hz. Although such a relaxation time is close to the range assigned from NMR studies to water molecules under various degrees of restricted motion (10) and to the tumbling rate of protein molecules (40) the relaxation frequency is below that associated with the dielectric relaxation of the "bound water" of protein solutions (the δ -dispersion, 92 and 93) being closer to the β -dispersion of cells and tissues, whose origin is not believed to arise directly from relaxation of intracellular water (92).

Our interpretations of these preliminary experiments are obviously tentative and highly speculative since we have been limited to frequencies well below those at which "bound" and ordinary water relax, and because the cyst is clearly an ex-

tremely complex system. Both considerations severely restrict interpretation. However, we are in the process of extending these studies well into the Giga-Hertz frequency range, and hope to attach more specific meaning to the results obtained thus far.

Data from sorption isotherms and derived thermodynamic quantities (done in my laboratory) and from pulsed NMR spectroscopy (done by Patsy Seitz, a doctoral student in the laboratories of Dr. Carlton Hazlewood) have also been obtained but space limitations prevent their coverage at this time. The results are, in general, consistent with some of our findings from DSC and dielectric studies: at cyst hydrations in the vicinity of 30 and, in some cases, at about 60g H₂O/100g cysts, more or less abrupt changes have been observed for NMR relaxation times (T_1 and T_2) and for the self-diffusion coefficient of water; changes in the differential and integral net heats and entropies accompanying the water sorption process as calculated from the temperature dependence of sorption isotherm measurements also reveal similar relationships. Thus, four different kinds of physical measurements reveal correlations between transitions in metabolism and the physical properties of the cysts at the critical hydrations. Future work will involve completion of these studies and an evaluation of possible causal connections between these correlations.

D. *Summary of Work on Artemia: Working Hypothesis*

Based on the results of this research, the following working hypothesis for interactions between water and metabolism in *Artemia* cells is proposed.

Up to a hydration of about 30g/100g all of the water is apparently very tightly and intimately associated with the cellular components and little, if any, "bulk water" is believed to be present in such cells. Nevertheless, we have obtained direct evidence that several metabolic pathways are initiated under these conditions (Figure 2; Table II). Therefore, these pathways are proposed to have a strictly interfacial location within the cells since an appreciable bulk aqueous phase apparently does not exist. This metabolic activity is considered to be "restricted" in space in the sense that sufficient water is not present to permit "long range" transfer of metabolites and so forth between the various organelles, and other compartments within the cells. It is this consideration that I believe prevents the initia-

tion of a full-blown metabolism at 30g H₂O/100g cysts. However, as more water is acquired, over the hydration range 30-65g/100g, interactions between water and cellular components become increasingly less intense and, at the upper limit of this range, all interfacial sites finally achieve maximal hydration. At this point (about 65g/100g) I suggest that sufficient water now becomes available to provide channels of continuity for the transfer of metabolites, energy sources, etc. between the various membrane-bound organelles and other intracellular compartments, allowing for their integration into what I have referred to here as "conventional metabolism" (Figure 2). Once this critical hydration is achieved, the further addition of water contributes to what may be taken to be "bulk aqueous phases" within the cells, although it is not established that this water is identical to ordinary water. In any event, water contents in excess of ~ 0.6 seem to play little, if any, qualitative role in ongoing metabolism. Consequently, the possibility is advanced that even in fully hydrated cells interfacial events dominate ongoing metabolic activity, regardless of the possible presence of "bulk aqueous phase". This possibility is consistent with the findings of Kempner and Miller (7) on stratified *Euglena* cells, and additional evidence that has been discussed in earlier sections of this paper; namely, that the soluble phases of cells may actually contain only very small amounts of enzymes and other large molecules in free solution, the latter being "associated" with various intracellular surfaces.

The work performed on the *Artemia* system, in conjunction with the considerations taken up in the preceding sections of this article lead me to propose a more general model of the intracellular environment which I suggest may be applicable to all eucaryotic cells.

VIII. THE VICINAL WATER-NETWORK MODEL

Consider any ultrastructural surface in cells, and refer to Figure 7 which I will use as a diagrammatic and highly simplified representation of what I suggest might be happening there. There is adequate reason to believe that the surface (membrane, microfilament, etc.) will influence water molecules in its vicinity in such a way that their properties will differ significantly from bulk water. This is the "vicinal water" as described by Drost-Hansen (17). While there is no direct and concrete evidence regarding the maximum depth of

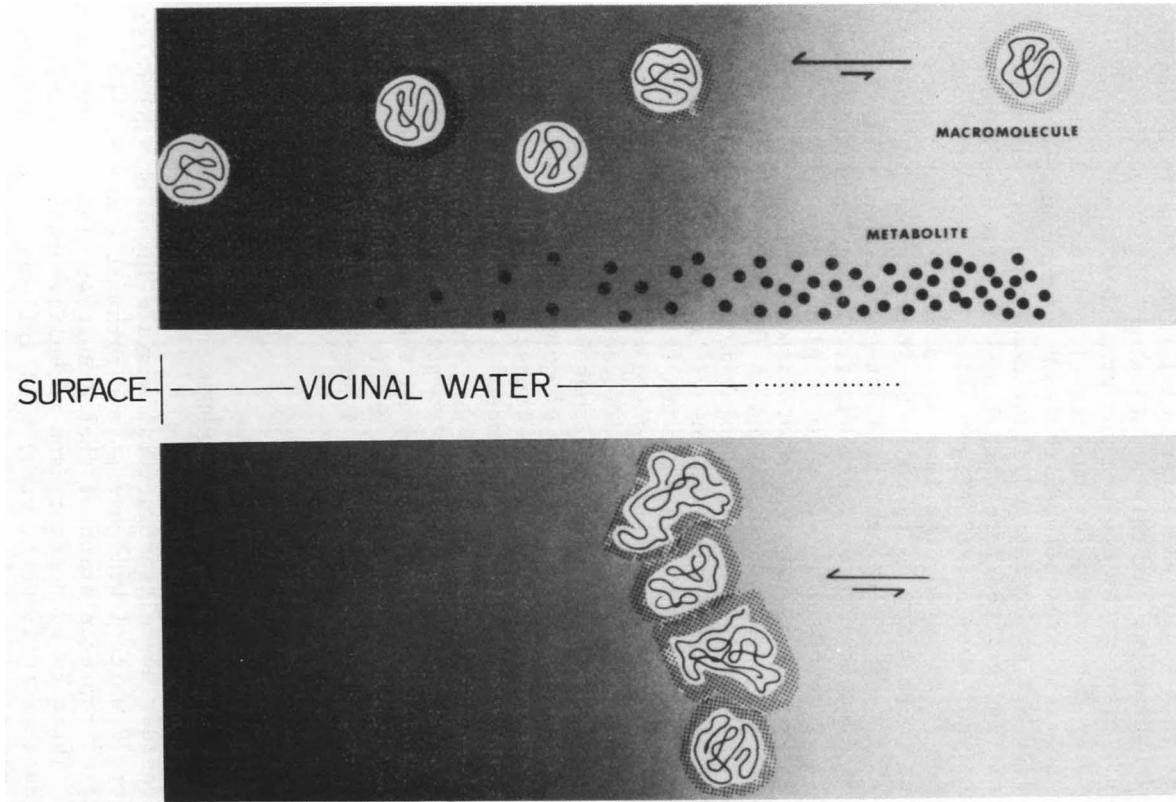


FIGURE 7. Diagrammatic representation of some features of the Vicinal Water-Network Model. See text for discussion.

the vicinal water layer at such intracellular interfaces, there is good reason to believe that it consists of 5-10 layers at certain interfaces (18) and perhaps much more (17). The shading in the figure indicates this uncertainty and also suggests that the probability of the presence of vicinal water decreases with distance from the surface. The model neither requires nor excludes the presence of bulk water; however, in its absence the properties and structure of the vicinal water must change as some function of distance from the surface, a reasonable possibility in my opinion. Articles by Drost-Hansen (17) and by Peschel and Belouschek in the present volume, summarize the current status of the structure and properties of vicinal water.

The water adjacent to the surfaces of dissolved macromolecules (i.e., molecular weights in excess of several thousand Daltons) will also be referred to here as vicinal water. Call it what you will, there is ample reason to believe that such water differs from bulk water in a number of ways (10,40,68,93). An important feature of my model is that the vicinal water of the macromolecules is hypothesized to have more affinity for the vicinal water of the ultrastructural surface than for bulk water, such that the macromolecule finds itself somewhere within (or on) the vicinal water layer more often than not. Of course, this will minimize the total vicinal water surface, which might be related to the mechanism of association, a topic I will consider elsewhere. Such features of the model do not demand the presence of bulk water; however, in its absence, it is suggested that various macromolecules will achieve some sort of distribution within the vicinal water layers (of the intracellular surface) which themselves are likely to exhibit different properties as a function of distance from the surface.

