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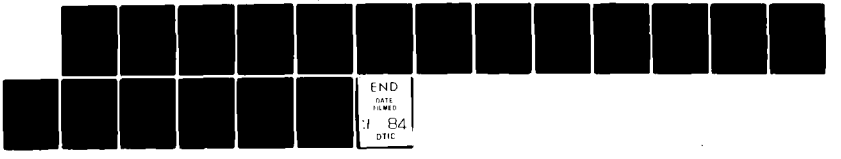

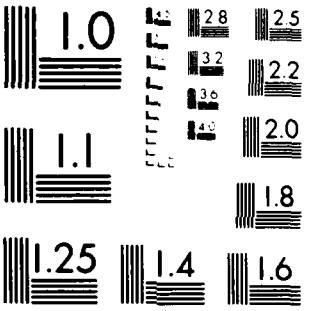

MECHANISM OF SOLUTE EXCLUSION FROM CELLS: THE ROLE OF PROTEIN-WATER INTERACTION

by

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A fundamental requirement of life is that its basic unit, the living cell, be separate from yet maintain a finely tuned continuity with its environment. This continuity is shown by the presence within the cells of ions, sugars, and other essential ingredients of life that are derived from the external medium but are in some way held at lower or higher levels.

To explain the low levels of permeant solutes like Na^+ and the high level of other solutes like K^+ in living cells, proponents of the membrane theory postulated specific energy-consuming pumps [1, 2]. In contrast, the association-induction hypothesis (AI hypothesis) attributes the high level of K^+ to specific adsorption on β- and γ-carboxyl groups carried on cell proteins and the low levels of Na^+ and other solutes to a single cause, i.e., reduced solubility within cell water which exists in the state of polarized multilayers [3-6]. As of this time, many believe that overwhelming evidence exists in support of the pump hypothesis. They cite, for example, the observation that Na, K-activated ATPase, when incorporated into phospholipid vesicles and supplied with ATP as an energy source, can apparently transport K^+ and Na^+ in opposite directions across the vescicular membrane and against concentration gradients [7-9], for critique see [10]. Others, however, believe that there is evidence decisively in support of the association-induction hypothesis [11-15], for critique see [16] and [17]. One way to help resolve these conflicting views is to carry out stringent tests of the most basic postulations of the theories. For example, in the association-induction hypothesis cell proteins maintain the polarized-multilayered state of cell water and that in this state water has reduced solubility for Na^+, sugars, and free amino acids. The question one asks is: "Can proteins outside the living cells indeed create such major changes in the solvent properties of bulk phase water?" In this report we present results of experimental efforts designed to answer this question.

The AI hypothesis assumes that the bulk of cell water is polarized in multilayers primarily by the exposed NH and CO groups of certain as yet unidentified intracellular proteins. In this state, water has decreasing solubility for hydrated ions and molecules of increasing size and complexity on both an enthalpic and an entropic basis[5, 6, 18]. This concept is different from that of "bound water," in that water molecules in the state of polarized multilayers are quite free to exchange or evaporate; it also differs from the concept of "non-solvent water," because no water in the system is considered to be categorically non-solvent to all solutes.

In order to exercise long-range effects on the physical state of water, proteins must be extended, with the alternating sequence of negatively charged CO groups and positively charged NH groups directly exposed to the water. In this conformation the protein chain constitutes part of a regular matrix of parallel extended protein chains between which the bulk of water is found. H-bonding of the NHCO groups either to the protein's own CONH groups (e.g., α helix) or to CONH groups of other proteins (e.g., interchain H bonds) annuls their abilities to polarize water in multilayers. Therefore, most native proteins with their CONH groups locked in α-helical and other intra- and intermolecular H bonds would have little or no long-range effect on water structure.

