THE PHYSICAL STATE OF WATER IN LIVING CELLS AND MODEL SYSTEMS. XII. THE INFLUENCE OF THE CONFORMATION OF A PROTEIN ON THE SOLUBILITY OF Na^+ (SULFATE), SUCROSE, GLYCINE AND UREA IN THE WATER IN WHICH THE PROTEIN IS ALSO DISSOLVED

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In this report, we describe the result of on extensive investigation of the effects of the conformations of proteins on the solvency of the bulk-phase water in which the proteins are dissolved. The concentrations of the proteins used were usually between 20 to 4%; the temperature was 25° ± I" c.

To probe the solvency of the water, the apparent equilibrium distribution coefficients (or ρ-values) of 4 solutes were studied: Na^+ (sulfate), glycine, sucrose, and urea From 8 to 14 isolated proteins in three types of conformations were investigated: native; denatured by agents that unravel the secondary structure (e.g., u-helix, β-pleated sheet) of the protein (i.e., 9 M urea. 3M guanidine HCl); denatured by agents that only disrupt the tertiary structure but leave the secondary structure intact or even strengthened (i.e., 0.1M sodium dodecylsulfate or SDS, 2M n-propanol).

The results are as follows: (I) as a rule, native proteins have no or weak effect on the solvency of the water for all 4 probes; (2) exposure to 0.1M SDS and to 2M n-propanol, as a rule, does not significantly decrease the ρ-value of all 4 probes; (3) exposure to 9M urea and to 3M guanidine HCl consistently lowers the ρ-values of sucrose, glycine and Na^+ (sulfate) and equally consistently produces no effect on the p-value of urea. Sucrose, glycine, and Na^+ ore found in low concentrations in cell water while urea is not.

These experiments were designed and carried outprimarily to test two subsidiary theories of the AI hypotheses: the polarized multilayer (PM) theory of cell water; and the theory of size-dependent solute exclusion. By and large, the relevant theoretical expectations have been confirmed:

(1) a protein can effectively change the solvency properties of the bulk phase water only when the protein exists in the fully extended conformation (as in urea- ahd guanidine HCl-denatured proteins) with theirpeptide NHCO groups directly exposed to the bulk phase water but not when it exists in the globular or other conformation, in which its backbone NHCO groups are internally locked in intro- or inter-macromolecular H-bonds (as in native and in SDS and n-propanol denatured protein):

(2) the ρ-values of different solutes in general follow the "size-rule': i.e., the ρ-values of molecules in water existing in the state of polarized multilayers (referred to also simply as polarized water) decrease with increasing molecular weight (or more accurately, molecular volume) of the solute.

To help the reader in assessing accurately the true significance of the findings described. we have also provided some historical highlights. Thus, it is pointed out that although colloid chemistry was named after gelatin, the original workers did not have the necessary peripheral information to understand what makes gelatin so extraordinary. It was this failure that had hamstrung the further development of the colloidal approach to cell physiology at that time.

With the understanding provided by the AI hypothesis and by knowledge acquired by other investigators on gelatin, we believe that we are now able to pinpoint why gelatin is so extra-

ordinary: a major part of gelatin exists permanently in *the fully extended state. In consequence, bulk phase water in the vicinity of the gelatin chains exist as polarized multilayers, and as such the water associated with gelatin assumes properties different from those of normal liquid water but more in common with water of the* living cells.

With this new understanding it has become possible to redefine colloid. Such a redefinition is offered.

According to the polarized multilayer (PM) theory of cell water, the bulk of water molecules in living cells exists in the dynamic structure of polarized multilayers. The assumption of this dynamic structure follows in consequence of the interaction of the water with the exposed NH and CO groups of a matrix of fully extended protein chains present pervasively in all living cells as part of, and/or anchored (directly or indirectly) onto a cytoskeletal framework (Ling, 1962, 1972, 1984). A matrix of fully extended protein chains, with their alternatingly positive (NH) and negative (CO) sites at proper distances apart is called an NP-NP-NP system, where N and P represent the negative and positive sites respectively.

A subsidiary hypotheses of the PM theory is the hypothesis of size-dependent solute exclusion from polarized water (i.e., water existing in the state of polarized multilayers, Ling, 1965a, 1972, 1984). This hypotheses of solute exclusion is founded on both energy and entropy. considerations: (1) enthalpy mechanism: it costs more energy (or enthalpy) to excavate a hole in the polarized water to accommodate the solute than the energy gained in tilling the hole left behind in the normal liquid water of the external medium, wherefrom a solute is transferred to the polarized water; (2) entropy mechanism: the polarized water is, so to speak, more "sticky" than normal liquid water. That is, a water molecule or a dissolved solute in the polarized water is more strongly associated with its immediate neighbors and is directly or indirectly anchored to, and thus immobilized by, the fixed protein matrix and cytoskeletal network. The resultant restriction of translational and especially rotational motional freedom reduces the entropy of the solute molecule in the polarized water. Again, the larger the solute molecule, the greater the proportional loss of entropy in being transferred from the external medium of normal liquid water to the polarized water within a living cell or model system (Ling, 1984, p171). Since both the enthalpy and entropy mechanisms of solute exclusion are stronger for the larger solute molecules than smaller ones, a dependence of the solubility of the solute in polarized water on the molecular size of the solute or the "size rule" is the consequence. More precisely, this rule states that the (true) ***equilibrium distribution coefficient,*** or ***q-value*** of a solute between water in a cell (or model system) and normal water in the external medium tends to decrease with increasing molecular size or volume of the solute.

The molecular volume is equal to the sum of the volume equivalents of individual atoms comprising the molecule (Glasstone, 1946, p 524). Since in organic chemicals in general and homologous members of simple aliphatic compounds in particular the molar volume increases with the atomic weights, the "size rule" in fact predicts an inverse relationship between the q-values of these solute molecules and their respective molecular weights.

However, there are also exceptions to the "size rule". The size rule does not apply to molecules comprising different kinds of atoms with widely different atomic volume vs atomic weight relationships. Another exception comes from the fact that some molecules, due to their special molecular structure and steric

configurations, fit into the dynamic water structures so well that their replacement of water molecules actually leads to further stabilization and enhancement of the dynamic water structure. Under these conditions, the q-values of these solutes may then equal or exceed unity.

The data to be described below are results of a major effort carried out over a long period of time. The aim of our efforts was to test the hypothesis that all proteins have the ***inherent*** ability of reducing the solvency of bulk phase water for those large solutes that are, as a rule, found in low concentrations in the water of living cells (e.g., (hydrated) Na^+ salts, sucrose, glycine), and that in most native globular proteins this inherent ability is masked. It becomes revealed, however, whenever a protein assumes the *fully extended* conformation with the backbone NH and CO groups directly exposed to the bulk-phase water.

To achieve the goal described we designed and carried out experiments aimed at testing the following specific predictions:

(1) Since the NHCO groups of most ***native*** proteins are locked in intra- or intermacromolecular H-bonds (e.g., as in a-helical or β-pleated sheet conformation), they are not expected to have a strong influence on the solvency of the probe molecules or ions like sucrose, glycine, and Na^+ salts in the water of a native protein solution. In other words, the q-values of sucrose, glycine, and Na^+ salts in water are influenced little or not at all by the presence of native proteins in the water.

