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INFLUENCE OF CYTOMATRIX PROTEINS ON WATER AND ON IONS IN CELLS

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Abstract

This review concerns the influence that cytomatrix proteins have on the motional properties of water and on the major inorganic ions in cells. The techniques we used for study of water in cells and on the cytomatrix proteins include: pulsed NMR of water protons, quench cooled ice crystal imprint size, and osmotic behavior. The technique for study of ions involved use of electron-probe X-ray microanalysis of thin cryosections of cells. The cytomatrix was found to play the major role in determining the extent of hydration water in cells. The amount of hydration water varied greatly between cell types (e.g., lens fiber cells have no detectable bulk water) and varied in the same cell type studied under different states (e.g., unfertilized and fertilized sea urchin eggs). Aggregation of cytomatrix proteins (actin in particular) is a determinant of the extent of hydration water in cells. Hydration water appears not to participate in the ideal osmotic equation of van't Hoff. The ionic content of cells does not accurately predict the chemical activity of the ions in cytoplasm. A major proportion of intracellular K^+ and a substantial fraction of Cl^- was found to be influenced by the cytomatrix such that their diffusion was impaired. The cytomatrix is responsible for the decreased motional properties of a substantial portion of cellular water and ions.

Key Words: Protein hydration, Proton NMR, Sodium, Potassium, Chloride, Electron probe X-ray microanalysis, Enzyme activity, Monovalent ions.

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Introduction

This review deals with the influence that macromolecules in cells have on the motional properties of water and ions. The paper is divided into two major sections. The first section deals primarily with interactions of water with proteins. The questions addressed in this section are: What is the extent of hydration water on specific macromolecules and in cells? Does the docking of macromolecules (i.e., aggregation) change the extent of hydration water? Does hydration water participate in the ideal osmotic equation of van't Hoff? Can hydration water explain the failure of cells to act as perfect osmometers? The second section of the report deals with the ionic environment within subcellular compartments. The questions addressed in this part of the review are: What is the content of ions, specifically Na^+, K^+ and Cl^-, in the cytoplasm of marine and non-marine plant and animal cells? Does the ionic content, expressed on a wet weight basis, accurately reflect the chemical activity of these ions? What mechanisms are involved in the maintenance of ion content gradients between the intra- and extracellular environment and between subcellular compartments?

The answers to these questions indicate that the cytomatrix proteins, and especially actin, play a key role in the determination of the extent of hydration water and also indicate that the cytomatrix is responsible for a reduced chemical activity of ions in the cytoplasm.

Hydration Water on Macromolecules and in Cells

It is generally agreed that some water molecules associated with proteins and other macromolecules are slowed in the motion (both rotationally and translocationally) from that of bulk water. This fraction of motionally perturbed water has been studied by a number of methods including: NMR titration (Fullerton et al. 1986), X-ray diffraction on protein crystals, sedimentation, isopiestic

isotherm, differential scanning calorimetry, non-freezing fraction, dielectric attenuation, solute exclusion and osmotic behavior. Several models have been proposed to interpret the data from these methods. The two extremes in these models are as follows: a small fraction of water which is greatly restricted in its movement (implying a long correlation time) is bound to protein and this fraction is in fast exchange with a much larger fraction of bulk water which has a short correlation time (10^{-12} sec); the other extreme model suggests that the water associated with a protein surface has a continuum of correlation times with no distinct boundary between the bound fraction and bulk water.

Our NMR relaxation time measurements on water protons associated with globular proteins and in cells, when studied as a function of dehydration, indicates that a model with three water compartments, each of which is in fast exchange with the next most outer water compartment, is both necessary and sufficient to explain all of the varied hydration measures (Fullerton et al. 1986, Merta et al. 1986).

At present there are no universally accepted terms which describe hydration of proteins or cells. We therefore define and use the following terms: 1) Bulk water - water so distant from the solute surface that its molecular motion is determined only by the interaction characteristics of the water molecules themselves; 2) Bound water - water molecules which are either hydrogen bonded to fixed polar sites (electric dipole) or to ionic charge sites; 3) Structured water - water molecules that are motionally perturbed from that of bulk water; and 4) Hydration water - all water molecules for which motion is perturbed from that of bulk water (hydration water is the sum of bound and structured water). The method we have used to determine the extent and the correlation times of the above fractions on proteins and in cells is referred to as the NMR titration method. Briefly the specimen to be analyzed is subject to a proton T_1 (spin-lattice or longitudinal) relaxation time measurement and is then weighed. This NMR measure and weighing procedure is repeated numerous times during stepwise dehydration of the specimen. When the specimen is completely dry, the data are plotted as the relaxation rate (reciprocal of the relaxation time) against the mass of solute per mass of water (Ms/M). What has been found for the globular protein lysozyme and in several cell systems is a series of linear slopes with distinct transition points, as illustrated in figure 1. The extrapolation of the data on the outermost water compartment to the relaxation rate intercept indicates that this compartment has a relaxation rate similar to that of bulk water. A distinct transition in the slope of this outer compartment with the next most inner compartment is seen in the data. The intercept of these two compartments at a specific Ms/M value can be used to define the extent of the water compartments. The intercept of the second

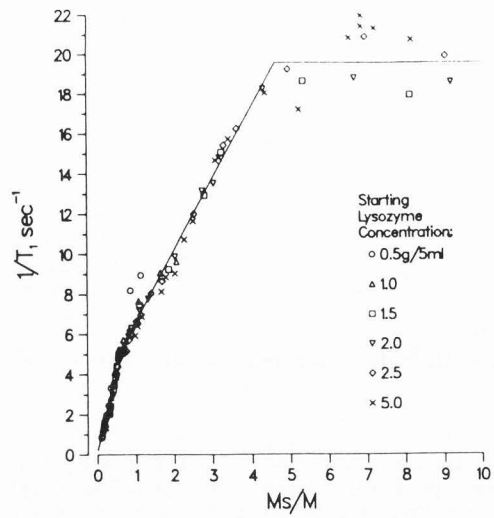

Fig. 1. A dehydration from dilute solution study of the spin-lattice relaxation rate ($1/T_1$) versus concentration (M_s/M) for six different initial concentrations of lysozyme. The lack of dependence on initial concentration shows the 'equilibration' time is not an important parameter (From Fullerton et al., 1986, with permission of the publishers, Elsevier).