In addition to these water-water interactions, other forces may obviously contribute, depending on the nature of the particular macromolecule or surface, that will tend to influence the association. Thus, some of the macromolecules may subsequently interact and be held directly by groups on the ultrastructural surface, whereas others may be held quite loosely, or even be repelled. This variability in strength of association is consistent with the highly variable results of cell fractionation studies as mentioned earlier. However, I advance the possibility that vicinal water interactions in intact cells may suffice to "hold" the molecule within at least part of the vicinal water structure, independently of other considerations, and for an appreciable time on a molecular scale. It should be pointed out that although a very

substantial amount of work has been done on ligand binding to proteins, including protein-protein interaction, it is my impression that the details of solvent (i.e., water) involvement on these interactions are not very well understood (for review, see 69). Thus, I know of no solid data that would rule out the proposed vicinal water interaction, admitting at the same time that I have no *direct* evidence at present that it actually occurs. The reader should recall at this point the extensive actin-microfilament network found in a wide variety of *non-muscle cells* (96). It is notable that these microfilaments are composed of actin-like protein subunits and that a number of the glycolytic pathway enzymes have been shown to be "associated" with the actin filaments of muscle cells (6). It may well be that the microfilament networks of *non-muscle cells* play an important role in the micro-localization of so-called "soluble" metabolic pathways, a notion fully consistent with the model described here. In the future we intend to examine such interactions in model systems.

The proposed association of macromolecule with interface via vicinal water interactions between the two is considered to be a very subtle relationship, highly sensitive to alterations in the intracellular environment. Such variables as ionic strength and pH are likely to strongly influence the vicinal water structures, as well as water-water interactions of the form described here. Consequently, the results of experiments dealing with these two variables need not be interpreted exclusively in terms of direct interactions between sites on the surface and macromolecule. It is possible, in my opinion, that water-water interactions could be profoundly important in this regard.

It is further hypothesized that macromolecules also make loose association with each other by means of their mutually-compatible vicinal water structures. This would provide a means by which some or all of the component enzymes of a given pathway might be brought in proximity to one another for appreciable periods, either before or after they become associated with the vicinal water of the surface. This possibility is illustrated in the lower diagram of Figure 7. Although such an association could be viewed as a *very* loosely associated multi-enzyme complex, the model stresses that vicinal water, and not customary intermolecular interactions, plays the key role in the association.

This feature obviously places great importance on the

nature of the enzyme surface, and implies that natural selection would have generated such "compatible" surfaces for self-association of enzymes in a given pathway during the course of evolution. This prediction of the model is consistent with the findings of Mowbray and Moses (4) on glycolytic enzymes in *E. coli*. It seems possible that one reason why enzymes that function intracellularly are usually so much larger than those whose function is performed solely outside cells: "largeness" would be required to generate compatible surfaces. Although it is clear that the need for "largeness" in many enzymes can be definitely related to their allosteric regulation, this need not be the only reason. The intracellular surface(s) with which the enzymes interact could also provide specificity for mutual associations of component enzymes of a given pathway at certain intracellular locations, and not at others.

By confining a pathway to an essentially 2-dimensional vicinal water location many significant advantages would be gained by the cell compared with the existence of a 3-dimensional metabolism based on mass-action laws, free diffusion of the participants, and so on. As Dirac (70) said, albeit in a much different context: "*Under these circumstances one would be surprised if nature had made no use of it.*"

I will not consider these advantages here since most are rather obvious and have been enumerated by several others (3,4,6). But in addition, I should mention the excellent article by Adam and Delbrück (71) on the nature of dimensionality in diffusion processes. Their calculations provide a quantitative description of how reduction from three to two dimensions can increase the efficiency of transfer by diffusion within cells. Also worth particular mention in this regard is the conclusion by Pollard (72) from his careful analysis of collision kinetics in cells, that some sort of submicroscopic order *must* exist to account for the kinetics of metabolic reactions in cells whose diameter exceeds 0.1-1 μm . In other words, essentially all eucaryotic cells! In my opinion, this submicroscopic order could arise from the structuring of intracellular water at interfaces, its ordering influence on the "soluble" enzymes of cells, and as a sort of "metabolite partitioning" device which will be described next.

The model indicates, on the basis of solute distribution studies, that molecules not large enough to generate their own vicinal water structures might tend to be partitioned between bulk and vicinal water. Virtually all of the cellular metabolites are in this category. However, based on

the work of Garlid (33, and the present volume) the partition coefficients could vary widely and, depending on the particular solute and the participating surface, may be less than, greater than, or equal to one (33). The diagram (Figure 7) shows only one such case, but it is certainly not intended to be limited to this. The ion-partition studies of Wiggins, mitochondrial experiments of Garlid, and the work of Peschel and Belouschek (all in this volume) are consistent with such a view. However, the chapter by Ling (this volume) presents an alternative in terms of solute partitioning.

I find it highly provocative that a distinct gap in the distribution of molecular weights of cellular molecules occurs in the region of about 1,000 to 5,000 Daltons, very few molecules being present in cells within this range according to the detailed complication of Anderson and Green (1). This is fascinating since the lower end of this molecular weight range appears to be, albeit on indirect evidence, about the minimum size required for a molecule to generate its own vicinal water (17). I suggest that this somewhat puzzling hiatus in molecular size distributions could be an extremely important but overlooked feature of the intra-cellular environment in a metabolic context. In terms of the model given here this distribution could reflect a device that cells use to partition enzymes and their substrates in such a way as to achieve favorable concentrations of each in a relatively easy fashion, while at the same time avoiding solubility (solvent) problems. Atkinson, and Sols and Marco (73) have considered in some detail the considerable problems that cells face in this regard, and I believe the model discussed here provides a plausible account of how cells may have solved at least some of those problems. The topic considered in the articles by these authors has not been given the attention it deserves.

It seems appropriate at this time to briefly consider the vast literature on the subject of metabolic pools and compartmentation in cells in the context of the model. It is, of course, well known that in certain cases metabolic intermediates that are believed to exist in free solution in cells do not behave this way, but instead appear to be restricted in time or space or both. There are a number of ways that this could come about, all of which have been treated in the recent excellent review by Kempner (67). Most of these proposals, and particularly the "micro-environment" compartmentation idea advanced by Srere and Mosback (74), seem to me to be reasonably consistent with the model I have presented. However, it should also be recognized that the study of sub-

cellular compartmentation and metabolic pools has been carried out largely by kinetic analysis involving tracer techniques, usually involving a number of assumptions which, according to Smith and Mohler (75) are somewhat questionable. In any event, the observations that such metabolites often do not behave "ideally", as described above, can only be made consistent with the concensus view of the soluble phases of the intracellular environment with some difficulty. On the other hand, such findings seem more understandable in terms of the "partitioning" aspect of the crude model described here.

Another consideration of the model has to do with the possible significance and consequence of enzymatic reactions occurring in vicinal water compared to those in bulk aqueous solution. This is a complex question and will be treated only briefly here.

It can be expected *a priori* that the behavior of a given enzyme will be influenced by significant changes in its micro-environment, including the surrounding solvent (68,76, 77). The ways by which water might enhance or depress the catalytic behavior of enzymes has been considered, among others, by Somogyi and Damjanovich (78,79,80). Their analysis, called the "energy funnel" model of enzyme action directly implicates the solvent water molecules in the environment of the enzyme as major participants in energetic events accompanying formation and breakdown of enzyme-substrate complexes. Somewhat related are two important papers by Low and Somero (81,82) in which it is proposed that side chains and peptide linkages on or near the surface of enzymes change their exposure to water during conformational events associated with catalysis. These protein group transfers are believed to be accompanied by appreciable volume and energy changes that result from alterations in the organization of water around such groups. The authors have offered an explanation as to how such changes could contribute significantly to the free energy of activation, as well as to the activation volume of the reaction. While the foregoing studies admittedly do not directly provide insight into the nature of enzyme reactions occurring in bulk solution compared with those taking place in the vicinal water of surfaces, they do emphasize the potentially profound role that water might play in enzymic catalysis, and also provide some rationale as to how vicinal water might be involved as a major metabolic determinant.

One final consideration involves the relatively high lability of intracellular enzymes and the considerable

energetic cost of their replacement. Since enzymatic reactions can result in the liberation of large amounts of highly localized energy, one might suppose that it would be advantageous for cells to have acquired the most efficient means of dissipating this localized energy from the vicinity of the enzyme surface as rapidly as possible. Since vicinal water appears to have an appreciably greater heat capacity and heat conductivity than ordinary bulk water (see Drost-Hansen (17) and Figure 3 and Table II in the present paper), it is reasonable to suggest that enzyme reactions occurring in the former might be advantageous in terms of enzyme stability, compared with those same reactions taking place in bulk water. If this were the case, very strong selection pressure would be generated for enzymes that preferentially associate with the vicinal water of intracellular surfaces, regardless of the mechanism of association. However, it is possible that "loose" associations of the type proposed here might provide greater metabolic flexibility than "rigid" ones involving covalent bonds, or other direct interactions between enzyme and surface.