(2) Denaturants like 9M urea, 3M guandidine HCl (GuHCl) are ***known*** to unravel the secondary structure (e.g., a-helix, B-pleated sheet) and thus expose the NHCO groups directly to bulk phase water. Exposure to 9M urea and 3M GuHCl is therefore expected to cause a decrease of the q-values (and ρ-values*) for large probe molecules (e.g., sucrose, glycine) but to cause little or no change of the q-value (and ρ-values) for solutes that are small and/or can fit into the dynamic water structure (e.g., urea).

(3) Denaturants like 0.1 M Na^+ dodecylsulfate (SDS) and 2M n-propanol are known to unravel only the tertiary structure, leaving the secondary structure unperturbed or even strengthened. 0.1M SDS and 2M n-propanol should, therefore, have little or no effect in reducing the q-values (or p-values) of the probe molecule large and small, below those in solutions of native proteins.

Earlier confirmations of these theoretical predictions based on the limited data then on hand have been reported briefly elsewhere (Ling, et al., 1980a). It is in the present document that the entire experimental work is presented in full.

Under "Discussion" the significance of thii and other very recent findings is considered in their historical background that spans a major portion of the 20th century.

MATERIALS AND METHODS

Materials. Spectra/Pa 2 dialysis tubing with a molecular weight cutoff value at 12,000 to 14,000 were used in all studies.

The following proteins were from Sigma Chemical Co., St. Louis, MO: bovine serum albumin, Cat. # A-4503, Lots 88C-0252, 95C-0191, 10603447; egg albumin, Cat. # A-5503, Lots 18C-8035-1, 105C-8022; α-chymotrypsinogen, Cat. # 4879, Lots 75C-8380, 106C-8030; fibrinogen, Cat. # F-4000, Lots 86C-0118, 876-0307; bovine y-globulin, Cat. # B-611, Lots 116C-0147, 126C-03062, 116C-

*The (true) equilibrium distribution coefficient, or q-value equals the ratio of the equilibrium concentration of the probe molecule exclusively found in the water of a cell or model system over that in the water of the external medium. When the probe solute is known to be or may be partly adsorbed or otherwise amplexed to intracellular macromolecules, the ratio is referred to as the *apparent equilibrium distribution coefficient, or ρ-value.* While q-values cannot exceed the value of I; p-values can. In the context of its usage in this paper, the p-value measured is not expected to be very different from the q-value.

0147; bovine hemoglobin, Cat. # H-2500, Lot 66C-8092; histone, Cat. # H-9250, Lots 115C-0142, 27C-0381; Cat. #H-7755, Lot 55C-8190; β-lactoglobulin, Cat. # L-6879, Lots 86C-8065, 106C-8070; lysozyme, Cat. # L-6876, Lots 57C-8025, 75C-8483; pepsin, Cat. # P-7012, Lots 26C-8045, 18C-8080; protamine (free base) Cat. # P-4005, Lot 104C-9550; protamine sulfate, Cat. # P-4255, Lot 84C-9540. Other proteins used were edestin, Control # 12624, 17256 (U.S. Biochemical); trypsin, Lot 7017 (Miles Lab.); trypsin inhibitor, Lot 7046.

Radiochemicals from New England Nuclear, Boston, include: 'H-glycine, Lots 929-042, 1012-017; "C-sucrose, Lots 919-169, 870-25T; ^{14}C-urea, Lot 952-178, THO, Lot 56502. ^{24}Na, Lot 093, was from New England Nuclear; ^{22}Na, Lot 39, from ICN Biomedicals.

Thymol, Lot 603050, Baker; penicillin G, sodium, Lot 5K620, Squibb, streptomysin sulfate, Lot 6UR94A, Lilly.

Methods. The immediate object of these studies was to determine the apparent ***equilibrium distribution coefficients*** (or ***p-values)*** of three neutral probe solutes (sucrose, glycine, and urea) and one ion, Na^+ in solutions of native proteins and of proteins denatured by urea, guanidine HCl, SDS and n-propanol. To obtain the p-values, we relied on the method of equilibrium dialysis. Radioactively labeled sodium, sucrose, glycine, or urea (often in pairs of two) were allowed to reach diffusion equilibrium (in a constant temperature room kept at 25°C ± 1°C), between a protein solution kept in a dialysis sac and its external bathing solution. Dividing the molar equilibrium concentration of the labeled solute in the water (or solvent) within the sac by that in the water (or solvent) in the external bathing solution yields the p-value.

Preparation of Protein Solutions and Loading of Dialysis Sacs. A weighted sample of protein was gently floated (without stirring) on top of a measured volume of water in a Nalgene cenrifuge tube (height, 6 cm; I.D. 2.5 cm). The tube and its content were gently shaken in a rotary shaker overnight in a cold room. Only when the protein failed to dissolve completely, was a plastic or glass rod used to facilitate the dissolution. To prepare solutions of denatured protein, native proteins were dissolved in a similar manner, except that the solution contained a suitable concentration of the denaturant (e.g., 9M urea).

Approximately 1 ml of the native or denatured protein solution prepared was then taken up in a tuberculin syringe (capacity, 1 ml) from which it was projected into a 10 cm length of wetted Spectra/Por 2 dialysis tubing, tied at one end with 4-O Ethicon surgical silk. A rubber-tipped "bull dog" clamp was used to maneuver and keep the protein solution toward the tied end of the dialysis sac. Four more ligatures were then made with the surgical threads at approximately equal distances apart to produce a tandem of four similar-sized sacs, each tightly-filled with about 0.25 ml of protein solution. The protein solution in each sac was totally separated from that in the immediately neighboring sac(s). The tandem of sacs was then introduced into a screw-cap tube containing 15cc of the incubation solution. The standard incubation solution used contained Na_2SO_4 (100 mM), glycine (10 mM), sucrose (10 mM), $MgSO_4$ (10 mM), thymol (0.15 mg/ml), penicillin (0.1 mg/ml), streptomycin (0.1 mg/ml) (see below) and one or two radioactively labeled probe(s) at 0.2 μC/ml. The pH of the incubation solution was adjusted with H_2SO_4 and/or NaOH to 7.5.

Other incubation solutions contained in addition, 9M urea, 6M guanidine HCl, 0.1M sodium dodecyl sulfate (SDS) or 2M n-propanol.

Control of Bacterial Growth. An experiment was carried out to determine the necessary agents required to check bacterial growth. Four dialysis sacs were filled with 40% egg albumin. Each sac was introduced into a screw cap tube containing 15 ml of incubation solution. A 0.05 ml of a suspension of gram-negative bacillus and gram-positive staphylococcus, collected from a blood agar plate, was added to each screw cap tube. To some of these tubes was then added thymol alone (15 mg/100 ml); to others, a mixture of thymol(15 mg/100 ml), penicillin G, Na^+ (10 mg/100 ml) and streptomycin sulfate (10 mg/100 ml) was added. Samples of the external solution were then taken out of different times and plated on blood agar plates. After 24 hours of incubation, bacterial growth occurred in both the control tubes containing no bacterial control agents and in the thymol (only) -containing tubes. No growth occurred in the tubes containing thymol, penicillin, and streptomycin up to 69 hours of incubation, but some streptococcus and staphylococcus were seen after 91 hours of incubation. Table I shows that the p-value of both glycine and sucrose reached equilibrium before 22 hours (see below) and stayed essentially constant in both the thymol series and the thymol-penicillin-streptomycin series despite demonstrable bacterial growth in the thymol (only) series. In all experiments described in this report, the thymol-penicillin-streptomycin combination was used routinely.