linear segment to the relaxation rate intercept indicates the relaxation rate of this compartment. The intercept of this second linear segment with the third linear segment defines the extent of the innermost water compartment and its intercept with the relaxation rate axis gives its relaxation rate. Thus this NMR titration method gives evidence for distinct water compartments, which we designate as bulk, structured and bound, respectively. The extent of the bound water compartment on lysozyme, as determined by the NMR titration method, is in excellent agreement with the other methods listed above (Fullerton et al. 1986). Prior to introduction of the NMR titration method, there had been little evidence presented for a structured water compartment. The extent of the hydration water compartment on proteins is greater than in the bound water compartment and includes water perturbed in structure from that of bulk water. The latter compartment is referred to as structured water.

The assumptions needed for use of the NMR titration method are that fast proton exchange exists between the compartments and that the relaxation characteristics of the compartments at different steps in dehydration do not change the interaction of water and solute (protein) from that in solution. We have recently developed and reported a

quench cooled ice crystal imprint size method for estimation of hydration water of proteins which are in solution as a way of checking on the validity of the latter assumption (Cameron et al. 1985 and elsewhere in this volume). This method gives data quantitatively similar to those obtained from the NMR titration method. Thus the estimated extent of hydration water was similar using a method requiring dehydration and a method not requiring dehydration below the solution condition.

One of our earliest applications of the NMR titration method to cells has been to study hydration water characteristics in unfertilized and fertilized sea urchin eggs. Table 1 summarizes the results of measures of the extent of bound, of structured and of hydration water compartments in both unfertilized and fertilized sea urchin eggs and on lysozyme. The data from NMR titration studies in sea urchin eggs give clear evidence for three water compartments similar to the data from NMR titration studies on lysozyme.

The fertilization of the sea urchin eggs did cause a marked decrease in the extent of the bound and the structured water compartments (Table 1) and because the egg volume remained the same after fertilization, it became clear that fertilization also caused an increase in bulk water. Indeed the hydration water compartment decreased 50% after fertilization. Can the extent of hydration water in the viable unfertilized egg be estimated by a procedure which does not require dehydration? The exposure of viable eggs to various dilutions of sea water and the measurement of the amount of water per unit dry mass in packed eggs at each dilution allowed us to estimate the amount of water in the eggs which does not participate with the same osmotic characteristics of bulk water. To assess the hydration water by this method requires the measurement of the osmotic pressure of each dilution of sea water and that the reciprocal of this osmotic pressure value be plotted against the g H_2O/100 g dry egg mass of tightly packed eggs. The intercept of the linear slope on the g H_2O/100 g dry mass axis indicated that 131g H_2O/100g dry mass of unfertilized eggs had an osmotic activity unlike that of bulk water. This value is in good agreement with the extent of hydration water as determined by the NMR titration method (128 g H_2O/100 g dry mass). This finding suggests that hydration water does not participate in the ideal osmotic equation of van't Hoff. This observation helps explain why most cells do not act as perfect osmometers.

What happens upon egg fertilization to account for the decrease in extent of hydration water? A clue to what may happen came from the work of Otto et al. (1980). Prior to fertilization of sea urchin eggs only 9% of the total actin was found to be assembled, but shortly after fertilization this percentage increased to 35%. We therefore began a series of *in vitro* and *in vivo* experiments to determine if the assembly of the major intracellular protein species (actin) could be linked to the extent of hydration water. We reasoned that each monomeric unit of globular actin had a hydration shell and that the assembly of actin would release hydration water from between the docked molecules to become bulk water. Such a model would help explain the hydration water decrease at fertilization and would be in agreement with the increased polymerization of actin at fertilization as reported by Otto et al. (1980). We have been testing this model by both *in vitro* and *in vivo* experiments.

The addition of KCl to a solution of highly purified actin at slightly below the critical concentration of actin results in assembly of multimers as shown by gelation (increased viscosity). The actin assembly was also confirmed by more sensitive measures including significant changes in the proton T_1 relaxation time, an increase in particle size as measured by analytical ultracentri-

Table 1. NMR characterization of hydration water compartments in sea urchin eggs and lysozyme. Grams water/100 g dry mass (g/100g), and correlation time (CT)[a]

Compartment	Unfertilized		Fertilized[b]		Lysozyme	
	(g/100g)	(CT)	(g/100g)	(CT)	(g/100g)	(CT)
Bound	33	2×10^{-9} sec	21	2×10^{-9} sec	25	2×10^{-9} sec
Structured	95	1×10^{-10} sec	34	1×10^{-12} sec	105	5×10^{-11} sec
Hydration	128		56		130	

[a] Sea urchin egg data from Merta et al. 1986 and lysozyme data from Fullerton et al. 1986. The correlation time of bulk water was 6×10^{-12} sec in both studies.

[b] Taken 25 min after fertilization of *Strongylocentrotus purpuratus* eggs.

fugation and an increase in ice crystal imprint size as measured in quench cooled samples (Fullerton et al. 1987, Cameron et al. 1987a). That actin assembly was responsible for these changes was confirmed by the fact that an inhibitor of actin assembly, cytochalasin B at 10µg/ml, prevented these changes.