Although the foregoing discussion has focused heavily on the behavior of "soluble enzymes", it applies equally well to any molecule capable of generating vicinal water; for example, the various species of transfer-RNA's. Moreover, I should stress again that the model is meant to be applicable to the nucleoplasm and the interior compartments of cytoplasmic organelles, as well as to the cytosol. Finally, it is also proposed that vicinal water interactions may be important to metabolic reactions occurring in *particulate* cellular components insofar as such enzymes are in contact with an aqueous phase. This consideration will be dealt with in more detail elsewhere.

This view of the intracellular environment is admittedly crude and over-simplified, and the model I advance will obviously require revision as more information appears. Indeed, because of the complexity of the topic dealt with here, it may be that some of my proposals are naive. I submit, however, that the model is based on a considerable amount of evidence from a wide variety of independent sources, accounts for the variability of cell fractionation studies, can incorporate what is known about metabolic regulation and intracellular transport of metabolites, and provides a metabolic explanation for the occurrence of thermal anomalies in biological systems as described by Drost-Hansen (17). Perhaps most important, the key features of the model are capable of being disproven by experiment. Finally, I suggest that the model attempts to provide a more realistic picture of the intra-

cellular environment than does the consensus view, particularly in terms of the well-documented complexity of cellular ultrastructure, its enormous surface area, and the perturbation of water that is vicinal to such surfaces.

I should point out here that the importance of various sorts of interfacial phenomena in cells has long been emphasized by many others, notably Weiss (83) and Danielli and Davis (84), to name but a few. Moreover, many of the features of the model proposed here are similar to the intracellular compartment models derived from tracer studies (see 67). I also believe the model is fully compatible with Wiggins' ideas on ion distribution mechanisms in cells, and with Garlid's description of solute distribution and water structuring in mitochondria (both studies reported in this volume). In other words, I do not claim the model to be entirely unique or original, with the exception of a single feature: the metabolic importance assigned to vicinal water, both that of intracellular surfaces and macromolecules. The model places extreme emphasis on this consideration. In fact, it holds that vicinal water is the basic cellular matrix within which most of metabolism takes place, and that the overall regulation of cellular activity is inexorably linked to this matrix. Inasmuch as the model attempts to relate recent observations on the ultrastructure of the intracellular environment to the dynamics of intermediary metabolism, its evaluation may prove useful in integrating structure with function at the *sub-organelle level* of organization.

This picture of the intracellular environment also implies that early evolution at the metabolic level may have operated within this framework. For example, if the notion is correct that interfacial physics and chemistry were crucial considerations in the evolutionary origin of metabolism (reviewed by Good, 85; also see 86 and 87), then one might suppose that the basic metabolic plan, once adopted, would likely be maintained and perfected through selection and other evolutionary forces, but would nevertheless retain its fundamentally interfacial locus, even in the presence of a surrounding "dilute solution" or "bulk aqueous phase". The latter would allow for transfer of metabolites, as well as for the various ion-associated cell functions. In this view of things the model suggests that the importance of vicinal water and intracellular surfaces to metabolism in contemporary cells could well be a reflection of the nature of the evolutionary origin of cellular metabolism.

IX. CONCLUDING COMMENTS

Almost 50 years ago Peters (88) suggested, without experimental evidence, that cells contained a "coordinating fluid cytoskeleton" based on the chemistry dependent upon interfacial molecular structure. He later developed this idea further (89) and it came to be known as the "Network Theorem" (90) whose relation to other early views of subcellular morphology is well described in a provocative article by Dean and Hinshelwood (91).

This view emphasized the role of protein in the network, but clearly recognized the importance of water interactions. I believe Peters raises an issue of enormous importance when he says (90, p. 11):

"Though doubts as to the existence of internal structures (in cells) have gone, there still remains the puzzle as to how the cell is integrated on a molecular basis, and adjusted to environmental stimuli, and otherwise. Every change in the individual reactions of a cell is based upon some phase of chemistry or physical chemistry. Can we still believe, however, that the whole living cell is merely an extremely complex chemical equilibrium, or have we still to look for some tenuous coordinating structure, fulfilling the role the nervous system does in the animal?" (my emphasis).

I propose that such a coordinating system could well exist in cells in the form of a network of vicinal water structures generated by intracellular surfaces. Because the latter can be presumed to be under cellular control, the extent and disposition of the vicinal water network can also be regulated accordingly. Although the numerous membrane systems and the microtubular-microfilament array in cells may be vital in this regard (96), it seems likely that the microtrabecular network (20) could be a key surface component. This water network could exercise its coordinating role by providing the fundamental intracellular framework or matrix within which most of cellular metabolism takes place. Its role as a partitioning device for metabolites between bulk and vicinal water may also be crucial to intracellular homeostasis and "solvent capacity" problems. Any influence that sufficiently alters the delicate and subtle balance between these relationships can be expected to result in metabolic imbalance and

loss of control which, if left uncorrected, could result in various cellular pathologies. Consider, for example, the well known fact that most types of cancer cells contain significantly more intracellular water than normal cells (12); indeed, this has been known since at least 1916 (94). Furthermore, NMR work indicates that cancer cell-water exhibits significantly less structure than normal cells (see articles by Hazlewood and Beall, this volume). It is important in this regard to emphasize that the malignant transition is also often accompanied by dramatic changes in the amounts and disposition of cytoplasmic microfilaments and other "cytoskeletal" structures (95,96). In other words, marked changes in intracellular surfaces. The possibility of causal connections between all these events demands further attention. It may also be noted that the model I have presented here is consistent with such causality.

Peters' early work (88,89) is rarely, if ever, cited in contemporary literature dealing with the intracellular environment. In my opinion, his insight was extraordinary when one considers the state of cell biology at that time (1929). As I have said, his view came to be known as the Network Theorem. I will call the model I have discussed here the "vicinal-water network model" in recognition of Sir Rudolph Peters' early contributions, and those of Professor Walter Drost-Hansen who, since 1956, has championed the occurrence and importance of vicinal water in biological systems.

NOTE ADDED IN PROOF

A. *Physical State of Water in Biological Systems*

Since this article was completed (November 1976) a rather large amount of literature has accumulated on the broad topic of the physical state of water in biological systems. Because other authors in this volume have considered this literature (particularly Ling, Hazlewood, and Beall) I will not repeat that effort here. However, I should mention and recommend the excellent collection of papers in "*A Discussion on Water Structure and Transport in Biology*" organized by Richards and Franks (97). Also, Somero, Low, and co-workers have extended their interesting analysis of the importance of hydration changes in the energetics of enzyme catalysis (98,99). The review articles by Richards (100) and by Makinen and Fink (101) on several aspects of the crystal structure of various enzymes and other proteins

are most interesting in terms of protein hydration and the role of "solvent" in crystalline enzyme reactions. In addition, the analysis by van Oss and Neumann (102) of the involvement of water in antigen-antibody reactions should be cited since it could bear in an important way on "water-water interactions" of the type I hypothesize to occur in the model advanced in this chapter. Finally, I will briefly describe some unpublished findings of Hansson Mild, Løvtrup and Forslind (103) which I believe are of some importance. They used an isotopic water exchange technique ($^{2}\text{H}_2\text{O}$ and H_2^{18}O) employing an automatic diver balance [see Hansson Mild and Løvtrup (104) to measure the density of water in living cells (amphibian eggs). They observed that the average density of the total intracellular water was appreciably greater than unity, and tentatively concluded that all (or part) of intracellular water must differ in its properties from ordinary bulk water. To my knowledge these measurements constitute the first to be made of water density in intact, living cells. [Although Pocsik (105) estimated water density in muscle, it seems unlikely that the tissue could be considered to be living under his experimental conditions]. This method seems to be a very powerful one that previously has not been used in studies of intracellular water.

B. Sub-cellular Localization of "Soluble" Enzymes

Several review articles have appeared that are of major importance to the view developed in this chapter; namely, that there is an increasing reason to doubt the notion of "soluble pathways" as bulk solution phenomena in cells. These review articles by Masters (106,107) and by Ottaway and Mowbray (108) make a very strong case for the view that the tendency to over-generalize about the nature of the aqueous intracellular environment may lead us to overlook subtle, but important metabolic consequences, a view I strongly endorse. These reviewers have compiled a large amount of evidence, from different laboratories, demonstrating that a number of enzymes previously believed by many to be "soluble" are, in fact, "bound" to various intracellular surfaces. In my view, however, it remains to be seen what forces are involved in the "binding process". The latter is quite interesting since in many cases it appears to be fully reversible and strongly influenced by such considerations as substrate and/or co-enzyme concentration. These reviews certainly justify the need to re-evaluate these aspects of intracellular metabolism. As Masters (107) puts it: "*For our concepts of metabolic regulation to be meaningful, they must be firmly*

based on the physiological realities of the microenvironment". I would add that they should also include a careful consideration of the potential role that intracellular water might play; it is, after all, the basic framework of the micro-environment.

The theoretical analysis of Welch (109) dealing with the energetics of intermediary metabolic processes also leads to the conclusion that the organization of the aqueous intracellular environment is much more important than the "consensus view" maintains. Welch suggests from this analysis that cellular evolution obviated such problems as transit times, enzyme competition for common substrates, metabolite concentrations and the like by "... compartmentalizing much (if not all) of intermediary metabolism via multi-enzyme complexes and/or membrane-associated schemes".