Duration of Incubation. To determine the time of incubation necessary for the probe molecules to reach diffusion equilibrium, we relied on two methods: the "simple efflux method" and the "converging influx and efflux method".

(1) Simple efflux method: A radioactively labeled solute was added to the protein solution inside a dialysis sac. The sac was then washed in successive portions of nonlabeled incubation solution. From the radioactivity in each washing solution and radioactivity remaining in the sac at the conclusion of the experiment, an efflux curve was constructed in which the logarithm of the concentration

TABLE 1. Time courses and changes of the ρ-values for glycine and sucrose in dialysis tubes containing 40% egg albumin, with no antibiotics, no thymol (control), with thymol alone (thymol) or with penicillin and streptomycin in addition to thymol (thymol + antibiotics) (25°C). Each set of means ± S.E. from 4 sacs.

		22¼ hrs	45% hrs.	69% hrs.	91 hrs.
Control	$\rho_{Glycine}$	0.900 ± 0.012			
	$\rho_{Sucrose}$	0.853 ± 0.007			
Thymol	$\rho_{Glycine}$	0.891 ± 0.016	0.915 ± 0.06	0.921 ± 0.016	0.929 ± 0.006
	$\rho_{Sucrose}$	0.883 ± 0.024	0.856 ± 0.011	0.945 ± 0.024	0.826 ± 0.028
Thymol + Antibiotics	$\rho_{Glycine}$	0.902 ± 0.010	0.902 ± 0.009	0.911 ± 0.016	0.909 ± 0.007
	$\rho_{Sucrose}$	0.904 ± 0.014	0.908 ± 0.009	0.886 ± 0.021	0.856 ± 0.008

of the labeled probe molecule remaining in the sac at time t was plotted against t From this type of efflux curve, the time for the loss of 99% ($t_{0.99}$) of the radioactivity initially introduced into the sac was determined. The results are shown in Table II.

Note that in solutions of native proteins, the relative values of $t_{0.99}$ varies with the kind of protein involved. $t_{0.99}$ for the smaller glycine (molecular weight, 75.07) is about twice as fast as that for the larger sucrose (M.W., 342.30). Urea denaturation lengthened the $t_{0.99}$ of sucrose for all proteins studied* but proportionately more so for those proteins which in their native state gave rise to shorter $t_{0.99}$'s (for both glycine and sucrose).

(2) "Converging influx and efflux method": In this method, two sets of time courses of radioactive label movements were followed. In one, the radioactive label was added to the inside of the sac; in the other, it was added to the outside of the sac. The minimal time needed for the p-values of the labeled probe in both sacs to reach the same value indicates the minimal time needed for diffusion equilibrium to be reached.

From the date of Table I and Table II on glycine and sucrose movements derived from simple efflux studies and from other data from studies described below (p 32) using the "converging influx and efflux method" we concluded that for native proteins, 24 hours of incubation is adequate for these probes. For the sake of extra safety, however, we routinely incubated for 48 hours. For solutions of denatured proteins, we incubated 72 hours, which was also longer than the minimal time needed for equilibration. Earlier reports show that these schedules are entirely applicable to the study of the p-value of Na^+ in native and urea-denatured proteins (Ling et al., 1980a).

TABLE IL Summary of the time (in hours) needed for 99% exchange of labeled glycine and labeled sucrose between the external bathing solution and in 40% solution of 10 native and urea-denatured proteins held in dialysis sacs (WC). Data were obtained from studies using the simple efflux method

Time for 99% Exchange (hrs.)			
	Glycine	Sucrose	
	(Native)	Native)	(Urea Denatured)
Albumin (Serum)			50.3
Albumin (Egg)	2.3	5.0	35.3
α-chymotrypsinogen			46.7
Edestin			43.1
Fibrinogen	7.3	18.0	38.2
γ-globulin			51
Hemoglobin			35
β-lactoglobulin	2.6	6.7	50.8
Lysozyme			14.7
Pepsin	2.3	5.3	

Extraction of probe materials from sac contents. We used three methods for the extraction of radioactive probes from protein solutions collected from the sacs at the conclusion of an experiment: (1) The hot HNO_3 method: Samples were introduced into the HNO_3 solution (1N) and heated in a 85°C water bath for 20 minutes in 15 cm long test tubes topped with a marble. In shorter (Nal-

*Taken as a whole, these findings suggest that denaturants like urea that unravel secondary protein structure exercise powerful effects on the rate of diffusion of the probe molecule sucrose through the wall of the dialysis tubing. One may suggest the following interpretation for the observed phenomenon. Protein molecules inside the dialysis-tubing pores underwent conformation changes in response to urea. As a result, the protein unfolds and water in the dialysis membrane pores becomes polarized in multilayers. The rate of diffusion of probe molecules through the pore wafer was reduced in the size-dependent manner-in harmony with earlier findings demonstrating size-dependent diffusion rates through membrane models containing intensely polarized water (Ling, 1973; Ling and Ochsenfeld, 1980b).

gene) centrifuge tubes (12 cm), the evaporation loss was 2%; in 15 cm tubes, the loss was much less (0.2%). A correction of suitable magnitude was routinely made for this evaporation loss. (2) The NaOH method: In this method the sac contents were introduced into 0.1 N NaOH, shaken vigorously with a vortex shaker, and then allowed to stand overnight. (3) LiOH-Trichloroacetic acid (TCA) method: In this method, the sac content was first dispersed in 1 volume of 0.3 N LiOH. After overnight incubation, 2 volumes of 10% TCA was then added before centrifugation and collection of the supernatant for assay.

Table III shows that all three methods proved equally effective in extracting labeled glycine and labeled sucrose from 40% solutions of native and urea-denatured egg albumin. The data presented in Table III also corroborates results of our main efforts in studies of the effect of urea denaturation on the p-values of glycine and sucrose to be described under RESULTS.

The determination of water contents. Since the p-value is the molar ratio of the concentration of the probe solute in the sac solvent (i.e. water) and in the external solution, the accuracy of our data critically depends on the accuracy in the assay of the water (or solvent) contents. The determination of water (or solvent) content was difficult due to the presence of high concentration of (heat labile) urea in a major portion of the data collected. To insure accuracy we evolved three independent methods to assay the water contents of both the sac contents and the external solution, and used two or even all three methods on aliquots of the same sample to make certain that no significant error was committed.

TABLE III. Demonstration of the effectiveness of three different methods for the extraction of labeled glycine and labeled sucrose from dialysis sacs containing (initially) 40% solution of native and urea-denatured egg albumin that had been incubated in the labeled solutions. For details of each method of extraction, see text. Each set of mean ± S.E. is from 4 similarly treated sacs.

		$\rho_{Glycine}$	$\rho_{Sucrose}$
Control	LiOH-TCA	0.935 ± 0.007	0.933 ± 0.034
	NaOH	0.923 ± 0.007	0.924 ± 0.018
	HNO_3	0.928 ± 0.005	0.893 ± 0.011
Urea	Li-TCA	0.773 ± 0.023	0.676 ± 0.017
	NaOH	0.717 ± 0.040	0.667 ± 0.057
	HNO_3	0.756 ± 0.010	0.664 ± 0.025

(A) Drying ***method.*** Drying at 80°C in vacuo (20 mm Hg vacuum) for 68 hours was the standard procedure most often used. It was quite adequate in dealing with the great majority of samples including those containing guanidine HCl, SDS, sucrose and glycine.