Native Globular Proteins Have No Effect While Gelatin and Two Synthetic Polymers Do Have Effect of Water Solvency

To test these theoretical deductions, we filled ¼-inch dialysis tubing with solutions of proteins (or melted gel) at concentrations similar to those found in cells (15 to 25%). The sacs were tied at both ends and were incubated at 25°C in 1.5 M Na_2SO_4 solution containing radioactive ^{22}Na for from 2 to 3 days, a period of time longer than that required for equilibrium of Na_2SO_4 between the inside and the

outside of the sac (40 to 60 min). The equilibrium Na^+ concentration in the water within the sac was then assayed and divided by the Na^+ concentration in the external solution. This ratio, called the apparent equilibrium distribution coefficient, or ρ-value, is to be distinguished from the q-value, or the true equilibrium distribution coefficient, which refers only to solute dissolved in the water within the sac[5, 6, 18]. If any part of the solute is adsorbed to the protein, the ρ-value will be larger than the actual q-value.

Table 1A shows that all 13 globular proteins studied exhibit ρ-values of Na^+ equal or close to unity, indicating that under this particular set of conditions there was little or no water with Na^+ solubility different from that of normal liquid water. Table 1B shows, in sharp contrast, the ability of water in the gelatin-water system to exclude Na^+; the ρ-value for Na^+ was only 0.537 ± 0.013 in a total of 37 determinations in 8 separate experiments on 4 varieties of commercial gelatins. (Indeed the ability of gelatin to exclude solutes has been known since Holleman, Bungenberg de Jong and Modderman in 1934[19]. This phenomenon was cited by Troschin in his theory of solute exclusion on the basis of the existence of gelatin and living cells in the state of colloidal coacervates[20].

Why is gelatin an exception in relation to the other proteins? It is well known that repeating sequences of glycine-proline-hydroxyproline in gelatin prevent it from assuming the α-helical conformation; also, the denaturation process used in preparing gelatin disrupts a major portion of the chain-to-chain H bonds present in native collagen[21]. As a result, in a gelatin-water system, portions of the polypeptide chains exist in an extended conformation with exposed NHCO groups, thereby satisfying the requirement of the AI hypothesis for long-range effects on water structure.

While the data of Table 1A and B agree with the theoretical expectations, one may argue that gelatin differs from the 13 globular proteins in that it alone

exists as a gel or a coacervate[22] and that it is really the gel or coacervate state that accounts for the low ρ-value for Na^+. To test this alternative explanation, we studied the Na^+ distribution in another polymer-water system, a solution of polyvinylpyrrolidone (PVP)[23 - 25]. PVP does not form a gel and is not in the form of a coacervate under the conditions of the experiment, but it does contain imino groups on bulky pyrrolidone rings similar to those present in gelatin and is therefore also unable to fold into α helices. Table 1C shows that PVP indeed acts like a gelatin and is in fact even more effective than gelatin in reducing water solvency for Na_2SO_4; the ρ-value for Na^+ at 25°C is only 0.239 ± 0.005. Since PVP does not contain proton-donating groups, these findings establish that the properly spaced oxygen atoms are the primary sites of water interaction, in full accord with the conclusion drawn from Wolfenden's vapor-phase analysis of the amide-water system that the peptide oxygen is the major site of water interaction[26].

The data presented so far correlate the water solubility effect with the exposure of NHCO or simply CO groups. However, to ensure that this effect is not due to the presence of the pyrrole rings in both gelatin and PVP, we studied yet another linear polymer, poly(ethylene oxide) (PEO)[27]. Unlike gelatin and PVP, PEO contains no side chains. Being a chain of oxygen atoms interspersed between pairs of ethylene groups, it is probably the simplest model available for the role of the water-polarizing intracellular proteins as visualized in the AI hypothesis. Table 1D shows that PEO, like PVP, does indeed have strong effects on the ρ-value of Na^+ in the form of citrate. Even more dramatically, PEO demonstrates that the basic requirement for bulk-phase water polarization and solvency is a properly spaced sequence of exposed oxygen atoms, be it in the form of carbonyl or ether oxygen.