The reference lists of the articles referred to in this section include the names of many others who adopt a similar position, and whose works I cannot consider here, solely because of space limitations. It is my distinct impression that what I have referred to in this chapter as the "consensus view" (when I wrote the main text of the article two years ago) is rapidly becoming the "minority view"! It seems safe to say that the idea of the "reality of the cellular microenvironment" is, indeed, currently being recognized as important. It appears now that we must attempt to elucidate exactly what those realities are.

C. The Cytoskeleton and Intracellular Surface Area

There has been vigorous interest in the study of the so-called "cytoskeletal" structures in cells: microtubules, actin-microfilaments and the intermediate (10 nm) filaments. Indeed, the literature has become so extensive that it is not feasible to cover it here. The reader is directed to the published proceedings of a major conference (110) and some recent reviews (111). I believe it is fair to say that one general picture emerging from these studies is that the "actin microfilament bundles" (stress fibers on actin cables) are ubiquitous and that these and the "intermediate" (10 nm) filaments are prime candidates for a device by which cells may alter their intracellular surface area (112). Furthermore, it is highly significant that these two cytoskeletal structures have almost always been found to decrease in extent during the malignant transition (see, for example, 113-116). Thus, I would suggest that a decrease in the amount of

vicinal water should also occur as a result. There seems little doubt now that a highly significant connection exists between the "cytoskeleton", the physical state of intracellular water, and normal cell structure and function. In the context of my model (Figure 7) the connection would lie in the causal relationship between intracellular surfaces, the extent and distribution of vicinal water, and the organization and regulation of enzymes of intermediary metabolism as well as the partitioning of metabolites (see Sections VIII and IX).

Finally, it should be mentioned that one of the cytoskeletal elements, the microtrabecular lattice described by Porter and associates (pp. 1-28 in reference 110) is believed to consist of channels on the order of 500-1000 Å. These dimensions should be viewed in terms of the similar distance over which vicinal water might be generated by surfaces (see chapters in this volume by Etzler and Drost-Hansen and Peschel and Belouschek). In addition, I suggest that these microtrabecular channels could represent an ultrastructural basis for what I have described in my model as "channels of continuity" for metabolite transfer (Sections VII-D and VIII).

It would be of some interest to know, even roughly, how much surface area is provided by the cytoskeletal elements. Certainly it must be vast, and much greater than the crude estimates for rat liver cell membrane surfaces presented in this article (Table I), and elsewhere (117) where some very preliminary calculations of the surface areas of microfilaments were reported.

D. Water and Metabolism in *Artemia* Cells

Research referred to earlier in this article on the relationship between intracellular water content and nucleic acid and protein synthesis, as well as the free amino acid pool, has been published (118,119). Most of our recent efforts have been devoted to the physical measurements. Thus far, papers dealing with the residual water content of the cysts (120) and an analysis of sorption isotherms (121) have been published. In addition, significant progress has been made toward a description of the state of water in these cysts by NMR spectroscopy and microwave dielectric analysis. These results are currently being prepared for publication. The NMR research is currently available in the doctoral dissertation of Seitz (122). Briefly, the dielectric study

(done in collaboration with Professor E. H. Grant and his research group in the Physics Department, Queen Elizabeth College, University of London) involved the measurement of ϵ' and ϵ'' , as a function of cyst hydration, over a broad frequency range extending up to 70 GHz. Detailed interpretation of these data must await further study of the extent of cyst-packing in the dielectric cells. However, it appears now that the values of ϵ' , and the nature of the permittivity dispersions we have observed, suggest quite strongly that at least a large fraction of the total intracellular water does not behave like bulk water in its dielectric properties, even in maximally hydrated cysts. To my knowledge, these measurements of ϵ' and ϵ'' are the first to have been carried out on living cells at frequencies as high as 70 GHz. On the basis of measurements of conductivity and permittivity up to 10 GHz, Schwan and Foster (123) recently proposed that the vast majority of tissue water behaves exactly like bulk water in its dielectric properties. However, because their data were limited to frequencies of 10 GHz or less, their conclusion required a considerable extrapolation to higher frequency which seems to me to be questionable. In my opinion, the extent to which intracellular water exhibits the microwave dielectric properties of bulk water remains an open question at present and one that is certainly worthy of more detailed study.

Additional aspects of the physical status of water in *Artemia* cells, its relationship to metabolism, and other features of this system can be found in a recent symposium article (124).

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AN ENTROPY-ENTHALPY COMPENSATION LAW
MODEL ANALYSIS OF THE THERMAL SHOCK
BIOASSAY PROCEDURE FOR CERTAIN
SPECIES OF FISHES¹

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I. INTRODUCTION

Fry (1) has described the experimental protocol for the thermal shock bioassay system used to evaluate the thermal tolerances of aquatic organisms. The procedure is paraphrased here because of its relationship to model analyses described later in this paper.

After a suitable period of acclimation, organisms of a given species and life stage, are transferred from a laboratory system maintained at one temperature, T_0 (an acclimation temperature), to a similar laboratory system maintained at a different temperature, T^* . The difference between the new exposure temperature and the original acclimation temperature is the "thermal shock". The shock is a "step change" in temperature. Depending on the sensitivity of the life stage of the species being tested, it is possible to observe either no noticeable biological effect in the test animals, some acute or chronic damage in the animals, or death from thermal causes. Most of the classical studies with fishes have emphasized thermal death. In this paper, the mortality

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endpoint is chosen also. The aquatic taxa to be discussed, fishes, are chosen mainly because of the large available data base for potential study.

The early Canadian investigators (e.g., Hart, Fry, Walker, Brett and their co-workers) have studied the lethal temperature limits of fishes. They found that the mortality data obtained using the thermal shock bioassay procedure are often well correlated by the following empirical regression equation:

$$\log_{10} t_{\frac{1}{2}} = a_0 + b_0 \theta \quad (1)$$

in which $t_{\frac{1}{2}}$ is the time to death of 50% of the test animals (usually in minutes), θ is the exposure temperature (in degrees Celsius)², and a_0 and b_0 are empirically fitted constants that depend on species, life stage, geographical area and habitat of origin, and prior thermal history. Each pair of values of a_0 and b_0 is associated with a given acclimation temperature.

Not all data from thermal shock bioassay studies are successfully correlated by equation (1). If more than one mechanism has been manifested in the test organisms with intensities comparable to thermal shock, then the dose-response equation will reflect these additional routes to mortality. Additionally, for some species, Coutant (2,3) has suggested that a cubic equation provides a better empirical fit to the dose-response temperature data. He has suggested an equation of the form:

$$\log_{10} t_{\frac{1}{2}} = a_0 + b_0 \theta + b_1 \theta^2 + b_2 \theta^3 \quad (2)$$

in which b_1 and b_2 are additional fitted constants. Coutant has used equation (2) to evaluate endpoints other than mortality, notably "loss of equilibrium" (or loss of orientation of the animal in avoiding predation following thermal shock).

Coutant has used equation (1) to correlate as much of the thermal shock data for fishes extant as of 1970. He has tabulated values for a_0 and b_0 for various species of fishes in Appendix II-C of *Water Quality Criteria, 1972*, a publication of the National Academy of Sciences and National Re-

²In this paper, T refers to temperature in the Kelvin (absolute) scale, and θ refers to temperature in the Celsius scale. A subscript of "0" refers to an acclimation condition.

search Council (4). Since then, several investigators have utilized the procedure to a limited extent.

Coutant's purpose in preparing the data tabulation was to provide the values of a_0 and b_0 which are needed for the prediction of the effects of short-term temperature exposures on fishes under situations of entrainment or passage through discharge canals. Two basic situations are important: (1) a population of fishes of a given species is caught in the thermal plume of a heated discharge from a power plant and tries to escape; and (2) a population of fishes of a given species is caught in the intake flow to the cooling water system of a power plant or industrial complex, and because of entrainment, is carried through the cooling system where it experiences shock temperature exposures before being released to the ambient water body at the discharge point.

From an engineering point of view, equation (1) is simple to apply to these situations, but there are some practical problems with both equation (1) and its supporting data base. The obvious problem of an inadequate number of species for which values of a_0 and b_0 are available is mentioned first.

Other problems are:

1. Because equation (1) is empirical, there is no fundamental interpretation of the values of a_0 and b_0 . Both quantities are merely statistically fitted parameters.

2. There is no obvious reason why a thermal dose-response equation should necessarily be logarithmic in time and arithmetic in temperature. There should be some fundamental interpretation to the nature of the overall mathematical function chosen.

3. A different pair of values of a_0 and b_0 exists for each life stage of a given species from each different type of habitat and geographical region of origin, and for each different acclimation temperature history and each experimental protocol. Potentially many sets of values of a_0 and b_0 are needed to characterize the thermal shock response characteristics of a given species. This frustrates the possibilities of extrapolation of data and generalization of results and principles about thermal responses of fishes. Coutant himself has warned about the extrapolation of data for a given life stage of a given species to thermal condi-

tions other than those from which the original experiments were performed.