As mentioned above, the presence of urea in the samples presents problems: even at a temperature of 80°C and thus substantially below the boiling point of water, some loss of urea occurred when the samples were heated in aluminum-foil pans (A.H. Thomas, Philadelphia, Catalog No. 3844-F10). For a 68 hour drying period, the error amounted to about 1%. A correction factor of this magnitude was applied routinely to all samples dried in aluminum pans. However, in later studies, we discovered that little or no loss of weight occurred when the drying was carried out in predried, 5 ml Pyrex glass beakers. These vessels were clearly superior to aluminum pans for the present purpose.

n-Propanol has a boiling point of 97.2°C and thus close to that of water. Drying at 80°C removes, besides water, all n-propanol from the samples. Thus in n-propanol containing solutions, the total solvent contents (water plus n-propanol) were assayed and the equilibrium distribution coefficients were calculated on the basis of total solvent rather than water alone.

(B) ***THO method.*** Tritiated water was included in the original incubation solution in experiments where only ^{14}C-labeled probe

molecules (alone) were used. Radioactivity from tritium (and from ^{14}C) was assayed in sample extracts and sorted out by standard dual channel counting procedures. The tritium counts were then used to calculate the water contents of both the sac contents and the final external solutions.

(C) Weight change method. The total weight of a filled dialysis sac can be separated into 5 sources: (1) surgical thread, (2) dialysis tubing, (3) protein(s), (4) small molecular weight solutes, and (5) solvent (e.g., water or water + urea). The water (or solvent) content of the final sample was obtained from the difference between the total weight of the sample and the sum of the final weights of the "on-water components. The final weights of the surgical thread and dialysis tubing could be estimated from their initial weights and from the estimated percentage weight changes from control runs of similarly treated thread and empty dialysis tubing. The final weights of the protein in the sac were determined by subtracting from the initial weight of the protein introduced into the sac, the amount of

TABLE IV. A comparison of the ρ-values of sucrose in solutions of fourteen native and urea-denatured proteins (25°C). In the parenthesis following the mean ± S.E. of the ρ-values for sucrose for each protein are in the order from left to right: the total number of independent assays performed; the total number of sets of experiments performed; the final percentage water content (× 100) of the sac content; the final pH of the external bathing solution. The last column lists the probability (P.) for the occurrence by random chance of the observed difference between the sucrose of the solution of native protein and urea-denatured proteins. The last row gives the mean ± standard error of the means of the sucrose for the fourteen proteins studied. Note that the water contents of the urea-denatured proteins are much lower than their native counterparts. This disparity arises from the large properties of urea both inside and outside the sacs. Since the ρ-value for urea is very close to one (see Table VI), essentially the ρ-values for sucrose will be obtained if instead of calculating the sucrose concentrate on the basis of water content, one chooses the total solvent (water + urea) as the basis for calculation.

	$\rho_{sucrose}$		
	Native	Urea-denatured	P
Albumin (serum)	0.979 ± 0.013 (23,6;77;5.4)	0.697 f0.010 (28,7;40;7.0)	< 0.001
Albumin (egg)	0.914 ±0.008 (44,8;68,5.0)	0.710 ± 0.010 (32,7;38;6.9)	< 0.001
α-chymotrypsinogen	0.900 ± 0.10 (24,6;61;3.9)	0.707 ± 0.012 (20,5;40;6.4)	< 0.001
Edestin	0.858 ± 0.018 (15,4;51;5.6)	0.759 ± 0.007 (24,6;42;7.2)	< *0.001*
Fibrinogen	0.929 ±9.017 (20,5;68;7.0)	0.771 ± 0.014 (20,5;40;7.8)	< 0.001
γ-globulin	0.910 ± 0.007 (32,7;66;6.6)	0.660f0.013 (34,8;37;7.6)	< 0.001
Hemoglobin	0.967 ± 0.013 (16,4;72;4.9)	0.685 ±0.020 (15,4;43;7.0)	< 0.001
Histone	0.910 ± 0.015 (20,4;72;7.3)	0.770 ± 0.009 (16,4;44;7.6)	< *0.001*
β-lactoglobulin	0.927± 0.014 (32,8;70;5.6)	0.658 ± 0.010 (36,9;44;7.6)	< *0.001*
Lysozyme	0.942 ± 0.020 (20,5;62;5.3)	0.850 ± 0.015 (24,6;43;7.0)	*0.005 > P > 0.001*
Pepsin	0.947 ± 0.012 (24,6;69;4.5)	0.918 ± 0.009 (16,4;47;5.3)	0.20 > P > 0.10
Protamine	1.150 ± 0.009 (8,2;83;11.1)		
Trypsin	0.924 ± 0.021 (8,2;76;3.9)	0.809 ± 0.016 (14,3;47;6.0)	< 0.001
Trypsin inhibitor	0.979f0.012 (9,2;84;6.6)	0.870 ± 0.009 (14,3;50;8.7)	0.05 > P > 0.025
Mean ± S.E.	0.945 ± 0.034 (14)	0.773 f0.025 (14)	

proteins that had leaked out from the sac into the external solution and chemically determined. The initial and final weights of the small molecular-weight solutes in the incubation solutions were very small, and were ignored. The final solvent contents were thus obtained by subtracting from the final sac weights, the final weights of the remaining protein, that of the dialysis tubing and of the surgical threads.

Assay of probe solute concentrations. All assays of probe solute concentrations were made with radioactive tracer methods. Single or dual channel counting of samples were carried out on a β-scintillation spectrometer on samples mixed usually with Bray's scintillation cocktail (Bray, 1964). The quenching factor for each sample was individually determined by counting each sample a second time after the addition from a Hamilton micropipette of a carefully measured small volume of either ^3H or %-labeled solute (to match that of the sample), containing 5 to 10 times more counts than thd original sample. An appropriate quenching correction for each sample was then made on the basis of the ratio of counts given by the same volume of additional radioactive material added to the sample extract and that added to the supernatant solution.

The amount of probe solute in both the samples and final supernatant solution or solvent (e.g., water + urea; water + n-propanol) were expressed in molality (i.e., moles of solutes per 1000 ml. of water or solvent, e.g., water + urea; water + n-propanol).

It should be pointed out that whenever the amount of an incubation solution is measured by volume (rather than by weight), while its water content is determined by *weight difference* of the sample before and after drying and expressed as *weight* percentage, the initial sample volume must be converted to a weight basis (by multiplying the volume by the density of the sample) for the accurate determination of p-values.

TABLE V. A comparison of the p-values of glycine in 12 native + urea denatured proteins (.25°C). See legends of Table IV for explanation of figures noted in this table.