Having established that assembly of actin in vitro, at concentrations similar to those found in the sea urchin egg, caused significant changes in the proton NMR T_1 relaxation time and in the quench cooled ice crystal imprint size and that these changes could be inhibited by cytochalasin gave us the information and the tools needed to evaluate actin assembly in vivo. We therefore investigated changes in T_1 relaxation time and in quench cooled ice crystal imprint size during the first cell cycle following fertilization of sea urchin eggs. To link significant changes in the T_1 relaxation time or in the ice crystal imprint size to actin assembly we first made sure that the water content of the eggs and the extracellular space of packed eggs remained constant during the first cell cycle following fertilization. These facts permitted us to attribute changes in these parameters to macromolecular changes rather than to changes in water content. Our results showed that an increase in T_1 relaxation time and in ice crystal imprint size occurred shortly after egg fertilization (figure 2). This change was followed by a significant shortening in T_1 time and in ice crystal imprint size at the time of mitosis and finally by an abrupt increase in T_1 time and in ice reticulation size at the time of cell division (cytokinesis) (see figure 2). Exposure of fertilized eggs to cytochalasin B at between 25 to 35% of the first cell cycle did not prevent the shortening of the T_1 relaxation time at mitosis but it did prevent the return of the T_1 time to longer duration and also prevented cytokinesis. The cells treated with cytochalasin B became binucleate but their T_1 relaxation time did not increase as long as they remained in the presence of cytochalasin B (Cameron et al. 1987b, Zimmerman et al. 1986). Finally a significant pattern of actin disassembly and reassembly at mitosis and at cytokinesis, respectively, was found by studies on total amount of monomeric actin (G actin) using the DNase I assay (Cameron et al. 1987b).

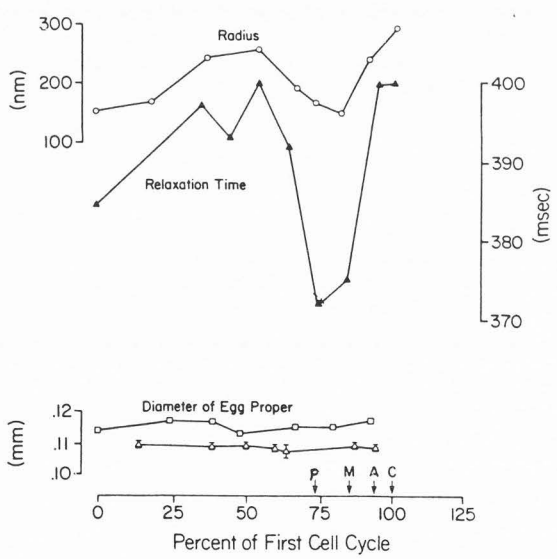

Fig. 2. Cell cycle dependent changes in mean ice crystal imprint radius (top), in proton NMR T_1 relaxation time (middle) and in diameter of fertilized sea urchin (Lytechinus variegatus) eggs during the first cell cycle. The time of cell cycle stages are indicated as follows: P=prophase, M=metaphase, A=anaphase and C=cleavage furrow. Significant decrease in ice crystal imprint size and in T_1 time occurred during mitosis and a significant rise occurred during cleavage. The T_1 time data were obtained from centrifugally packed eggs which had their fertilization membrane and hyaline layer removed shortly after fertilization. No significant cell cycle change in egg diameter occurred in either batch of eggs which were measured (the open triangles were measurements taken on the demembranated eggs).

Because the assembly and the disassembly of microtubules into a mitotic apparatus also parallels the decrease and increase in the proton T_1 relaxation time and the decrease and increase in the ice crystal imprint size changes, we undertook to determine if tubulin assembly or disassembly was involved in the changes that took place at mitosis and cytokinesis. We first established that assembly of tubulin extracts caused significant changes in the proton T_1 relaxation time and that these changes were inhibited by cold exposure, colchicine and high pressure (treatments known to inhibit tubulin assembly, Zimmerman et al. 1985). We established that the inhibitors of tubulin assembly did prevent the increase in T_1 relaxation time. We then applied these inhibitors to sea urchin cells which had a well established mitotic apparatus. Although the apparatus was seen to disappear upon treatment with the inhibitors, the T_1 relaxation time was not lengthened. The conclusion drawn was that tubulin disassembly was not responsible for the T_1 relaxation time changes at mitosis and cytokinesis but that actin disassembly at mitosis and repolymerization at cytokinesis was implicated in the observed changes in ice reticulation size and in the T_1 relaxation time. The failure of the sea urchin eggs to show a significant T_1 relaxation time response when exposed to inhibitors of tubulin polymerization as compared to the

significant T_1 relaxation time response to inhibitors of actin assembly may be due to the much greater abundance in mass of actin versus tubulin in these cells.

In summary, there is good evidence that 54% (128 g H_2O/100g dry mass) of intracellular water in unfertilized sea urchin eggs differs from bulk water. Shortly after fertilization, the hydration water compartment decreased from 128 g H_2O/100 g dry mass to 56 g H_2O/100 g dry mass. The shorter water proton T_1 relaxation time that occurred at mitosis and the increase in T_1 relaxation time at cytokinesis was not due to microtubule assembly and disassembly but was related to actin disassembly and reassembly, respectively.

From the above we conclude: 1) that the extent of hydration water varies in the same cell in different states (phases of the cell cycle); 2) that hydration water does not appear to participate in the ideal osmotic equation of van't Hoff; and 3) that molecular assembly of globular proteins can account for the observed T_1 relaxation time, ice reticulation size and hydration water changes seen at fertilization and during the first cell cycle of sea urchin eggs.

The Ionic Environment Within Cells and Within Subcellular Compartments.

To address the question of what is the intracellular content of Na^+, K^+ and Cl^- in the cytoplasm of marine and non-marine plant and animal cells, we assembled representative data from various sources as summarized in table 2. Most of the data in this table are based on flame photometry or atomic absorption or on coulmetric analysis of muscle tissue or of centrifugally packed cells. In most cases assessment was made of the extent of extracellular space using a non-permeable radioactive marker. Knowing the ionic content of this extracellular space and its volume fraction allowed calculation and use of a correction factor so that the data could be expressed as the intracellular concentration of the ion expressed as mM/l cell water or as mM/kg wet weight. Information on the subcellular concentration of ions using subcellular fractionation techniques is subject to severe criticisms due to the problem of diffusion of small ions in disrupted cells. The method of choice for measurement of ionic content at a subcellular level of resolution is electron-probe X-ray microanalysis performed on appropriately quench cooled specimens that are cryosectioned and freeze-dried at temperatures below -80°C. Such thin cryosections can be analyzed in either the hydrated or in the frozen-dried condition (Smith and Cameron 1987). The analysis of hydrated sections, if properly done, allows expression of data on a wet weight basis while analysis of freeze-dried sections allows expression of data only on a dry wt. basis (unless an independent measure of the water content is available).