4. Unfortunately, not all of the parts of values of a_0 and b_0 which have been tabulated are statistically significant regression parameters. Thus, in some cases, equation (1) is itself not a valid regression equation for predictive use.

5. The "current wisdom" among performers of experiments in fish toxicology, including those performing experiments to determine thermal tolerances, is that one tests the most sensitive or critical stages of species subject to the toxicant in nature. For entrainment problems, small fishes, juvenile or fry stages, are of greatest concern since they are the most likely to be entrained. They are not necessarily the most sensitive thermal forms. Many of the studies reviewed by Coutant were more than thirty years old before publication of *Water Quality Criteria, 1972* (4) and these studies reflected an older stage of knowledge on the culture and laboratory maintenance of species. Greater advances in culture and management of species in the laboratory have allowed investigators now to be selective about species tested.

In this paper, the emphasis is on providing a fundamental physico-chemical interpretation of the regression coefficients, a_0 and b_0 , and thus, to show that the particular dose-response curve has a theoretical rather than purely empirical interpretation. It also considers means of demonstrating the consistency of various kinds of experimental results on thermal studies of fishes. To achieve these objectives, only statistically valid and tested values of regression coefficients were used.

II. THE DEVELOPMENT OF THE MODEL

The purpose of this paper is to provide a physico-chemical interpretation of the regression parameters a_0 and b_0 . This is to be accomplished by construction of a model which leads to equation (1). The structuring of the model is in terms of assumptions, parameters and arguments with recognizable biological interpretations and subject to testing with data.

The model proposed is simple. Thermal death of a species during the shock bioassay experiment does not depend

on the number of animals tested. All physiological processes are assumed to have Arrhenius factors to express temperature dependency. Because shock temperature tests involve step changes in the exposure temperatures, it is assumed that for a period of time following the temperature change, the physiological parameters of the test organisms remain those associated with the previous acclimation temperature. However, the rates of physiological processes contain the new exposure temperatures. This combination of new exposure temperatures with physiological parameters associated with a previous acclimation temperature reflects a state of physiological imbalance in the test organisms. Failure of the test organisms to adapt and remove this imbalance leads to organism death. There is also a time period, t^* , after which death cannot be assumed traceable to thermal causes. Brett (5) has suggested that 7 days or 10^4 minutes is the time period. Although the value of t^* is not essential to the derivation of the model presented here, it is important in applying the model to the analysis of temperature thresholds of thermal death. (That application is the subject of another paper, in preparation).

If N is the number of test animals in the experiment, t is time, T_o is the acclimation temperature, μ is the death rate constant associated with T_o , and ΔH_o is an enthalpy of activation of the death:

$$-\frac{1}{N} \frac{dN}{dt} = \mu_o \exp[-\Delta H_o/RT_o] \quad (3)$$

Following a step change in temperature, the death rate is:

$$-\frac{1}{N} \frac{dN}{dt} = \mu_o \exp[-\Delta H_o/RT] \quad (4)$$

Because the temperature of the system is independent of time, equation (4) may be integrated. The limits are $N = N_o$ at $t = t_o$, and $N = N_o/2$ at $t = t_{1/2}$. The integration, followed by algebraic rearrangement of terms, leads to the expression:

$$t_{1/2} = \left[\frac{0.693}{\mu_o} \right] \exp \left[\Delta H_o/RT \right] \quad (5)$$

Because of the equivalencies of the Kelvin and Celsius scales (one is a translation of the other), $T - T_o = \theta - \theta_o$. Substitution of the value of T into equation (5), and expansion of the Arrhenius factor in the exponential as an infinite series:

$$\Delta H_o/RT = \Delta H_o/RT_o \left[1 - \left(\frac{\theta - \theta_o}{T_o} \right) + \dots \right] \quad (6)$$

Equation (6) contains the infinite series previously mentioned. If the series is truncated at the linear term, equation (1) is obtained; if the series is truncated at the cubic term, equation (2) is obtained.

In data which have been tested, the range of T is 273°K to 313°K (0°C to 40°C). Temperatures outside of this range are rarely relevant to studies of the thermal physiology of fishes. Therefore, a "worst case" analysis is an acclimation temperature of 0°C, and the thermal shock of 40°C. The ratio of $(\theta - \theta_o)/T_o$ is 40/313 or 0.146. On order of magnitude grounds alone it is necessary to carry the series to the quadratic term, and study a second degree equation. For most situations, however, order of magnitude analyses of the truncation error allows the linear approximation as satisfactory. Therefore, truncating the series at the linear term, and taking logarithms to the base of ten of the terms in equation (5):

$$a_o = \log_{10} \left(\frac{0.693}{\mu_o} \right) + \frac{0.434 \Delta H_o}{RT_o} \left(1 + \frac{\theta_o}{T_o} \right) \quad (7.1)$$

$$b_o = - \frac{0.434 \Delta H_o}{RT_o^2} \quad (7.2)$$

$$\log_{10} t^{1/2} = a_o + b_o \theta \quad (7.3)$$

The assumption about ΔH_o and μ_o being related to T_o , and the validity of truncating a series at the linear term provide the mathematical rationale for the form of equation (1) (now repeated as equation 7.3).

Equations (7.1) and (7.2) provide the first step in the physico-chemical interpretations of a_o and b_o . It is clear that b_o is only a function of the enthalpy of activation of the presumed overall death process and the acclimation temperature of the organism. On the other hand, a_o depends on both the enthalpy of activation with acclimation temperature and the death rate constant for a given acclimation temperature. In some ways, a_o may be thought of as a function of both "thermodynamic type and kinetic type parameters".

With the last comment in mind, the analysis of the model proceeds with the development of the thermodynamic nature of b_0 . The reader will recognize that this approach implies the use of the Eyring model from chemical kinetics. Herein, a higher-order "analogy" to the Eyring model is being invoked. Eyring's model explicitly concerns a single chemical reaction. Thermal death, however, is a complex combination of chemical reactions or disrupted chemical processes, rather than a single process. Equation (3) really gives a function which is an integration of these various sub-processes in thermal death. Therefore, the word *analogy* is used because the mathematical methods of Eyring's model are adapted, but there are basic differences in its foundations, vis-a-vis this application to thermal death. In this case, it is possible to think of the Eyring theory applied to the whole organism level rather than the prebiotic cellular level.

The "thermodynamics" of b_0 are based on the assumption that the "thermodynamic functions" obey standard thermodynamic equations and relationships: Maxwell equations, van't Hoff equations, Gibbs equations.

The first step is to place all of the functions on a basis of a common reference temperature, T_r . The problem with the data obtained experimentally is that each value of b_0 is associated with a different acclimation temperature. Each acclimation temperature is a reference temperature. Therefore, all values of b_0 must now be referred to a common reference temperature basis. This basis, according to thermodynamics, is arbitrary, but once chosen, must be used consistently.

A thermodynamic function which is independent of the reference temperature is a specific heat. A specific heat at constant pressure is chosen because the organism can change cellular volume and density under thermal stress, but does not change cellular pressure significantly. A specific heat of activation, equation (8.1), can be defined as the temperature derivative of the enthalpy of activation. A fundamental question here is which temperature is to be used on the differentiation process? Careful reflection of the experimental protocol suggests that the appropriate temperature variable is the acclimation temperature. The model assumption that organisms retain the physiological properties (associated with prior acclimation temperatures) following a thermal shock is equivalent to noting that T_0 is the "reaction variable" in the kinetic equations. Organisms which

successfully adapt to the new temperatures assume the new temperatures as their acclimation temperatures. Essentially, therefore, one has moved along a scale of acclimation temperatures by a constant value, a simple linear translation of coordinates. The derivative of the enthalpy of activation with the general experimental temperature variable is thus numerically equal to the derivative with respect to acclimation temperature, since a derivative with respect to the constant of translation is zero.

The "complete" thermodynamics includes not only specific heat of activation, but entropy of activation, enthalpy of activation (independent of reference acclimation temperature) and a free energy of activation:

$$\Delta C_{po} \neq = -2.306 R \frac{\partial}{\partial T_o} (b_o T_o^2) \quad (8.1)$$

$$\Delta S_o \neq = \int_{T_r}^{T_o} \left\{ \left[\frac{-2.306R}{T_o} \right] \frac{\partial}{\partial T_o} (b_o T_o^2) \right\} dT_o \quad (8.2)$$

$$\Delta H_o \neq = \int_{T_r}^{T_o} \left\{ -2.306R \frac{\partial}{\partial T_o} (b_o T_o^2) \right\} dT_o \quad (8.3)$$

$$\Delta G_o \neq = \Delta H_o \neq - T_o \Delta S_o \neq \quad (8.4)$$

It is important to consider some special values of T_o which are encountered when actual bioassay data for fishes are evaluated. These values of T_o occur when equation (8.1) has a numerical value of zero. Thus, the enthalpy of activation, and entropy of activation, have critical points on a T_o axis.