	$\rho_{glycine}$		
	Native	Urea-denatured	P
Albumin (serum)	0.971 ± 0.022 (11,3;76;5.3)	0.787 ± 0.006 (16,4;39;6.9)	< 0.001
Albumin (egg)	0.926 ± 0.006 (32,5;67,4.8)	0.792 ± 0.007 (36,6;38;6.8)	< 0.001
α-chymotrypsinogen	0.991 ± 0.004 (12,3;61;4.3)	0.831 ± 0.021 (12,3;41;6.5)	< 0.001
Edestin	0.922 ± 0.013 (8,2;48;5.6)	0.892 ± 0.007 (12,3;41;7.1)	< 0.001
Fibrinogen	0.957 ± 0.014 (8,2;67;7.1)	0.837 ± 0.014 (12,3;40;8.0)	< 0.001
y-globulin	0.897 ± 0.011 (20,4;66;6.3)	0.757 ± 0.008 (24,6;39;7.4)	< 0.001
Hemoglobin	0.977 ± 0.816 (8,2;70;-)	0.728 ± 0.015 (19,3;39;6.9)	< 0.001
Histone	1.070 ± 0.025 (8,2;68;7.7)	0.929 ± 0.020 (12,3;44;7.9)	< 0.001
β-lactoglobulin	0.924 ± 0.024 (20,5;69;5.7)	0.691 ± 0.010 (36,9;38;6.9)	< 0.001
Lysozyme	0.025 ± 0.017 (8,2;60;5.6)	0.948 ± 0.014 (16,4;43;7.2)	0.005 > P > 0.001
Pepsin	0.992 ± 0.023 (11,3;70;4.6)	0.933 ± 0.009 (±6,4;46;5.3)	0.1 > P > 0.05
Protamine	1.191 f0.013 (8,2;91;12.2)	0.991 ± 0.007 (16,4;49;9.7)	> 0.001
Mean ± S.E.	0.987 ± 0.04 ± (12)	0.843 ± 0.026 (12)	

Calculation of the apparent equilibrium distribution coefficients. As mentioned earlier, the ultimate goal of the present study was to determine accurately the true equilibrium distribution coefficients or q-value of various probe solutes in protein soluions. However, from the experiments carried out and described in this paper, we could only determine the apparent equilibrium distribution coeficient or p-values. Anticipating results described in a sister publication of the series (Ling and Hu, 1988), we may mention that there is evidence that the p-values obtained here are in most cases equal to or at least close to the true equilibrium distribution coefficients or q-values.

A q-value or p-value equals the ratio of the molar concentration of the probe solute in the water (or solvent) in the sac over that in the bathing solution. However, due to the presence of large quantities of proteins in the sac, it is impractical to measure the molar concentration of the probe solutes in the sac content. To overcome this difficulty, we first determined the molal concentration (i.e., moles per kilogram of water or solvent) of the probe solute and then obtained the p-value after converting the molal concentration of the probe into its molar concentrations. Since the conversion factor from molal to molar concentration does not depart very far from a constant, one can usually obtain the p-value directly by calculating the ratio of the molal concentration of probe solute in the sac content and that in the external solution where the constant conversion factor could then cancel out.

RESULTS

The p-values of glycine, sucrose, and urea in solutions of native and urea-denatured proteins. Tables IV, V and VI summarize the extensive experimental data we collected in

TABLE VI. A comparison of the ρ-value of sucrose and the ρ-value of urea in the same solutions of (10) urea-denatured proteins (25° C). For explanations of the significance of the figures cited see Legend of Table IV. The ρ-values for urea and for sucrose were obtained from the same samples in the same experiment where ^{3}H labeled urea and ^{14}C labeled sucrose (or vice versa) were added to the bathing solutions. The ρ-values were calculated from the ratios of the concentrations in the sac contents and in the external bathing solution at the conclusion of the experiment. Data show very marked lowering of the ρ-values for the larger sucrose occurring at the same time with a near unity ρ-value for the smaller urea, which apparently can fit into the dynamic water structure.

	ρ_{urea}	$\rho_{sucrose}$
Albumin (serum)	1.02 ± 0.010 (20,5;41;7.0)	0.697 ± 0.010
Albumin (egg)	1.00 ± 0.015 (24,4;40,6.9)	0.710 ± 0.010
α-chymotrypsinogen	1.02 ± 0.030 (12,3;44;6.7)	0.707 ± 0.012
Edestin	1.00 ± 0.013 (8,2;41;6.7)	0.759 ± 0.007
Fibrinogen	0.964 ± 0.012 (12,3;43;8.1)	0.771 ± 0.014
y-globulin	1.05 ± 0.015 (14,3;40;7.8)	0.660 ± 0.013
Hemoglobin	0.984 ± 0.008 (32,8;41;6.9)	0.685 ± 0.020
β-lactoglobulin	0.972 ± 0.010 (20,5;40;7.2)	0.670 ± 0.010
Lysozyme	1.01 ± 0.014 (12,3;43;7.3)	0.855 ± 0.015
Trypsin	1.04 ± 0.022 (12,2;47;5.7)	0.809 ± 0.016
Mean ± S.E.	1.006 ± 0.008 (10)	0.732 ± 0.019 (10)

our studies of the effects of some 10 to 14 native proteins and urea-denatured proteins upon the p-values of sucrose, glycine and urea in the water (or solvent) phase of the protein solutions. The parentheses following each set of [means ± standard errors of the means (S.E.)] are from left to right: the total number of individual assays, the total number of sets of experiments performed; the average percentage (X 100) of water in the protein solutions; and the pH of the external bathing solutions at the conclusion of the experiments.

The data presented shows that exposure to 9 M urea reduces very significantly the ρ-value of both glycine and sucrose in the water (or solvent) phase of the protein solutions (Tables IV and V). The exceptions are pepsin and trypsin inhibitor. In these cases, the reduction of the p-values was of marginal significance. The insensitivity of pepsin to urea denaturation is well known (Putnam, 1953, p. 858).

In solutions of the 10 ureadenatured proteins, the ρ-value of urea itself averages 1.006, thus essentially unchanged following urea denaturation, while the average p-values for sucrose has fallen to 0.732 (Table VI).

These data affirm the first two predictions of the Polarized Multilayer theory mentioned above: (1) native proteins exercise a minimal effect on the p-value of the larger solutes, glycine and sucrose; (2) in the water (or solvent) phase of urea-denatured proteins, both glycine (M.W. 75.05) and sucrose (M.W. 342.30) are excluded and in degree following the size rule. Urea, being the smallest with a M.W. of 60.06 has the highest p-value.

As mentioned above, Table IV, V and VI present overall average ***p-values*** obtained with the aid of several different techniques. Since it is impractical to present all the data from all the 10 to 14 proteins studied, we choose to provide some idea of the scope of all the study conducted, by presenting all the data collected on a single protein, β-lactoglobulin. This protein is also the one we studied most thoroughly (Table VII). Note for example, in the evaluation of the water (or solvent) contents of the samples, all 3 methods described earlier were used: drying at 80°C (A); THO method (B); and weighing method (C), with consistent results.

The overall average observed p-values for glycine and sucrose in solutions of native β-lactoglobulin (0.942, 0.914 respectively) and in solutions of urea-denatured β-lactoglobulin (0.691, 0.670) given in Table VII are by and large in accord with the average of all 12 to 14 proteins described in Tables IV and V. But there are also individual differences. For example, the p-values for glycine and sucrose, in solutions of native and urea-denatured proteins are much closer together in the case of β-lactoglobulin (see Table VII and VIII below) than in that of egg albumin (Table III) and many other proteins (Tables IV and V).

In addition, the ***p-value*** of 0.972 for urea in solution of urea denatured β-lactoglobulin is close to but also somewhat lower than the grand average of 1.006 for all ten urea-denatured proteins studied and described in Table VI.

An important finding mentioned above is the substantial lowering of the p-values for glycine and sucrose following urea denaturation and a lack of change for the p-value for urea itself in the same solutions.

Since small molecules as a rule diffuse faster through the dialysis tubing wall, and since urea reduces the rate of solute passage out of the dialysis tubing as our data presented in Table II has shown, one asks, could the observed differences in the p-value merely reflect a systematic and reproducible error from the failure to reach diffusion equilibrium for the larger solute molecules, its degree increasing with the increasing molecular weights under study? The answer is, No.