Protein Denaturation and Effect on Bulk Phase Water Solvency

Further verification of the theoretical linkage between exposure of NHCO groups and solvency change of bulk-phase water was achieved by altering the conformations of native globular proteins through denaturation. This investigation has additional potential significance. If such a conformation change can indeed alter the solvency property of water, it would offer support for another main theme of the AI hypothesis, i.e., reversible changes of cell water between polarized multilayered state and a more random state as the basis of many physiological manifestations of the living cell[3, 28]. To guard against possible interference from electric charge effects, we chose one neutral and another effectively neutral probe molecule, sucrose and glycine, respectively, in an environment of 0.1 M Na_2SO_4. Sucrose and glycine, like Na^+, are as a rule excluded from water in living cells[3, 6, 18, 29, 30].

Four denaturants were chosen: Urea (9 M), guanidine HCl (6 M), sodium dodecyl sulfate (SDS) (0.1 M), and n-propanol (2 M). The first two are well known for their ability to unravel the NHCO bonds maintaining the secondary structure of proteins[31 - 32]; the last two are now believed to have little or no effect on the secondary structure and may even promote α-helix formation, but they disrupt and unravel tertiary and quaternary structure.[33- 38]. We expect therefore that the ρ-values for sucrose and glycine should decrease as a result of denaturation by urea and guanidine HCl but not as a result of denaturation by SDS and n-propanol.

Since urea has a propensity to decompose at the elevated temperatures necessary for sample drying, these experiments were carried out using 3 different procedures, which ultimately provided consistent results: prolonged drying at 80°C; labeling water with tritiated water; and careful measurement of total weight changes and subtraction of other components determined chemically or with isotopes. Urea

substantially increased the time for probe molecule equilibration. To ensure accuracy, we conducted dual sets of experiments in which the radioactive labels of sucrose and glycine were initially added either outside or inside the sac; agreement of results ensured equilibrium. The details of the extensive data regarding sucrose and glycine distribution in 10 to 15 proteins that were studied will be presented elsewhere. We present here one specific example, sucrose distribution in a bovine serum albumin-water system (Fig. 1A), and the averages of all data on sucrose distribution for 10 to 15 proteins studied (Fig. 1C).

The ρ-value for sucrose in a native bovine serum albumin (bsa) solution containing 78.1% $\pm$ 2.25% (mean $\pm$ S.E.) water is 0.947 ± 0.012 (n = 24) while the ρ-values of urea- and guanidine-HCl-denatured bsa were reduced to 0.697 ± 0.010 (n = 24) and 0.679 ± 0.034 (n = 4), respectively, with p far below 0.001 (t - test). On the other hand, neither SDS nor n-propanol produced a significant fall of the $\rho_{sucrose}$. Figure 1C shows that essentially the same results occur with other proteins studied in regard to sucrose distribution. Although space does not permit a presentation of the less complete glycine data, it may be stated that they are qualitatively similar to the results obtained for sucrose. It may be mentioned that the ρ-value of sucrose and glycine in urea-denatured proteins can be calculated on the basis of sucrose and glycine distribution either in water alone in the protein-water-urea system or in water plus urea, with essentially the same results, since the ρ-value for urea itself is very close to unity (0.991 ± 0.0085, from all 15 sets of data).

Together, these data suggest that the criterion for reducing the solvency for the probe molecules is indeed the presence of extended polypeptide chains with the NHCO groups free to interact with water, independent of the means by which the chain extension is brought about.

The data given in Figure 1 also permit us to make an estimate of the minimum amount of water affected by the extended protein chains. To facilitate this assessment, we introduced an artificial device - the separation of water into (1) a

normal category, with normal solvent properties, and (2) a totally "non-solvent" portion, given in grams of H_2O per gram of dry protein - thereby providing an index of the apparent minimum amount of water affected by the proteins (AMINOW). Thus if $\rho_{sucrose}$ for a protein-water system containing 18 percent proteins and 80 percent water is 0.45, the AMINOW is 0.80 x (1-0.45)/0.18 = 2.44 gm H_2O/gm protein. Since the part of the water affected is not likely to be totally nonsolvent, this figure is a minimal estimate of a larger amount of water actually affected.