Thermodynamic consistency of data requires that $b_o T_o^2$ and $b_o T_o$ share common values of T_o which produce maxima or minima. Furthermore, when the entropies and enthalpies of activation are maxima and minima, the free energies of activation also exhibit critical values. Since the temperature derivative of a free energy is the negative of the entropy, it is clear that when entropy and enthalpy have a common maximum or minimum value, the $\Delta S_o \neq$ must vanish in the free energy expression.

The change in entropy may vanish in equation (8.4) in two ways: either ΔS_0^\neq is zero, or it acquires a numerical coefficient which becomes zero. This latter situation is of special interest although it is soon shown that from an experimental point of view, both types of zero conditions arise concurrently.

One of the ways to make the entropy function vanish at that acclimation temperature where the free energy has a critical point is through the use of an entropy-enthalpy compensation rule. Lumry and Rajender (6) have discussed the systems for which such rules appear to apply. Drost-Hansen (7) has discussed the conditions and controversies when these rules have been challenged.

Entropy-enthalpy compensation rules are extrathermodynamic expressions which relate linear changes in enthalpy to linear changes in entropy. Because they are extrathermodynamic, the rules cannot usually be derived from first principles except for some mathematically trivial cases. Nevertheless, when these rules are operational, they are powerful tools to correlate data.

The interpretation of compensation rules presently rests on the hypothesis that they reflect the interaction of the solvent medium with the processes exhibiting the compensation. The compensation parameters are themselves considered properties of the solvent. Since the solvent medium of the cell is water, the presence of a compensation rule is circumstantial evidence to implicate the participation of water molecules in the molecular level processes. Intuitively, such a situation would be reasonable. Theories of thermal death are often discussed in terms of denaturation and breakdown of enzymes. Usually when complex proteins and macromolecules are formed or destroyed in a cell, the water molecule is somewhere in the chemical reaction or cellular pool. The special role of water outside of its "reagent characteristics" is however relatively recent in biophysical thinking. This special role can be very neatly ascribed to the "solvent" nature of the molecule in the cellular system.

The compensation rule is expressed by the relationship:

$$\Delta H_0^\neq = \alpha + \beta \Delta S_0^\neq \quad (9)$$

in which α , β are the compensation parameters and are constants, and β is referred to as the "compensation temperature". Substitution of equation (9) into equation (8.4)

shows that the entropy term will vanish when the acclimation temperature equals the compensation temperature:

$$\Delta G_o \neq \alpha + (\beta - T_o) \Delta S_o \neq$$
 (10)

Once again, recalling that the temperature derivative of the free energy function is the negative of the entropy function, then substitution into equation (10) of expressions in terms of b_o leads to the result that:

$$\frac{\partial b_o}{\partial T_o} = -\frac{2}{T_o}$$
 (11)

Equation (11) is suitable for numerical and statistical testing. Within the range of uncertainties and variations of values of b_o (estimated as a regression parameter from thermal bioassay data), equation (11) holds for many cases, over a relatively broad range of temperatures. It is not always valid, and this is not disappointing: because compensation rules are extra-thermodynamic, there is no inherent reason to assume that they are always operable.

Experimental factors and thermodynamic consistency requirements of data dictate that the reference temperature be chosen at the compensation temperature which is where the function $b_o T_o$ vs. T_o has zero slope.

The model has several limitations. As previously mentioned, compensation rules cannot be assumed *a priori* to be operable for a biochemical system. A second problem is the considerable debate about the statistical validity of compensation parameters which have been derived for particular experimental systems. Any value of a compensation temperature which can be assigned to a species as a result of work reported herein, must pass statistical tests of suitable rigor. A third problem is that one does not know with certainty that the information derived from the study fixes the role of water in the thermal death process. The current interpretation of compensation rules favors this thesis, but the evidence remains strongly circumstantial. Therefore, it is felt that the success of the model is supportive of the role of water in cellular processes leading to thermal death, but not proof of the role. Other analyses are required once a possible compensation temperature has been derived. A fourth problem is that the assignment of a compensation temperature to a species must be shown to be "unique, reproducible, and independent of investigator or protocol". Actually the uniqueness requirement may be refined to consider a par-

ticular life stage of a species from a given geographical area. In this regard, while the model has been examined conceptually for the case of heat shock, the same compensation temperature must be derivable from studies of cold shock.

III. ASSIGNMENT OF COMPENSATION TEMPERATURES FOR SPECIES OF FISHES

The essential data base was Appendix II-C of *Water Quality Criteria, 1972* (4). These data already contained tabulations of b_o vs. T_o and appropriate statistical information about b_o . Other sources of data, usually more recent and not available to Coutant at the time he processed the Appendix II-C (8) data, were also used. In all, data from over forty species of fishes were processed.

In order to estimate a compensation temperature, it was necessary that at least three statistically valid non-zero values of b_o be available. A numerical difference scheme was used, noting that a zero slope of the function $b_o T_o$ vs. T_o occurs when the numerical differences change sign on either side of that T_o which is the critical value. Once the location of the possible compensation temperature was obtained, numerical interpolation procedures were used to refine the estimate. The curvature of $b_o T_o$ vs. T_o in the vicinity of the critical point was approximated by a symmetrical parabolic segment. Only those critical points which were true maxima or true minima were considered. In some cases, a plateau region occurred (a place where higher derivatives of $b_o T_o$ are also zero). Such a situation is considered "unresolved" or ambiguous at this time; it is not known how to assign a compensation temperature in such cases. Other evidence, external to the mathematical methodology, must be invoked to resolve an ambiguous case.

Once a compensation temperature was derived, it was tested for statistical validity. Values of $b_o T_o$ on either side were compared to the critical value of $b_o T_o$ using one-tailed t-tests. If the assigned critical value was statistically less than neighboring values (a minimum) or greater than neighboring values (a maximum) at $Pr = 0.05$ or less, the compensation temperature was considered established. Those values which did not meet this test were considered potential compensation temperatures, but not established. The established values were then compared for consistency in

as many cases as possible. For example, if both heat shock and cold shock data were available, then it was ascertained whether both kinds of data produced the same result. If more than one investigator had run shock tests with organisms of a given species, the results of independent investigators were compared. Table I provides the results of these studies.

The assigned compensation temperatures in both confirmable and non-confirmable cases have a statistical distribution which shows a modal dominance (not very definitive) in the vicinity of 28-30°C. This pattern is consistent with much of the data collected thus far by Drost-Hansen (7) on systems with apparent marked changes in thermal properties in the vicinity of certain temperatures. Both of the modal temperatures are essentially the same as those found by Drost-Hansen with other kinds of data. While Drost-Hansen did examine at one point the upper thermal limits of certain marine species to locate a modal temperature in the vicinity of 30°C, he had not examined thermal bioassay data with fishes (both marine as well as fresh water) for the manifestation of the critical temperatures with similar characteristics.

It is also quite interesting to note that all five species of the North American fauna of the salmon genus *Oncorhynchus* show confirmable compensation temperatures. In most work on the thermal characteristics of aquatic species, any properties observed are usually assigned uniquely to the species. In this case there is some evidence that the compensation temperature model carries over to a higher taxonomic level, that of genus. Other species of a common genus are also represented in Table I; however, there is not a large enough sample of such species to apply the generalization further.

IV. SUMMARY

A model is proposed to provide a physico-chemical basis for the parameters and formulation of a specific type of thermal dose-response curve and is applied to the data for a variety of species of fishes. The model is suggestive of the operation of an entropy-enthalpy compensation rule in a thermal death process for some of the species tested. The model is by no means applicable to all of the species examined, but works in enough cases to be encouraging. The operation of an entropy-enthalpy compensation rule in a

TABLE I. Assignment of Compensation Temperatures to Species of Fishes from Thermal Shock Bioassay Studies

I. Species of fishes which can be assigned compensation temperatures which are statistically confirmable at $Pr = 0.05$ or less

Species and Life Stage		Compensation Temperature °C	Range of Acclimation Temperature °C
<i>Brevoortia tyrannus</i>	larval (a)	14	7-20
<i>Coregonus artedii</i>	juveniles	18	2-25
<i>Coregonus hoyi</i>	juvenile (b)	10, 18	5-25
<i>Esox lucius</i>	sac fry (c)	12	6-18
<i>Fundulus parvipinnis</i>	adult	20	14-28
<i>Girella nigricans</i>	juvenile (b)	23	12-28
<i>Ictalurus nebulosus</i>	(unknown)	15, 32	5-34
<i>Lepomis macrochirus</i>	sac fry (b,c)	26	12-33
<i>Lepomis magalotus</i>	juvenile	29	25-35
<i>Menidia menidia</i>	juvenile (b)	23	7-28
<i>Notemigonus crysoleucas</i>	adults (e)	18, 28	10-30
<i>Oncorhynchus gorbuscha</i>	fry	10	5-24
<i>Oncorhynchus keta</i>	juvenile (e)	11	5-23
<i>Oncorhynchus kisutch</i>	juvenile	15	5-23
<i>Oncorhynchus nerka</i>	juvenile (e)	12	5-23
<i>Oncorhynchus tshawytscha</i>	fry	10, 22	5-24
<i>Pseudopleuronectes americanus</i>	juvenile	14	7-28
<i>Rhinichthys atratulus</i>	adult	18	10-25
<i>Stizostidion canadense</i>	juvenile (c)	17	11-23

TABLE I (continued)

II. Species of fishes from which compensation temperatures can be derived but which cannot be statistically confirmed. These are possible temperatures and require that other kinds of evidence be marshalled before a confirmed assignment is made.