There are at least two types of evidence against such an interpretation. Firstly, the time for 99% exchange of the largest labeled

TABLE VII. A tabulation of the kind of detailed data surrounding the acquisition of the ρ-values for urea, glycine and sucrose obtained in the case of a single illustrative protein, β-lacto-globulin. This table is presented to illustrate the extensive efforts behind each set of mean ± S.E. numbers cited for each protein in the preceding three tables. Some minor discrepancies between number of assays cited here and those cited in Tables IV, V and VI come from the fact that all data collected were used in the averages found in Tables IV, V and VI while for various minor reasons

β-lacto-globulin

Expt. NO.	Sample NO.	Native						
		ρ_{gly}	$\rho_{sucrose}$			H_2O Content		pH
		A	A	B	C	A	B	
P16	C1	0.930	0.937			64.3		
	c2	0.942	0.935			64.3		
	C3	0.919	0.919			64.3		
	C4	0.950	0.955			64.3		
P24	P1	0.902	0.936			65.7		5.7
	P2	0.905	0.802			68.2		5.7
	P3	0.965	0.857			68.4		5.7
	P4	1.131	0.828			67.7		5.7
P42	1a							
	1b							
	1c							
	1d							
P49	9a	0.917	0.934			64.9		5.6
	9b	0.917	0.936			65.1		5.6
	9c	0.905	0.962			65.0		5.6
	9d	0.92,	0 927			64.9		5.6
P50	13a							
	13b							
	13c							
	13d							
P51	16a		0.930					5.55
	16b		1.088					5.55
	16c		0.982					5.55
	16d		0.957					5.55
P57	41a		0.911	0.882		67.7	71.5	5.7
	41b		0.912	0.889		67.7	69.6	5.7
	41c		0.907	0.890		67.4	68.8	5.7
	41d		0.894	0.864		67.3	70.0	5.7
P59	9A							
	9B							
	9C							
	9D							
P60	125A		0.935		0.878	75.66	77.90‡	5.55
	125B		0.936		0.878	78.25	81.28‡	5.55
	125C		0.992		0.935	86.10	88.35‡	5.55
	125D		0.935		0.886	72.57	74.05‡	5.55
P65	A1		0.855					5.6
	A2		0.853					5.6
	A3		0.880					5.6
	A4		0.883					5.6
P14	G1							
	G2							
	G3							
Mean + S.E.		0.942 ±0.031	0.914 ± 0.010					

some of the data are not presented here in this illustrative table. A. B. C. in the table refer to the three different methods used for the assay of water content: A, the drying method; B, the THO method; C, the weight change method. For an explanation of lower water content for the urea denatured contents see Legend of Table IV. *indicates total solvent content (water + urea).

β-lacto-globulin

			Urea Denatured					
ρ_{gly}	$\rho_{sucrose}$		ρ_{urea}			H_2O (or Solvent Content)		pH
A	A	C	A	B	C	A	C	
0.735	0.590					34.4		
0.706	0.598					34.4		
0.653	0.531					34.4		
0.661	0.546					34.4		
0.640	0.627					36.4		6.9
0.633	0.615					36.4		6.9
0.639	0.625					36.4		6.9
0.672	0.630					36.7		6.9
0.593			0.815			44.9		7.0
0.717			0.968			37.6		7.0
0.713			0.975			37.9		7.0
0.709			0.98"			38.0		7.0
0.729	0.636					38.0		7.0
0.749	0.651					38.0		7.0
0.781	0.684					37.9		7.0
			1.027	1.009		35.6		7.05
			0.945	1.005		36.4		7.05
			0.973	0.998		36.8		7.05
			0.990	0.983		36.4		7.05
	0.659							6.8
	0.663							6.8
	0.717							6.8
	0.686							6.8
	0.668					37.7		7.0
	0.676					35.8		7.0
	0.658					36.9		7.0
	0.695					36.8		7.0
	0.701	0.697	0.966		0.950	40.8	76.5*	7.4
	0.728	0.712	0.983		0.950	41.0	77.9*	7.4
	0.758	0.767	0.980		0.983	44.4	81.7*	7.4
	0.759	0.750	0.995		0.971	42.2	79.5*	7.4
0.713	0.666					36.9		6.75
0.700	0.674					38.7		6.75
0.693	0.644					40.1		6.75
0.698	0.679					37.2		6.75
0.691 ±0.045	0.670 ± 0.056		0.972 ± 0.041					

solute under study, sucrose (a. 50 hours) is shorter than the duration of incubation chosen (i.e., 72 hours). The second type of evidence is derived from an experiment documented in Table VIII. In this experiment, we prepared and incubated two sets of sacs in as closely similar a manner as possible. In one set, the radioactively labeled glycine (or sucrose) was added to the inside of the sac, while in the companion set, the radioactively labeled solute was added only to the external solution. After 72 hours of incubation, the radioactivities in both sac content and in the external solution were analyzed and compared. As shown in Table VIII, there is no significant difference between the two sets of values. Thus diffusion equilibrium has been reached and failure to reach diffusion equilibrium was not the cause of the observed differences in the levels of urea, glycine and sucrose reached.

Note also that in this series of studies (Table VIII), the indifference of pepsin to urea denaturation was observed once more.

The effect of urea denaturation on the ρ-value of Na^+ (as sulfate) in solutions of 8 proteins. As shown in Table IX, urea denaturation significantly reduced the p-values of Na^+ (sulfate) in 7 of the 8 proteins studied*. Tbe lone exception is α-chymotrypsinogen.

Although the overall average p-value of Na^+ (sulfate) for 8 native proteins, 0.964, is not too far from unity, it would probably be higher, had it not been for the low p-values for α-chymotrypsinogen, already mentioned, and for lysozyme.

In earlier reports, Ling and coworkers reported p-values of Na^+ of solutions of 13 native proteins. The p-values were also very

*It should be mentioned that the atomic weight of Na^+, at approximately 23, is relatively low. However, Na^+ does not exist as such in aqueous solutions. Rather. it exists as highly hydrated ions. Coated in a layer of tightly held wafer molecules, the hydrated Na^+ is in fact a de facto multiatomic entity, and as such behaves like the multiatomic molecules, glycine and sucrose. It is therefore not surprising that Na^+ is partially excluded by water in the presence of urea-denatured proteins.

TABLE VIII. Demonstration that similar ρ-values for glycine and for sucrose were obtained regardless of whether the labeled glycine or sucrose was added initially to the inside or to the outside of the sacs (25°C). These data offer further affirmation that the ρ-value obtained were truly (apparent) equilibrium distribution coefficients.

	$\rho_{glycine}$		$\rho_{sucrose}$	
	Added Inside	Added outside	Added Inside	Added Outside
Albumin (serum)	0.786 ± 0.017	0.787 ± 0.006	0.759 ± 0.014	0.697 ± 0.010
Albumin (egg)	0.819 ± 0.009	0.792 ± 0.007	0.749 ± 0.011	0.710 ± 0.010
α-chymotrypsinogen	0.913 ± 0.044	0.831 ± 0.021	0.776 ± 0.036	0.707 ± 0.012
Edestin	0.811 ± 0.007	0.892 ± 0.007	0.70 ± 0.012	0.759 ± 0.007
Fibrinogen	0.768 ± 0.007	0.837 ± 0.014	0.723 ± 0.008	0.771 ± 0.014
y-globulin	0.781 ± 0.007	0.757 ± 0.008	0.691 ± 0.005	0.660 ± 0.013
β-lacto-globulin	0.70, ± 0.005	0.691 ± 0.010	0.666 ± 0.008	0.658 ± 0.015
Pepsin	0.935 ± 0.004	0.933 ± 0.008	0.908 ± 0.007	0.918 ± 0.012
Mean ± S.E.	0.814 ± 0.026	0.815 ± 0.029	0.748 ± 0.029	0.735 ± 0.032

close to unity but more consistently so (Ling et al., 1980a). A major difference between the two sets of experiments lay in the protein concentrations studied: in the earlier report, the initial protein concentration was 20%, the final concentration being even lower. In the present series, the initial concentration was often as high as 40%, the final concentration being often close to 30%, as indicated by the last figure in the parentheses following each set of mean ± S.E.