Figures 1B and D present, in terms of AMINOW, the diverse effects of the 4 denaturants as well as the AMINOW in the native proteins. In the specific case of bsa, as well as in the average of all the 10 to 15 proteins studied, AMINOW rises in response to urea and guanidine HCl. Destruction of the tertiary and quaternary structures by high concentration of SDS and n-propanol produced little or no change in AMINOW.

Let us now compare these AMINOW values of the extended protein chains produced by urea and guanidine HCl with the value that would theoretically be expected to operate in living cells. The ρ-values for different probe molecules vary considerably from tissue to tissue. For safety we shall choose skeletal muscle as an example, since it tends to have a low q-value and hence a high AMINOW. In this tissue the q-value for sucrose is 0.18 [29] and for glycine about 0.30 [30]; its total protein content is about 20%. If all the proteins participate in the interaction with cell water, the AMINOW needed would be between 2.5 and 3.0, which is 2 to 3 times higher than that in urea-denatured proteins. In fact, not all intracellular proteins could be in an extended state. The required AMINOW must be still higher.

Several factors are likely to reduce the water-orienting property of proteins under the conditions of our experiment. First, proteins in solution are not likely to be themselves oriented in an orderly way, while maximal water polarization

will occur only when there is a more or less parallel orientation of the protein chains; this was demonstrated in an experiment that compared the exclusion of Na^+ from sacs of PVP solution that remained quiescent (Q) and from similar sacs that were constantly stirred (S). Since to-and-fro stirring of a linear polymer solution tends to align the chains in parallel (as witnessed by the phenomenon of flow birefringence), and since agitation would also tend to diminish interchain adhesion, we expect an increase of the solvent effect of extended polypeptide chains with agitation. As shown in Table 1E, this was indeed what we observed; the ρ-value of Na^+ is consistently lower in agitated samples. In a prior report, Woessner and Snowden described a long-range ordering effect on water by a bacterial polysaccharide Kelzan®. This ordering effect was also enhanced by mechanical stirring [39], although just the opposite might be expected.

A second factor that can alter the water-orienting properties of extended protein chains is the number of available NHCO groups. It is unlikely that urea or guanidine HCl will release all NHCO groups; for example, witness the inability of urea to denature poly-L-alanine [40] and pepsin [41-42]. A third factor is the tendency of the denatured protein chains to aggregate and clump together by forming side chain-to-side chain hydrophobic bonds, salt linkages, etc., rather than to distribute themselves away from each other at regular intervals [31, 43]. However, all three factors preventing water polarization can be expected to be reduced when water contains the simple polymer PEO, because (1) it possesses no side chain and therefore cannot undergo side chain-to-side chain interaction; (2) it possesses no H-donating group and therefore cannot form strong inter- or intracellular H bonds; and (3) it readily aligns itself in parallel, as witnessed by the remarkable property of a drop of PEO water to be pulled into an ever-lengthening, ever-thinning thread in a quiescent atmosphere.

As expected, the data in Table 1D of PEO in 0.5 M Na-citrate yield an AMINOW value of 4.00, a value approaching or equaling that envisaged in living cells [34].

The data presented in Table 1D for example, also permit calculation of the minimal average number of water molecules under the influence of each oxygen atom of PEO. At an external Na_2SO_4 concentration of 0.5 M this number is 14; multilayers of water molecules must be involved. Years ago, Brunauer, Emmett and Teller[44] showed that charged sites can influence more than one layer of adsorbed molecules only if these molecules possess a large permanent dipole moment, as it is the case with water, (μ = 1.834 X 10^{-18} e.s.u.). In other words, oxygen atoms in PEO and other polymers can influence distant water molecules only by a mechanism of propagated electrical polarization involving both induced and permanent dipole moments of the "target" as well as intervening water molecules. This is just another way of saying that these water molecules exist in the state of polarized multilayers.

Our data indicate strongly that a search for the specific cellular proteins involved in the polarization of cell water and a delineation of the factors acting to enhance this phenomenon are likely to be fruitful.