Table 2 summarizes the compilation of much data on both plant and on animal cells. The data on plant cells is much less abundant than that on animal cells. This difference is due, in large measure, to the fact that plant cells generally have large vacuoles which contain an ionic content distinctly different from the cytoplasm (Flowers, et al., 1977) plus the fact that it is difficult to assess the size and ionic composition of the extracellular environment in plants. Thus the plant data in Table 2 are derived primarily from electron-probe X-ray microanalysis from thin cryosections, a technique which has only recently become possible with plant cells (Cameron et al., 1984).

Table 2 lists the concentration of Na^+, K^+ and Cl^- for sea water in comparison to the cytoplasmic concentration of these ions in the cytoplasm of cells from marine organisms. Clearly Na^+ and Cl^- are in much lower concentration and K^+ in much higher concentration in the cytoplasm of marine cells than in sea water. In the non-marine organisms the situation is a bit more complex. The non-marine plant cells listed in table 2 often are maintained in a moist atmosphere of water vapor or directly in liquid water with few if any monovalent ions in the environment. In the vascular plants the ions within the growing root tip, cultured in distilled water, are obviously obtained by translocation from storage areas in the seed. The cytoplasm of growing cells in such plants must have mechanisms for the accumulation of ions. The cells from vertebrates are normally bathed in a fluid relatively rich in Na^+ and Cl^- and relatively poor in K^+. Such cells normally have lower concentrations of Na^+ and Cl^- than their extracellular environment and a higher concentration of K^+ than their extracellular environment. A genetically determined exception to this rule occurs in the low K^+ sheep red blood cell (Table 2). Notice that the sum of monovalent cations (Na^+ plus K^+) in mammalian cells is about equal to the sum of these monovalent cations in the plasma. The intracellular concentration of Cl^- in all mammalian cells is considerably less than in the plasma.

Intracellular-extracellular content gradients of specific ions clearly exist in the distribution of each ion in essentially every cell type studied. Before turning to the mechanism(s) responsible for the observed ion content gradients, let us stop and ask if the concentration of ions in these cells gives a true reflection of their chemical activity, as would be the case, assuming that these ions are in dilute solution. In other words, are all of the ions within the cells listed in table 2 free as they would be under dilute solution conditions?

We have resorted to an indirect approach to address this question. Specifically we

Table 2. Representative examples of the cytoplasmic concentration of ions in plant and in animal cells (mM/l H_2O or mM/kg wet wt.) Number of species sampled is given in parentheses.

	Marine Organisms					Fresh Water and Terrestrial Organisms			
	Na^+	K^+	$Na^+ \& K^+$	Cl^-		Na^+	K^+	$Na^+ \& K^+$	Cl^-
Plant cells (a)									
Dunaliella					mung bean				
parva in 0.4M	37	100	137	26	root tip	-	-	-	30
& in 1.5 salt	36	106	142	30	onion root	32.5	-	-	-
step up 5 min	152	84	236	111	onion meristem	10	83	93	4.5
Suaeda	150	50	200	-	*Euglena gracilis*	3.5	17	21	12
					corn root cap	1.5	116	118	5
Muscle cells (b)									
mollusca(9)	129±36	137±19	270±18	172±46	mollusca(2)	21±16	13±3	34±19	11
crustacean(3)	66±9	131±19	197±27	54	amphibian	11	126	137	11
fish	122	117	239	107	fish(2)	28±3	138±28	165±25	24
					reptile	45	122	167	30
					bird	21	83	104	13
					mammal	8	185	193	11
Other animal cells (c)									
crustacean					mammal				
axon	46	292	338	57	RBC(4)	15	143	158	88
mollusca					sheep low K^+ RBC	137	17	154	-
axon	52	233	284	114	mouse L cells	9	167	176	70
"	77	267	344	-	mouse Ehrlich cells	26	134	160	51
mollusca					protozoan				
neuron	25	232	257	45	ciliate	13	32	45	6
sea urchin									
egg,F	40	211	251	202					
UF	37	212	249	115					
					plasma				
sea water	470	10	480	548	mammal(5)	149	5	154	109

(a) *Dunaliella* data from Hajibagheri et al. 1986. *Suaeda* from Flowers et al. 1977. Mung bean, onion root and meristem compiled from Cameron et al. 1984. *Euglena* from Cameron et al. 1986a, and corn root cap, Cameron unpublished.

(b) All muscle cell data taken from Prosser, 1973.

(c) Crustacean, mollusca and mammalian cell data compiled from DeWeer, 1976. Protozoan data from Prosser, 1973. Sea urchin egg data, fertilized from Cameron unpublished and unfertilized from Shen, 1983.

asked ourselves how the ionic sensitivity of various *in vitro* enzymes and *in vitro* protein synthesis systems compares with the known ionic conditions found in cells as reported in table 2. Specifically we asked ourselves how does the intracellular ionic environment of the cells listed in table 2 (assuming the ions are all free as in a dilute solution) relate to the optimum ionic environment for enzyme activity as determined *in vitro*?

Table 3 summarizes representative examples of the sensitivities of various *in vitro* enzyme systems to ions (Na^+, K^+ and Cl^-). In general the enzyme systems demonstrate optimal activity at less than 100 mM Na^+ and/or K^+ and at less than 50 mM Cl^-. There appears to be no obvious tolerance of the enzymes from the marine plants or animal cells to higher salt concentrations; however, the enzymes from the halophilic bacteria are indeed exceptional in that they are not only tolerant of salt but require 1M or more of salt for their proper activity. All of the other enzyme systems show inhibition at concentrations of greater than 75 to 200 mM Na^+ and/or K^+ and at concentrations greater than 90 mM Cl^-. Table 4 summarizes an analysis of the cytoplasmic ion concentration data from plant and animal cells (listed in table 2) in comparison to the ionic concentration conditions required for optimal *in vitro* enzymatic activity (listed in table 3). The point to be made from the data in table 4 is that most of the cells have concentrations of ions which would