The p-value of sucrose in the water (or solvent) phase of solutions of 9 proteins denatured by guanidine HCl, sodium dodecyl sulfate (SDS) and n-propanol. In Table X, the first row under the heading "H_2O" refers to solutions of native proteins. The second row refers to solutions of proteins denatured by guanidine HCl (GuHCl), etc. The data show that exposure to GuHCl consistently lowers the p-value for sucrose in all 9 proteins studied. On the other hand, minor lowering or no lowering at all of p-values was observed in response to denaturation with either SDS or n-propanol. These results also confirm predictions described above.

DISCUSSION

The basic unit of all life is the living cell. There are two opposing theories of the living cell: the membrane (pump) theory and what one may call, collectively, the protoplasmic theories. To understand and correctly assess the significance of the findings described in this paper and in several other recent publications from this laboratory to be discussed, it is important to know the true current status of these two diametrically opposed theories. It is only in the protoplasmic theories that the physical state of cell water is a matter of central importance.

In the membrane-pump theory, the living cell is in essence a sackful of a dilute solution. In this view, water in living cells is just normal liquid water. The cell's unique chemical composition is delegated to the continual operation of a battery of pumps postulated to exist in all cell membranes.

TABLE IX. The ρ-values of labeled Na^+ (as sulfate) in solution of eight native and urea-denatured proteins (25° C). Numbers in parenthesis following each set of mean ± S.E. were from left to right: total number of independent assays; the number of sets of experiments performed; and the water content of the sac contents at the conclusion of the experiment. The cited lower water contents of urea-denatured proteins solutions were due to presence of large amount of urea as part of the solvent.

P represents the level of significance between the mean p-values of native and urea-denatured proteins. First number in the parenthesis gives the sample size(N).

	Native	Urea-denatured	P
Albumin (egg)	1.090 ± 0.017 (9;3;80)	0.999 ± 0.005 (4;1;43)	.005 > P > .001
α-Chymotrypsinogen	0.883 ± 0.010 (7;2;65)	0.811 ± 0.013 (4;1;45)	.10 > P > .05
Fibrinogen	0.933 ± 0.018 (3;1;68)	0.780 ± 0.003 (4;1;46)	< 0.001
γ-Globulin	0.929 ± 0.016 (6;2;77)	0.792 ± -.003 (4;1;43)	< 0.001
Hemoglobin	0.94 ± 0.016 (8;2;73)	0.768 ± 0.005 (4;1;45)	< 0.001
β-Lactoglobulin	1.084 ± 0.035 (7;2;69)	0.941 ± 0.009 (4;1;43)	0.01 > P > .02
Lysozyme	0.872 ± 0.007 (4;1;71)	0.775 ± 0.013 (4;1;45)	0.005 > P > .001
Protamine	0.975 ± 0.023 (11;2;89)	0.79 ± 0.012 (4;1;59)	0.005 > P > .001
Mean ± S.E.	0.964 ± 0.028 (8)	0.832 ± 0.029 (8)	

TABLE X. The effects of 3M guanidine HCl, 0.1 M sodium dodecylsulfate (SDS) and 2M n-propanol upon the ρ-values of sucrose in solutions of nine proteins.

For the majority of experiments, the mean and standard errors (S.E.) were from four independent assays in one set of experiments. However, in other cases the values were based on more assays and sets of experiments. For all samples of proteins treated with n-propanol, the total H_2O content is not merely water but the entire solvent, i.e., water plus n-propanol. For all other samples H_2O contents refer to the contents of water only. The low water contents of guanidine HCl experiments, like those of urea experiments, are due to the large amount of GuHCl present.

P represents the level of significance between the control (H_2O) and protein treated with denaturants. N is 4 in most cases.

		$\rho_{sucrose}$	H_2O Content* (%)	pH	P
Albumin (serum)	H_2O	0.946 ± 0.015	72.07 ± 0.012	5.2	
	G". HCl	0.607 ± 0.030	38.01 ± 0.45	5.6	> 0.001
Albumin (egg)	H_2O	0.877 ± 0.013	68.50 ± 0.38	5.8	
	Gu. HCl	0.569 ± 0.004	37.82 ± 0.086	6.1	< < 0.001
	SDS	0.943 ± 0.014	67.31 ± 0.26	6.4	0.01 > P > 0.005
	n-propanol	0.813 ± 0.009	61.01 ± 0.24	6.4	0.005 > P > 0.001
α-chymo-trypsinogen	H_2O	0.900 ± 0.013	59.46 ± 0.045	3.45	
	Gu. HCl	0.718 ± 0.015	41.56 ± 0.660	4.55	< 0.001
	SDS	0.847 ± 0.009	52.39 ± 0.605	3.90	0.025 > P > 0.02
	n-propanol	0.878 ± 0.002	51.55 ± 0.154	4.25	0.27 > P > 0.1
Edestin	H_2O	0.833 ± 0.030	50.58 ± 0.98	5.55	
	Urea	0.64, ± 0.016	41.26 ± 0.23	7.2	< 0.001
	Gu. HCl	0.641 ± 0.006	39.96 ± 0.044	6.2	< 0.001
	SDS	0.823 ± 0.002	46.28 ± 0.390	6.3	0.8 > P > 0.7
	n-propanol	0.820 ± 0.005	50.44 ± 0.610	6.0	0.7 > P > 0.6
Fibrinogen	H_2O	0.856 ± 0.01 I	66.85 ± 0.080	7.3	
	Gu. HCl	0.650 ± 0.004	39.62 ± 0.010	5.3	< < 0.001
	SDS	0.893 ± 0.007	58.92 ± 0.270	5.4	0.01 > P > 0.005
	n-propanol	0.874 ± 0.006	61.31 ± 0.075	5.2	0.4 > P > 0.3
y-globulin	H_2O	0.907 ± 0.009	65.66 ± 0.150	7.1	
	Gu. HCl	0.620 ± 0.01 I	38.88 ± 0.240	7.0	< < 0.001
	SDS	0.852 ± 0.008	59.94 ± 0.140	7.5	0.02 > P > 0.01
	n-propanol	0.866 ± 0.008	58.91 ± 0.360	7.1	0.1 > P > 0.05
Hemoglobin	H_2O	0.824 ± 0.018	68.47 ± 0.069	4.8	
	G". HCl	0.634 ± 0.002	41.58 ± 0.100	5.8	< < 0.001
	SDS	0.843 ± 0.012	63.56 ± 0.260	5.5	0.5 > P > 0.4
	n-propanol	0.743 ± 0.005	51.74 ± 0.17	5.5	0.005 > P > 0.001
β-lacto-globulin	H_2O	0.872 ± 0.008	68.80 ± 0.24	5.6	
	Gu. HCl	0.632 ± 0.014	39.11 f0.19	5.1	< < 0.001
	SDS	0.930 ± 0.020	66.27 ± 0.44	6.0	0.02 > P > 0.01
	n-propanol	0.864 ± 0.015	63.05 ± 0.37	5.7	0.5 > P > 0.4
Lysozyme	H_2O	0.992 ± 0.005	63.67 ± 0.015	5.0	
	Gu. HCl	0.635 ± 0.001	39.62 ± 0.010	5.3	< < 0.001
	SDS	0.929 ± 0.008	58.92 ± 0.270	5.6	0.005 > P > 0.001
	n-propanol	1.004 ± 0.013	61.31 ± 0.075	5.2	0.7 > P > 0.6