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Abstract

Studies of native and denatured proteins and two synthetic polymers, polyvinylpyrrolidone and poly(ethylene oxide), led to the conclusion that arrays of fully extended protein chains with their NH and CO groups directly exposed to bulk water reduce the solubility of $Na^{(+)}$, sucrose, and glycine within that water. These findings provide a basis for the view that exclusion of Na^{+}, sucrose, and amino acids seen in living cells has a common origin - the dynamic ordering of water by certain extended intracellular protein chains.

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Legends

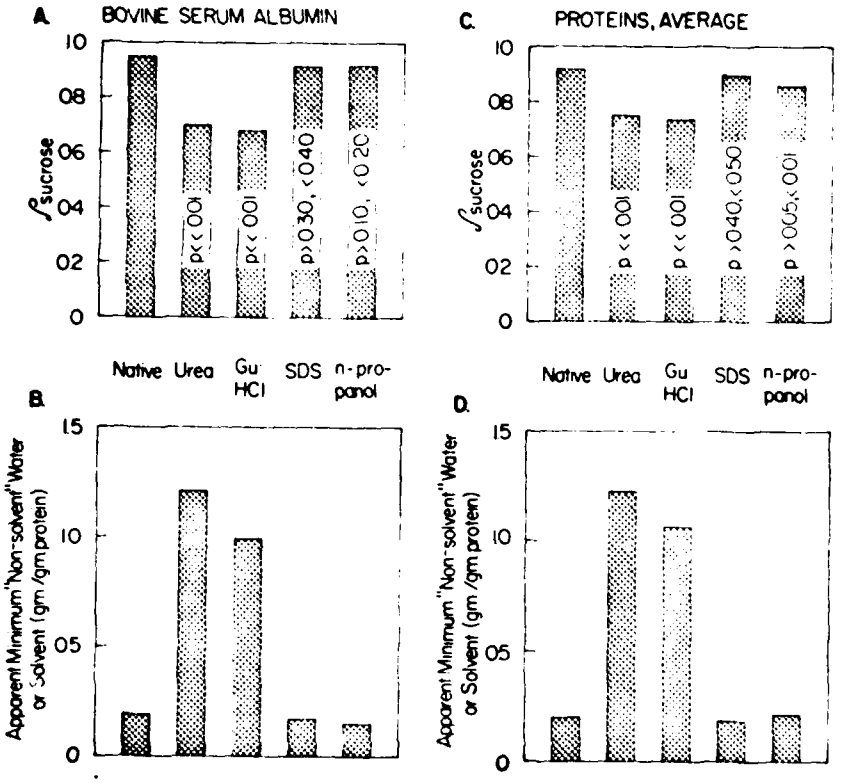
Table 1. ρ-values of Na^+ in water containing native proteins (A), gelatin (B), PVP (C, E) and poly(ethylene oxide) (D). In all the experiments the temperature was $25^{o}\pm1^{o}C$ and the test tubes were agitated with the exception of those in E, which were carried out at $0^{o}\pm1^{o}C$ but in which some test tubes, marked Q, were quiescent and unstirred. S represents sacs shaken in test tubes at 30 excursions/min (each excursion spans 1 inch) except the first set (S*) for which agitation was achieved by the to-and-fro movement of silicone-rubber coated lead shots within the sacs. The second column 1.5 (A) and 0.5 (B) indicate that the media contained initially 1.5 M Na_2SO_4 (A) and 0.5 M Na-citrate (B) respectively. In D, poly(ethylene oxide) (molecular wt 600,000) was dissolved as a 10% (w/w) solution, and the viscous solution was vigorously stirred before being introduced into dialysis tubing. In E, the quiescent samples also contained more water. This higher water content accounts for only a minor part of the difference, as shown by comparison of the 6th and 7th sets of data; even with a larger water content, the ρ-value is lower in the stirred samples (6th). Na was labeled with ^{22}Na and assayed with a γ-counter.