Table 3. Ionic sensitivity for *in vitro* enzyme activity and *in vitro* protein synthetic systems

System	Observation	Reference
in vitro protein synthesis wheat germ	Optimal below 150 mM Na^+ plus K^+ Optimal below 50 mM Cl^-	Gibson et al., 1984
	50 mM KCl optimal inhibited 80% by 200 mM K Cl or NaCl	Hall and Flowers, 1973
	128 mM K^+ optimal 90 mM Cl^- optimal	Wyn Jones et al. cited in Greenway and Munns, 1980
	50 mM KCl	Baliga and Munro, 1976
reticulocyte	75 mM K^+ optimal 76 mM KCl optimal	Schreier and Staehelin, 1973 Baliga and Munro, 1976
rat brain and muscle, human placenta	Optimal 40-150 mM KCl	Baliga and Munro, 1976
sea urchin egg	50 mM Na^+ optimal	Mano, 1970
bacteria *E. coli*	50 mM KCl optimal	Baliga and Munro, 1976
Euglena	4 mM KCl optimal	Baliga and Munro, 1976
Physarum	50 mM KCl optimal	Baliga and Munro, 1976
Soybean	16 mM KCl optimal	Baliga and Munro, 1976
malate dehydrogenase	high M.W. form activated at 100 mM NaCl, low MW form activated at 50 mM	Evans and Sorger, 1966
enzymes from halophytes, non-halophytes and commercial enzymes: aspartic transaminase, malate dehydrogenase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase	all show similar sensitivity to NaCl. All optimal below 170 mM Na^+ plus K^+ and below 50 mM Cl^-	Greenway and Osmond, 1972 Johnson et al., 1968
enzymes with anionic substrates to Cl^-	optimal below 50 mM Cl^-	Gimmler et al., 1984
phosphoribomutase	inhibited 50% by 50 mM NaCl	Axelrod, 1955
lysozyme	inhibited at 200 mM NaCl	Spizizen, 1962
esterase	inhibited 50% by 50-70 mM NaCl	Mandl, 1962
halophilic bacterial enzymes	require 1M or more NaCl	Lanyi, 1974; Larsen, 1967

be above optimal for enzyme activity, assuming the ions are free in solution. In fact, the reported concentration of ions in many cells would be strongly inhibitory to enzymatic activity if the ion content reflected their chemical activity. What conclusion is to be drawn from this apparent paradox? We favor the conclusion that the measured ionic concentration, expressed in mM/l water or mM/kg wet weight, does not accurately reflect the chemical activity of these ions if they were dissolved in bulk water. That intracellular ions have a reduced chemical activity compared to dilute solution conditions is not a novel idea (see Ling, 1984 for a review of this subject) but has not to our knowledge previously been assessed by the ionic sensitivity of enzyme activity measured under in vitro conditions.

Other data on concentration of Na^+ in the large fully grown Xenopus oocytes measured by flame spectrophotometry as compared to intracellular Na^+ activity measured with a Na^+ selective electrode showed an eight fold difference (Slack et al., 1973). Slack et al. conclude that "a large proportion of the intracellular sodium is held in an ionically inactive form."

The effects of ions on chromatin structure and on the extraction of chromosomal proteins is offered as another reason for thinking that the chemical activity of ions is not accurately reflected by the measured ionic content. For example, Cameron (1985) showed that the concentration of monovalent cations (Na^+ plus K^+) within the nucleus of the living Xenopus oocyte is greater than 120 mM. This concentration of monovalent cations in the medium was shown to cause a striking change in the structure of chromatin which had been isolated from cells under low ionic strength conditions. Actually exposure of chromatin isolated from chicken erythrocytes under low ionic strength conditions to this concentration of ions in solution extracts a specific set of proteins (Cameron et al., 1979). It was concluded from these observations that the chemical activity of monovalent cations (Na^+ plus K^+) must be lower than 120 mM in the nucleus of a living cell. Kellermayer and Hazlewood (1979) came to a similar conclusion based on different experimental results. To better judge if the nuclear K^+ in the Xenopus oocyte was free to exchange with its surrogate (Rb^+), we exposed fully grown Xenopus oocytes to amphibian Ringer's solution in which K^+ was exchanged with Rb^+ on a molar basis. Samples of the oocytes were removed from the Rb^+ Ringer's solution over a 40 h duration and the oocytes were processed for electron-probe X-ray microanalysis for determination of ionic content in subcellular compartments (Cameron and Hunter, 1985). About 20% of the intranuclear K^+ was exchangeable for Rb^+ over the first 3 to 6 h, however, exchange of the remaining K^+ was not observed even after 40 h. This suggests that the majority of intranuclear K^+ is not free to diffuse from

Table 4. Summary of cytoplasmic ion concentration (mM/l water or mM/kg wet wt.) in plant and in animal cells compared to the ionic concentrations required for optimal in vitro enzymatic activity (mean ± S.E., number of species given in parentheses).

	Sum Na^+, K^+	Cl^-
Plant cells		
Marine	179±24(4)	56±28(3)
Non-marine	77±29(3)	13± 6(4)
Animal cells		
Marine	264±14(17)	135±31(13)
Non-marine	130±15(13)	32± 9(10)
Samples of In vitro systems, optimal ionic condition for protein synthesis and for enzyme activity	< 73±12(7)	< 58± 6(6)

Results of ANOVA & S/N/K analyses: a b

a Means of the marine plant and animal cells are significantly higher than the means of the non-marine cells and the mean of the in vitro systems.

b Mean of the marine animal cells is significantly higher than the means of the non-marine plant and animal cells and the mean of the in vitro systems. No other means differed significantly.

the oocyte in exchange for Rb^+ over 40 h. Assuming that all of the intranuclear Na^+ measured in the oocyte is free and that only 20% of the K^+ is free, we estimate that the total concentration of monovalent cations is about 26 mM. This concentration is in the range that Kellermayer and Hazlewood (1979) found to maintain normal chromatin structure in mammalian cells. Again the evidence suggests that the true chemical activity of ions, i.e., Na^+ and K^+, is much less than predicted under the assumption that all intracellular ions are maintained under conditions which approximate dilute solution.