Species and Life Stage		Compensation Temperature °C	Range of Acclimation Temperature °C
<i>Catostomus commersoni</i>	adult	15	10-25
<i>Esox masquinongy</i>	juvenile	28	25-30
<i>Gambusia affinis holbrooki</i>	adult	20	15-35
<i>Notropis atherinoides</i>	juveniles	15	5-25
<i>Perca flavescens</i>	adult	11	5-25
<i>Pimephales notatus</i>	adult	10	5-25
<i>Salvelinus namaycush</i>	juveniles	15	8-20
<i>Sphaeroides maculatus</i>	adult	14	10-28

III. Species of fishes for which no compensation temperature assignment can be made within the range of acclimation temperatures available from thermal shock bioassay tests.

Species and Life Stage		Range of Acclimation Temperature °C
<i>Dorosoma cepedianum</i>	underyearling	25-35
<i>Ictalurus punctatus</i>	adults	25-35

TABLE I (continued)

Species and Life Stage	Range of Acclimation Temperature °C
<i>Micropterus salmoides</i>	underyearling
<i>Notropis cornutus</i>	adult
<i>Salmo gairdnerii</i>	juvenile
<i>Salvelinus fontinalis</i>	yearling (f)
<i>Semotilus atromaculatus</i>	adult

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- (a) Compensation temperature derived from cold shock data only.
 - (b) Compensation temperature derived from both heat shock and cold shock.
 - (c) Data from sources other than Appendix II-C of "Water Quality Criteria 1972" (4).
 - (d) Unless otherwise stated all compensation temperatures derived only from heat shock data.
 - (e) Assigned compensation temperatures are based on refined estimates.
 - (f) This species exhibits a plateau region in the curve of b_0T_0 vs. T_0 which is an unresolved case.

thermal death process is supportive of the possible role of water in the cellular processes involved.

Note: Readers interested in the extensive data tabulations and statistical tests, or requiring details on the procedures and data, sources, etc., are invited to communicate with the author.

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A SPECULATION ON THE RELATIONSHIP
BETWEEN WATER STRUCTURE AND THE
ZOOGEOGRAPHIC DISTRIBUTION OF
SPECIES IN AQUATIC COMMUNITIES

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Ecologists have occasionally classified biological communities by the climatic zones in which these communities exist. Of all of the climatological parameters used, temperature clearly is the most popular. Witness the following zonations: arctic, boreal, temperate, subtropical, and tropical. Further examination of these definitions or classifications suggests that for aquatic communities: arctic communities typically function from 0°C to 15°C, boreal and temperate from 10°C to 30°C, subtropical from 25°C to 32°C, and tropical from 30°C to 36°C. These limits are approximate, but illustrative.

In any given biological community, not all of the member species fit exactly into the temperatures ranges used in the classifications. Some species have broad tolerances to environmental temperatures and are found in several of these communities; other species have very restricted temperature tolerances and are found only within a restricted range within a given type of community. What is observed, nonetheless, is that the community, as a structural entity with properties greater than the sum of its species members' properties, seems to undergo abrupt or rapid changes within very short temperature intervals coincident with the upper end of the temperature range of the bulk of its members. These structural changes include replacements of dominant species, population shifts within species, and changes in the patterns of cycling of nutrients and energy. These changes are identi-

fiable. Very clearly the structural nature of the community has changed. It has become unstable at a certain temperature and a new structural arrangement replaces it.

A rather interesting observation is that the upper temperature limits of some of the zoogeographic classifications often coincide with those temperatures at which one observes changes in structural properties of interfacial water. The structure of vicinal water has been explored in great depth by many investigators, but Drost-Hansen and his co-workers have emphasized the changes which are associated with certain special temperatures. These temperatures occur within the range of 14-17°C, 15-18°C, and 30-32°C, with respect to aquatic systems of interest in this paper. These investigators have emphasized the properties of water structure on a molecular scale in biochemical and biophysical systems; however, they have not addressed themselves to the question of these structural characteristics on a higher biological scale; namely, that of communities and ecosystems. In this paper, a relationship between water structure and zoogeographic distributions of aquatic organisms in biological communities is addressed.

The approach is based upon a particular type of modeling scheme which has been applied by Prigogine and Glansdorff (2) to limited ecological situations. Prigogine and his co-workers have been interested in chemical analogues to the Lotka-Volterra model in ecology. (Lotka-Volterra dynamics or models are classical two species models of host-parasite or predator-prey interactions in which the various populations of the species have density-dependent behavior). Specifically, ecological systems are "redescribed" in terms of systems of chemical equations. Species become populations of a given molecular entity. Thus, one talks of molecules rather than organisms. From analogues of the ecological equations structured as chemical equations, thermodynamic functions for the molecular species such as chemical potentials or affinities, and thermodynamic functions such as reaction rate expressions are calculated. In non-equilibrium thermodynamics, the product of an affinity or chemical potential and a reaction rate is a time-dependent entropy function known as the "entropy production". The product of differentials of the affinity and reaction rate, a differential of the entropy production, has been shown to be a Lyapunov function in the mathematical sense. Thus, if the chemical reaction analogue model has been structured properly to mimic the ecological model, one can use this Lyapunov function to study mathematically the stability properties of the model.

Since the objective of models is ultimate prediction of new situations, the stability analysis can be invoked to hypothesize stability relationships in the real world natural communities being modeled.

Prigogine and Glansdorff (2) have provided "recipes" for constructing the appropriate chemical reaction analogue for an ecological situation; for example (a) autotrophic growth processes become "autocatalytic" reactions; (b) predator-prey processes become second-order chemical reactions or binary reactions in two molecular species; (c) density-dependent death processes are first-order chemical reactions.

To avoid any situations that violate biological laws, specifically modeling a situation such as "spontaneous generation", all chemical reactions are made *irreversible*. Recycling of the exogeneous or endogeneous products of organism death becomes a situation requiring a subtlety of reasoning. Basically for recycling, a chemical reaction analogue of the inverse of the death reaction would appear to be the proper model format. While the format is proper, the following cautions must be observed: (a) the ratio of rate constants for the generation of products that are not recycled and the recycling of these products is NOT a true thermodynamic equilibrium constant; (b) mechanisms of recycle are rarely the exact thermodynamic inverse of mechanisms of product generation for recycle. Thus, the important assumption of *microscopic reversibility*, applicable to true thermodynamic situations does NOT apply.

Use of the Prigogine-Glansdorff stability function depends on its numerical use. A positive or zero value means that the system being modeled is mathematically stable (this may not have anything to do with natural or ecological stability if the model is inappropriate); a negative value means that the system being modeled is mathematically unstable (again, this may not have anything to do with natural or ecological stability if the model is inappropriate). All stability is with regard to perturbations, and the perturbations are in those parameters which form the basis of taking the differentials or affinity and reaction rate.

A zero value of the stability function applies to thermodynamic equilibrium in a chemical system. For the ecological situation, however, a different interpretation is needed. It is the *threshold* of stability for a model and not an analysis of an equilibrium. For certain restricted

mathematical cases, the zero value may also represent an equilibrium analysis, but one is hard pressed to predict equilibrium in most dynamic ecological systems.

The zero value of the stability function, or threshold case, is the most important in this work. Only the perturbation of temperature is considered. Other perturbations, notably species populations, nutrient levels, habitat availability, etc. may be conveniently handled. If several different factors are capable of being perturbed independently, then each type of perturbation contributes a differential and a term to the stability function. The stability function becomes a summation, and one judges stability of the system being modeled by the numerical sign of the summation: positive, negative, or zero. Thus, a stable system may have one or more types of perturbations acting as destabilizing situations, but the overall function (stability function) remains positive because of the influence of the remaining stabilizing situations. In this work, these other perturbations are not involved so that their contributions to stability functions are zero.

Prigogine and Glansdorff have shown that under some circumstances, notably perturbations in population fluctuations, "death" tends to be a stabilizing process. When temperature is considered, however, the situation may change. If the temperature coefficient of the death rate suggests an exothermic situation, temperature perturbations may cause a destabilizing effect. The sign of the differential of the reaction rate is now negative, and if the temperature differential of the affinity function is positive, then the stability function will be negative.