Figure 1. The ρ-values of sucrose (A and C) and the apparent minimum "non-solvent" water (B and D) of native and denatured proteins. C and D represent the averages of 15 proteins studied: actin[1], albumin (bovine)[2], albumin (egg)[3], chondroitin sulfate[4], α-chymotrypsinogen[5], edestin[6], fibrinogen[7], γ-globulin[8], hemoglobin[9], β-lactoglobulin[10], lysozyme[11], myosin[12], trypsin[13], trypsin inhibitor[14], and histone[15]. Values for the native and urea denatured states were determined from all 15 proteins; guanidine HCl values from proteins 2 - 11, 13, 14; SDS and n-propanol values from

2 - 11. No significant p-value difference was observed in the native protein value whether it was determined from 15, 12, or 10 proteins. Incubating solutions contained Na_2SO_4 (100 mM), glycine (10 mM), sucrose (10 mM), and $MgCl_2$ (10 mM). In addition, urea (9 M) and guanidine HCl (6 M), sodium dodecyl sulfate (0.1 M), and n-propanol (2 M) were present as indicated. Incubation at $25^{o}\pm1^{o}C$ lasted from 28 to 96 hours, sufficient time to establish equilibrium. The test tubes were shaken (30 excursions per min, each excursion measuring 3/4 inch). Water contents were assayed with 3 different methods [28]; sucrose was labeled with ^{14}C or ^{3}H and extracts were assayed with a β-scintillation counter.

TABLE 1

	Polymer	Concentration of Medium (M)	Number of Assays	Water Content % (mean ± S. E.)	p-Value (mean ± S. E.)
A.	Albumin (bovine serum)	1.5 (A)	4	81.9±0.063	0.973±0.005
	Albumin (egg)	1.5 (A)	4	82.1±0.058	1.000±0.016
	Chondroitin sulfate	1.5 (A)	4	84.2±0.061	1.009±0.003
	α-Chymotrypsinogen	1.5 (A)	4	82.7±0.089	1.004±0.009
	Fibrinogen	1.5 (A)	4	82.8±0.12	1.004±0.002
	γ-Globulin (bovine)	1.5 (A)	4	82.0±0.16	1.004±0.004
	γ-Globulin (human)	1.5 (A)	4	83.5±0.16	1.016±0.005
	Hemoglobin	1.5 (A)	4	73.7±0.073	0.923±0.006
	β-Lactoglobulin	1.5 (A)	4	82.6±0.029	0.991±0.005
	Lysozyme	1.5 (A)	4	82.0±0.085	1.009±0.005
	Pepsin	1.5 (A)	4	83.4±0.11	1.031±0.006
	Protamine	1.5 (A)	4	83.9±0.10	0.990±0.020
	Ribonuclease	1.5 (A)	4	79.9±0.19	0.984±0.006
B.	Gelatin	1.5 (A)	37	57.0±1.1	0.537±0.013
C.	PVP	1.5 (A)	8	61.0±0.30	0.239±0.005
D.	Poly(ethylene oxide)	0.75 (A)	5	81.1±0.34	0.475±0.009
		0.5 (A)	5	89.2±0.06	0.623±0.011
		0.1 (A)	5	91.1±0.162	0.754±0.015
E.	PVP Q	0.2 (B)	4	89.9±0.06	0.955±0.004
	S*	0.2 (B)	4	87.2±0.05	0.865±0.004
	Q	0.5 (B)	3	83.3±0.09	0.768±0.012
	S	0.5 (B)	3	81.8±0.07	0.685±0.007
	Q	1.0 (B)	3	67.0±0.26	0.448±0.012
	S	1.0 (B)	3	66.6±0.006	0.294±0.008
	Q	1.5 (B)	3	56.3±0.87	0.313±0.025
	S	1.5 (B)	3	55.0±1.00	0.220±0.021


A
BOVINE SERUM ALBUMIN
10
08
06
04
02
0
$\int^{\rho}$ sucrose
p << 001
p << 001
p > 030, < 040
p > 010, < 020
C.
PROTEINS, AVERAGE
p << 001
p << 001
p > 040, < 050
p > 005, < 001
Native
Urea
Gu HCl
SDS
n-propanol
B.
D.
Apparent Minimum "Non-solvent" Water or Solvent (gm /gm protein)
15
10
05
0


MED
8