What are the mechanisms responsible for the extracellular and intracellular ionic gradients illustrated in table 2? Does the plasma membrane with its semipermeability and associated ion selective, active transport "pumps" play the main or exclusive role in maintenance of the ionic gradients? Is the cytomatrix responsible for the selective adsorption of specific ionic species? Does the hydration water compartment associated with the cytomatrix proteins have different solvation properties for the various ions as compared with the solvation properties of bulk water? There is no a priori reason why more than one of these postulated mechanisms for generation or maintenance of ionic gradients might not operate in the same cell or that different cell types might not show greater or lesser reliance on one of these proposed mechanisms. We and others have designed experiments to test which of these possible mechanisms is the main determinant of the observed ionic content gradients in a variety of cell types, as reviewed next.

The main method we have used to measure ion content gradients is electron-probe X-ray microanalysis (energy dispersive spectroscopy, EDS) on cryofixed, cryosectioned and freeze-dried thin sections of cells. The method requires the quench cooling of small (1-2 mm^3) samples in liquid propane at -169°C. The frozen samples of cells are thin-sectioned at -100°C and the water removed by freeze-drying prior to EDS. The ion distributions and content measures reflect those in vivo, as ion translocation due to diffusion is negligible at -100°C.

In one of our experiments we prepared mouse erythrocytes in plasma for EDS analysis. After processing some of the specimens at -100°C, the temperature was raised to -40°C. The higher temperature of the cryosectioning, we reasoned, might allow diffusion of free ions down their extracellular/intracellular concentration gradients. We found an influx of Na^+ and Cl^- down their extracellular to intracellular ion concentration gradient. However intracellular K^+ did not leave the erythrocyte in spite of a 15- to 29-fold K^+ content gradient. The conclusion we drew is that the maintenance of the Na^+ and Cl^- gradients was due, at least in part, to the intact plasma membrane, which was disrupted by sectioning in our experiment but that the maintenance of the K^+ gradient is best accounted for by its adsorption to cytomatrix proteins. These observations add to a growing list of reports that intracellular K^+ ions are not freely dissolved in cell water (Ling 1984, Edelman 1986, Cameron et al. 1986a,b).

We have recently completed, but have not yet published, experiments similar to those done earlier by Kellermayer et al. (1986). The rationale for the experiments is to see if disruption of the plasma membrane integrity by use of detergents would allow dissipation of the known extracellular/intracellular ion content gradients of Na^+, K^+ and Cl^- in the case of the nucleated chicken erythrocyte. Exposure of the erythrocytes to a 1% Brij 58 solution in the plasma was shown to produce disruption of the plasma membrane within one minute, as verified by TEM of fixed erythrocytes. If the ions were all free in solution, one might expect diffusion to allow ionic equilibration to occur through the holes in the plasma membrane within a few msec. What we observed was a significant but not instantaneous influx of Cl^- and Na^+ into the erythrocytes but no efflux of K^+ from the erythrocyte until the majority of the cytoplasmic proteins had been extracted. Again we conclude that the plasma membrane is in some way involved in the maintenance of the Na^+ and Cl^- content gradient but that the cytomatrix proteins are the main determinants of the K^+ content gradient in these cells.

Is there any evidence that intracellular Cl^- may not be free to diffuse within the cell? The experimental evidence that cellular Cl^- is not free to diffuse as it would in dilute solution is at present less than is the evidence that intracellular K^+ is not free to diffuse. We have however some evidence that at least some of the intracellular Cl^- is associated with elements of the cytomatrix. The cell studied in regard to Cl^- was the flagellate Euglena gracilis.

Studies have shown that the subcellular contents of Euglena can be stratified into six layers by means of ultracentrifugation, with the cells still remaining viable (Kempner and Miller 1968a,b, Kempner 1975). One of these layers was clear and was reported to be free of a number of components, including membranes, nucleic acids and enzyme, and to be almost free of proteins. This finding was used to support the idea that all enzyme activity in cells takes place on fixed cytomatrix surfaces (Clegg, 1984). We reasoned that a subcellular redistribution of ions associated with specific subcellular components might also be obtained by such an ultracentrifugal stratification procedure. If, for example, an ion, such as Na^+, K^+, or Cl^-, were bound to a given macromolecule, the ion might be expected to decrease in concentration in the layer of cytoplasm cleared of cytomatrix, ribosomes, proteins, and enzymes. However, if certain ions were free in solution they would not decrease in concentration in this cleared layer of cytoplasm. By com-

paring the distribution of ions at the subcellular level in stratified and non-stratified Euglena, one can obtain information on the mechanism(s) by which subcellular element concentration gradients are established and maintained.

Our ultrastructural observations on cytoplasm in non-stratified and stratified cells showed that a field of open cytoplasm free of almost all recognizable structures was indeed produced by the ultracentrifugation (Cameron, et al., 1986a). If any of the Na^+, K^+ or Cl^- ions were closely associated with the recognizable structures, it might be expected that they would be removed or decrease in concentration by ultracentrifugation. Analysis of the data revealed that the concentration of Cl^- was significantly decreased by this procedure ($p < 0.001$). The fact that the cleared layer of cytoplasm has been shown to contain little if any macromolecular material or enzyme activity, plus the fact that the Cl^- concentration is significantly decreased to less than half its initial concentration, indicates that more than 60% of the Cl^- in the open cytoplasm of non-stratified Euglena is adsorbed to the material that was removed by ultracentrifugation. The fact that over 60% of the cellular Cl^- may be closely associated with intracellular material is also supported by recently published Cl^--efflux studies on human lymphocytes (Negendank, 1984).

The electron-probe X-ray microanalysis studies which we have done support the hypothesis that much of the intracellular K^+ is adsorbed to the cytomatrix within living cells. Our studies add to the growing list of experimental proof that the majority of intracellular K^+ is in the adsorbed state. The unequal distributions of K^+, Cl^- and probably Na^+ between the subcellular compartments is perhaps best accounted for by the preferential distribution of macromolecules which bind such ions.