In general, "autotrophic" growth is destabilizing. If the building or repair of protoplasm is endothermic, then the temperature differential for a growth process will be positive. Consequently, perturbations in temperature will make contributions of stability terms for a given species to some overall stability function for a community, overwhelmingly negative. There will be a continuous prediction of instability. Yet the natural systems are observed to be stable. Therefore, some balancing must occur. This balancing of stabilizing and destabilizing temperature related terms must be complete at unique temperatures where the biotic community's structure collapses. If the stability function for the community is itself the summation of the stability contributions of its members and their interactions, then for a threshold of stability for the community to exist, *most* of

the species must share a common upper thermal limit.

It is possible to make the contributions of temperature related perturbations to the stability function positive, negative, or zero, as one desires merely by making the temperature coefficient of the death rate itself dependent on temperature in some complex manner. Among the "simplest" of the "complex" mechanisms to use is to make the enthalpy of activation of the particular process in the rate expression for thermal growth or thermal death or both obey a *linear entropy-enthalpy compensation law*. The choice of this procedure has been explored in a previous paper by this author with regard to statistical studies of thermal death processes in some aquatic fishes.

The present views on entropy-enthalpy compensation laws as they apply or are operable in various kinds of biochemical systems have been discussed by Lumry and Rajender (3; see also 4). They reason that these laws express "properties" of the solvent medium for the appropriate system. With reference to aquatic organisms in biological communities, water is a solvent in two different senses. First, it is the solvent medium of the cell, and thus, intimately related to internal biochemical processes leading to organisms' growth or death or both. Second, it is the solvent medium for the ecosystem, and thus analogously, it may be thought to be related to "internal ecological processes" which lead to instability ("collapse" or "break-up") in community structures of an ecosystem.

In the previous work of this author, one must be cognizant of the temperature which is used in formulating death rate functions. The "acclimation temperature" is the base temperature from which perturbations, fluctuations or changes in temperature are measured. The acclimation temperature is the temperature used in the death rate and growth rate functions. In natural ecosystems and communities, the environmental temperature becomes the acclimation temperature. Departures from this temperature, through sudden changes in climate or releases of man-made heated or chilled water discharges, become the perturbations. Thus, for a model of a natural ecosystem without man-induced temperature changes, only sudden climatic catastrophes are going to change temperature in a manner to destabilize the system. One can assume that the organisms present in a given area are tuned by evolution to the seasonal temperature cycle. Since the previous work involved man-induced temperature changes, mainly those associated with potential powerplant discharge

situations, the correct temperature to use in the growth and death rate functions is the environmental temperature prior to the perturbation. If, however, the temperature field remains relatively constant, then there is no perturbation at a given position and time, and one just considers the environmental temperature relative to other temperatures nearby to which the species may be exposed by currents, or movements.

For the stability function analysis, the temperature to be used in the modeling and taking of differentials is the acclimation temperature. One can always regard the environmental temperature as a linear transposition of this acclimation temperature, in which case the differentials are the same.

When the linear entropy-enthalpy compensation laws are now introduced into the growth and/or death functions, and temperature differentials are taken, it is immediately apparent that the differential is zero at the compensation temperature. Thus, the compensation temperature now becomes the *temperature threshold* for stability since it will make the stability function zero.

In order for the methodology to make ecological sense, the following pattern of transitions with increasing temperatures must be observed in aquatic ecosystems: (a) an arctic community should provide a positive stability function below about 15°C , and a negative stability function above 18°C ; (b) a temperature community should become unstable around 30°C , and be replaced either with a subtropical or tropical community. Tropical communities should provide negative stability function terms above about 37°C ; (c) a zero should occur at 45°C , an upper limit of protoplasm streaming and a death condition for most communities except those of thermal springs.

The suggested use of a compensation law in the stability function does indeed produce the correct behavior of the stability function provided some conditions are fulfilled: (a) the model must not be "global" in the sense that it encompasses all ecosystems possible through the entire temperature range of 0°C to 40°C of the aquatic communities of this planet. Rather the model should be restricted to the description of one or at most, two, communities; (b) almost all of the species in a community which have restricted temperature tolerance ranges appropriate to the community type being modeled should have coincident upper thermal limits with each

other and a compensation temperature for water molecules; (c) those organisms which have broad thermal tolerances must be in the minority and not exhibit broadly or strongly temperature dependent death rates or growth rates in general except near their upper thermal limit. If these species exhibit strongly temperature dependent growth and death rates at temperatures considerably below their upper thermal limits, their growth rates and death rates should be balanced so that these species contribute zeros or weakly positive terms to the overall stability function; (d) the stability function itself must consist of a summation of contributions of the species and their interactions.

The four previous conditions are basically common sense. Prigogine and Glansdorff's stability theory cannot predict "evolution". Thus, for condition (a), the "global" restriction just serves to prevent the modeler from predicting the evolution of systems and arrangements following instabilities. At the same time, knowledge of the behavior of some of the aquatic communities of this planet allows one to guess certain community patterns associated with certain temperature transitions. Condition (a) thus covers the possibility that at least one transition can be considered, but warns against more than one transition. Furthermore, if two transitions are required, one must be concerned about more than compensation temperature being built into the entropy-enthalpy compensation law. There are several empirical ways of doing this, but none of them is sufficiently satisfactory for theoretical or modeling purposes to be discussed in detail here.

Condition (b) is an expression of the "cooperativity" aspect of structural collapse of the ecosystem. If all or most of the species here did not have coincident thermal upper limits, a transition in community structure might be observed over several degrees Celsius, and the transition must be so subtle, that change in organization and structure of the community is missed. The fact that abrupt changes are observed is the guiding principle in the modeling.

Condition (c) is consistent with condition (b). If organisms of broad thermal tolerance dominated a community, transitions in community structure would not be observed.

Condition (d) is a logical extension of the Prigogine and Glansdorff concept that the stability function for systems must consider all of its species parts. Instead of worrying about two species systems of Lotka-Volterra dyna-

mics, one can now generalize the many species systems.

The particular model used to illustrate the situation is adapted from previous work of the author on studies of thermal death rates in fishes.

(1) The model considers temperature close to an upper lethal temperature of community members. Thus, death rates are faster than growth or reproduction rates. For simplicity, the growth rates are negligible. There are actual ecological situations of an upper temperature of "zero growth". This temperature is below the incipient lethal temperature of the organism and represents a transition point for a species relative to survival and longevity without reproduction.

(2) The death rate process is autotrophic. The temperature coefficient is expressed as an Arrhenius function. The enthalpy of activation is to obey the linear entropy-enthalpy compensation law.

(3) The environmental temperature is the acclimation temperature. Temperature perturbations away from this temperature come from man-induced thermal regimes such as power-plant discharge waters or effluents.

(4) Only temperature is considered. Chemical toxicity is not considered.

(5) Temperature perturbations are independent of population perturbations. This means that changes in organism numbers do not affect thermal perturbations or temperature fluctuations.

The Stability Function is $\delta A \delta r$. The chemical scheme of interest is:

- 1) $N + X \rightarrow 2N$ autocatalytic reaction (growth)
- 2) $N \rightarrow Y$ death (density-dependent)

$$r = \mu_o N \exp [-\Delta H/RT_o] \quad \text{chemical reaction 2}$$

$$A = RT_o \ln [N/Y] \quad \text{chemical reaction 2}$$

$$\Delta H = \alpha + \beta \Delta S \quad \text{compensation mechanism}$$

$$\delta r = \{\mu_o \exp [-\Delta H/RT_o]/RT^2\} \{(\Delta H) - T_o (\delta H)/\delta T_o\} \{\delta T_o\}$$

$$\delta A = R \ln [N/Y] (\delta T_o)$$

$$\delta A \delta r = \{\mu_o \exp [-\Delta H/RT_o]\} [\ln (N/Y)]$$

$$(\delta T_o)^2/T_o^2 \{\Delta H - T_o (\delta H)/\delta T_o\}$$

The symbols used are as follows:

A	Thermodynamic affinity
ΔH	Enthalpy of activation
N	Population of a molecular species (numbers)
R	Universal Gas Constant
ΔS	Entropy of activation
T	Absolute Temperature
X	Nutrient levels (concentrations)
Y	Products of exogenous or endogenous reactions
r	Reaction rate expression
μ	death rate constant
α	compensation law constant
β	compensation temperature

The subscript, o, refers to acclimation temperature.

All of the terms in the first set of inclusive brackets are positive definite, and their product is positive definite. For the case of $\ln N/Y$, this term must be positive definite or it implies that the affinity favors the reverse reaction, the unallowable spontaneous generation. The terms in the second set of inclusive brackets, however, may be numerically positive, zero, or negative. Sign of the second set of terms depends on the signs and numerical values of the enthalpy of activation and its temperature derivatives.

From previous work, it is known that when $T_o = \beta$, the second set of bracketed terms will be zero. This is the compensation law requirement. Remember, that the reference temperature for ΔH must be chosen at $T_o = \beta$ in order to assure thermodynamic consistency for the results from thermal death studies for aquatic organisms. Thus both ΔH and ΔS are zero at the compensation temperature. From the compensation law, the temperature derivative of H is itself an entropy term as well as a derivative of the entropy. Again, thermodynamic consistency requires that the temperature derivative of ΔH and ΔS be zero at the compensation temperature.

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