Concluding Remarks

Cellular membranes partition distinct subcellular compartments within the cell. Cell biologists now realize that subcellular compartments (i.e., the nucleoplasm, the mitochondrial matrix, the inside of lysosomes, the cisternal space, the open cytoplasm and the vacuole space) all have distinct and characteristic populations of proteins. Although we have made progress in defining the role that the various subcellular membranes play in the generation and the maintenance of different microenvironments, we still have little information concerning the role that the proteins within these compartments play in generating and maintaining these unique microenvironments.

Two extreme descriptions of these subcellular compartments have been offered (Clegg, 1984). A solution description states that the microenvironments can be considered to approximate a dilute solution with regard to the physicochemical properties of water and ions. It is fair but unfortunate to say, that most of the published papers and commonly used textbooks which are concerned with: the permeability of cellular membranes, the volume regulation of cells and with the transmembrane electrical chemical potentials of cells, make the tacit assumption that essentially all of the intracellular water and ions have the same motional, solvational and osmotic properties as they have in dilute solution.

In this review we presented several of our own experimental results concerning the physicochemical properties of water and of the major inorganic ions within cells. We sought to characterize the properties of water and ions at the cellular and subcellular level using techniques which are as non-perturbing as possible. In summary, our results support an organized description of the subcellular compartment by showing that the cytomatrix plays a major role in determining the extent of hydration water in cells. Our data also support the idea that cellular hydration water differs from bulk water in its response to osmotic force. The assembly of cytoplasmic proteins into multimer aggregates (actin in particular) appears to be a major mechanism which determines the extent of hydration water. The ionic content of cells, as determined by flame photometry or atomic absorption spectroscopy, does not appear to accurately reflect the chemical activity of intracellular ions. This may, in part, be due to the subcellular concentration of the ions in morphologically recognizable compartments, but the data from our electron probe experiments show that the major proportion of intracellular K^+ and a substantial fraction of intracellular Cl^- is influenced by the cytomatrix such that they do not have the same motional properties or activity coefficient as they would have were they in dilute solution.

In brief, the intracellular environment differs significantly from that of a dilute solution and it is wrong to assume that the intracellular environment approximates dilute solution conditions. As unpalatable as this situation may be to teachers and students of cell physiology, it must be accepted and dealt with if we are to understand cellular functions.

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Discussion with Reviewers

L. Edelman: I do agree that the presented NMR relaxation time measurements may be fitted by a 3 compartment water model; I doubt however that this model is suited to determine precisely the percentage of the so called bulk water. It has been shown by the quasi-elastic neutron scattering technique (Rorschach HE, 1985, Water in polymers and artemia cysts. In: Pullman A, Vasilescu V, Packer L (Eds.) Water and Ions in Biological Systems, Plenum Press, N.Y. 729-736) and by investigating solute exclusions properties of polyethyleneoxide solutions and other solutions (Ling GN, Ochsenfeld MM, 1983, Physiol. Chem. Phys. 15:127-136) that model systems with high water content exist in which most of the water must be influenced by macromolecules. Did you investigate such model systems with the NMR titration technique and did you find solutions containing no "bulk water"?

Authors: The NMR titration analysis, has been applied to: the artemia cysts (Fullerton et al. 1983 Physiol. Chem. Phys. Med. NMR 15, 489-499), unfertilized and fertilized sea urchin eggs (Merta et al., 1986), the mammalian lens (Fullerton and Cameron, 1987) and most recently to human erythrocytes and hemoglobin solutions (I Cameron, V Ord and G Fullerton, unpublished observations). In agreement with the data that you cite on the hydrated artemia cyst, the NMR titration analysis also showed no evidence of bulk water up to a hydration level of 1.2-1.4 g H_2O g dry mass (this amount of water equals full hydration in these cysts). Likewise the lens fiber cells showed no evidence of bulk water at the normal *in vivo* level of hydration (1.4g H_2O/g dry mass). However, the sea urchin eggs, human erythrocytes and hemoglobin solutions (all of which have a water content greater than 1.5 g/g dry mass) indicated that water contents greater than 1.4 - 1.5 g H_2O/g dry mass in these specimens have motional and osmotic properties which revert to those typical of bulk water. Thus the NMR titration method does appear suitable to determine the extent of bulk water.

C.F. Hazlewood: In your designations of the fractions of water into bound, structured, hydration, and bulk, do you consider that all of the water in each of these fractions has one correlation time (τ_c)? If your answer is yes please explain the rationale behind this position.

Authors: As indicated in table 1 of the paper, each of the designated water fractions has a very different correlation time. Because of the heterogeneous nature of cellular proteins, we are of the opinion that there is some dispersion of correlation times in each water compartment but that the correlation times in each compartment are far enough apart in magnitude as to allow distinction of the separate water compartments by the NMR titration method.

C.F. Hazlewood: How do you explain the sharp changes in relaxation rate as a function of Ms/M in Fig. 1? It is difficult for me to see how fast exchange between "the next most outer water compartment..." is sufficient to explain this observation. How fast is the "fast exchange" at each of these breaks in the relaxation rate? It seems that exchange rates between water fractions must be

different--have these been determined?
Authors: The NMR titration method we use relies on the assumption of fast proton diffusion or exchange between water proton compartments. In other words it is assumed that exchange between water compartments is fast with respect to the relaxation time. This is a widely used assumption and we know of no evidence to indicate that this assumption is not valid.

C.F. Hazlewood: Do you mean that all of the intracellular proteins are part of the cytomatrix or only that the three major classes of the filamentous proteins such as microtubules, microfilaments and intermediate filaments are associated with the cytomatrix? What evidence exists that the proteins are indeed freely dissolved in the aqueous phase of the cell?
Authors: Our conception is that most of the intracellular proteins can be associated with the cytomatrix under certain conditions; however any number of perturbations to a cell can cause the release or disassembly of aggregated protein complexes. This concept is based partly on our published observations (Cameron et al. 1987b, Fullerton et al. 1987) but also on the earlier observations of Kopac (1938, Biol. Bull. 75:351, as reviewed by James S. Clegg, 1986, in The Organization of Cell Metabolism, Plenum Press, N.Y., pp. 51-53). Thus we accept the prophetic conclusion from this earlier work, that under some situations the intracellular proteins are not free to diffuse and are aggregated together.

C.F. Hazlewood: From your view point do you consider that both G and F actin are the same in the living cell as in the test tube? In your view do the cellular proteins require an extended configuration in order to induce water structuring?
Authors: It is doubtful that test tube conditions seldom, if ever, are the same as conditions in the living cell. Nevertheless it seems likely that conditions which cause transitions of G to F actin in vitro, such as concentration of actin monomers, temperature and ionic conditions, may well play a similar role in vivo. To your second question, the answer is no but our recent studies, do show that aggregation and disaggregation of globular proteins does change the extent of hydration water in cells. Although denaturation and unfolding of globular proteins has been shown by Ling (1984) and others to increase the amount of water that is "structured", we are not aware of any proteins in cells that are naturally in an extended or unfolded configuration. We therefore regard assembly and disassembly of globular proteins as a more likely source of change in extent of cellular hydration water.

G. Lustyik: It follows from your data that newly fertilized sea urchin eggs increase the proportion of "osmotically active" bulk water in the eggs. Can this increase be estimated (as it was in your earlier paper with Merta et al. 1986) from osmotic behavior of the fertilized eggs incubated in diluted (and/or hypertonic) sea water?
Authors: We do think that a change in the amount of "osmotically active" or inactive water can be estimated from the osmotic behavior of newly fertilized sea urchin eggs by incubation in diluted and/or hypertonic sea water using the approach published in our earlier paper (Merta et al., 1986). Unfortunately such an analysis remains to be completed.

G. Lustyik: How is osmotic equilibrium between the extra- and intracellular space maintained if only 20% of the cellular potassium is free in the animal cells?
Authors: Different types of cells in different environments apparently use different strategies to maintain osmotic equilibrium with their environment. Vertebrates rely heavily on organ systems to maintain a relatively homeostatic osmotic environment for somatic cells and in general their somatic cells are not exposed to widely varying osmotic environments. Some cells in nature maintain osmotic equilibrium under widely varying osmotic environments. Review of a few of these systems may help answer your question. Sea urchin eggs, exist in osmotic equilibrium with sea water; while the dechorionated eggs of some fresh water fish can exist in osmotic equilibrium with distilled water. As indicated in Table 2 neither the sum of the concentration of the major intracellular monovalent cations or anions in the unfertilized sea urchin egg comes close to the sum of the concentration of the same major ions in the extracellular environment (sea water). How then does the sea urchin egg keep from osmotic shrinkage? Firstly, it can increase the number of osmotically active non-electrolyte particles, such as amino acids or polyols; secondly, it can reduce the amount of "osmotically active water." Others have demonstrated that non-electrolytes can be involved as a major mechanism for regulation of osmotic equilibrium (see Prosser for some examples, 1973). For example the bacteria B. subtitis, when exposed to 1M NaCl, increases its intracellular proline two orders of magnitude after several minutes then resumes growth (Gould and Measures, 1977, Philos. Trans. Roy. Soc. London B278,151). Regarding the osmotic activity of cellular water, our research (Merta et al. 1986) indicates that more than half of the intracellular water in the unfertilized sea urchin egg (the hydration water fraction) has reduced osmotic activity compared to bulk water. In addition to these two mechanisms, one may envision the existence of a plasma membrane bound energy requiring pump that moves non-water particles into or out of the cell to balance osmotic activity. Such a pump is the most commonly cited mechanism for

balancing osmotic activity. Aside from suggesting that the plasma membrane surrounding a dechorionated fish egg equilibrated in distilled water is impermeable to water such that it cannot swell, it seems difficult to explain osmotic equilibrium in distilled water on the sole basis of such a particle pump. On the other hand, if all or most of the water in such a fresh water fish egg were "osmotically inactive" and/or if all or most of the particles were adsorbed "osmotically inactive" there would be no need to use a particle pump as osmotic equilibrium with the environment would already exist. This brings us back to a main conclusion of our paper; it is misleading and quite wrong to continue to assume that the intracellular environment approximates dilute solution conditions such that all or most of the ions are free or that essentially all of the water has the motional or osmotic properties of bulk water.

G. Lustyik: Your Table 1 gives water correlation times (CT) belonging to different water compartments, but changes of these compartments in sea urchin eggs are discussed with the spin-lattice relaxation times (T_1). What is the qualitative relationship between CT and T_1?
Authors: The theory of Bloembergen, Pound and Purcell (1948, Phys. Rev. 73:679) directly relates hydrogen (proton) nucleus relaxation time to a specific correlation time (time for the proton to rotate or translocate to a specific degree). In pure aqueous water a measurement of the NMR relaxation time gives a direct determination of the correlation time of water molecules. This direct approach is not successful for aqueous solutions of protein because of the exchange of protons between different water compartments. Zimmerman and Brittin (1957, J. Phys. Chem. 6, 1328-1333) have however shown that one can easily account for the exchange of protons between hydration and bulk water compartments when the exchange between compartments is fast in relation to the relaxation time of the two compartments. If one can determine the size and relaxation rate ($1/T_1$) of the water compartments then the correlation time of each compartment can be calculated. The NMR titration method, which assumes fast proton diffusion or exchange between water compartments, has therefore proven successful for determination of the size, relaxation rate and correlation time of the individual water compartments as listed in table 1.

W. Negendank: In the NMR titration model, how can you be sure that the apparent compartments are not "created" by the drying process?
Authors: It seems likely in some specimens that the abrupt changes in NMR parameters do reflect comformational change at specific points in the drying process. However we can directly address your question regarding measurement of the total extent of hydration water on fully hydrated specimens using other methods (i.e. osmotic and quench cooled ice crystal imprint size. The extent of hydration water was in close agreement between the NMR titration method and these alternate methods. Thus in regard to the total extent of hydration water, we do have data from alternate methods, measured on fully hydrated samples, which agree with the NMR titration method.