Proponents of the membrane pump theory have not been able to rise to the challenge of the extensive and unequivocal evidence against the theory, which have been steadily gathering over more than a quarter of a century. (For documentation of this evidence see: monograph. Ling, 1984; review articles. Ling, 1952, 1960, 1965b, 1969, 1977a, 1981a, 1986, 1988, Ling and Negendank, 1980, Ling et al., 1973; Hazelwood, 1976, Edelmann, 1986. Key original articles include: Ling, 1965a, 1973, 1977b, 1978, 1980, 1981b, 1981c, Ling and Bohr, 1970, Ling and Negendank, 1970, Ling and Ochsenfeld, 1966, 1973, Ling and Walton, 1976, Ling et al., 1967).

Among the decisive evidence against the membrane pump theory are: (1) grossly insufficient energy to operate just one pump (the sodium pump at the plasma membrane), while many more pumps are required both at the plasma membrane and at the membranes of subcellular particles; (2) cells with intact and functional cell membrane (and hence intact postulated pumps) but without cytoplasm fail to pump Na^+ and K^+ against concentration gradients (see Ling and Negendank, 1980), while cells without a functional cell membrane and (hence postulated pumps) but with intact cytoplasm continue to function like normal cells in maintaining the low levels of some solutes (e.g., Nd) and high levels of others (e.g., K^+), as seen in normal cells (Ling, 1978).

The resistance to new ideas is all too easily understandable. However, poor understanding of history is unquestionably another contributing factor to the lack of enthusiasm for and interests in new ideas and new findings. It is for this reason that an historical background of the new findings presented here and elsewhere is given below.

History of the protoplasmic theories. The protoplasmic theories originated from early recognition that living matter or protoplasm is gelatin-like or colloidal, an adjective derived from the Greek word, κολλα meaning glue or gelatin (Graham, 1861).

Martin Fischer defined colloids in these words: "Colloid systems result wherever one material is divided into a second with a degree of subdivision coarser than molecules" (see Gortner, 1938). Gortner further modified Fischer's definition: "Colloidal systems result wherever one material is divided into a second with a degree of subdivisions either (a) coarser than molecules or (b) where the micelles exceed 1-15 millimicra in diameter," as illustrated in his diagram below (Gortner, 1938, p. 5).

Molecules and Ions	Colloids	Matter in Mass
not visible in ultramicroscope	visible in ultramicroscope	visible in microscope
	1 mμ 0.1μ	

Note that in their times, only small molecules are called molecules. Unfortunately, the emphasis on particle size in defining colloids might have inadvertently played a role in the decline of colloid chemistry. Thus, with the advent of macromolecular chemistry, colloidal chemistry with no better definition that the larger particle size, soon began to lose its identity. Yet to those who know colloid chemistry, it is obvious that colloid chemistry and macromolecular chemistry are not synonymous. What then is the *essence* of colloids that has not been recognized, and spelled out?

This essence is intimately associated with the namesake of colloids, gelatin — an insight that Graham recognized more than 100 years ago, an insight that we have further elaborated on, and in the Process envisaged a new definition of colloids (see below).

Although the particle-size definition of colloids provided no clear-cut connections between colloids so defined and gelatin, Gortner, among others, was eminently aware

of the special ability of gelatin to bind water (Gortner, 1938, p. 278). The water so bound was at that time called "bound water". Two characteristics of "bound water" were stressed: bound water does not act as a solvent for dissolved solutes and is therefore "nonsolvent"; bound water does not freeze at temperatures below the normal freezing point of liquid water and is therefore "nonfreezing".

Based on these assumptions, a number of experimental methods were devised to measure the amount of "bound water" in biological fluids including the living cells. Among these methods are the "cryoscopic method" of Newton and Gortner (Newton and Gortner, 1922, Gortner and Gortner, 1934) and the "calorimetric method" of Rubner (Rubner, 1922, Thoenes, 1925). In the cryoscopic method, a measured amount of sucrose is added to a biological fluid of known weight and the freezing point of the fluid measured. Lowering of the freezing point beyond what would be expected if all the water in the sample is normal liquid water provides the data basis for the estimation of the "non-solvent" bound water. With this method, Newton and Martin (1930) found that from 0.96 to 2.05 grams of water is bound to 1 gram of gelatin.

In the calorimetric method, the biological material, including living tissues, is cooled to -20" or -30°C and held at that temperature until all free water is frozen. Since in the melting of 1 gram of water, 80 calories of heat is absorbed, the amount of heat needed to bring the sample back to a temperature above 0°C permits the calculation of the amount of bound water which was not frozen. Using this method Thoenes (1925) found that from 1.86 to 2.14 grams of water is bound to 1 gram of dry gelatin. Similar amounts of water per gram of dry matter were found to exist in muscle tissues, equivalent to some 20 to 25% of the total muscle tissue water.

The conclusions derived from these studies were seriously challenged. Blanchard (1940) pointed out that pure water could be supercooled to −20°C or even lower temperatures without freezing, and that the presence of high concentrations of proteins in living cells delays the onset and propagation of ice formation, thereby making it inherently impossible to assess the physical state of water in living cells. However, Blanchard was apparently not aware of other important experimental data already available then (and to be described below) that dampens his criticism.

The attacks on the conclusion from the cryoscopic studies was at that time far more difficult to rebut. Thus MacLeod and Ponder (1936) showed that ethylene glycol distributes equally between water in red blood cells and the surrounding medium. A. V. Hill, then at the height of his fame and influence, showed that urea distributes equally between water in frog muscle and the bathing Ringer solution (Hill, 1930). Soon afterward the whole colloidal and protoplasmic approach to cell physiology apparently came to a halt.

Yet, there was no question that even then there was enough valid information to refute at least Blanchard's criticism. In 1926, Moran had shown that a 65% gelatin could not be frozen at temperatures as low as that of liquid air (ca. -190°C) (Moran, 1926). There was absolutely no way for normal liquid water to be supercooled to this temperature.

The apparent triumph of the proponents of the membrane (pump) theory was short-lived. New evidence against this theory soon made it no longer tenable (see above). To present the reader with an overview panorama of the growing and waning of major theories of the living cell, we return to the fundamentals.

The association-induction hypothesis and its subsidiary hypotheses. Hydrated Na^+ as a rule, exists at a concentration in the cell water only a fraction of that in the external medium (Ling, 1962, p. 217). This type of phenomena has far reaching significance in the very existence of the living cells, which are at once continuous and separate from their environ-

ments. In general terms, there are only three kinds of mechanisms to account for lasting differences in the concentrations of a substance in two contiguous spaces:

MECHANISM 1, an insurmountable energy barrier (e.g., an absolutely impermeable membrane prevents the traffic of Na^+ between the two spaces);

MECHANISM 2, a continually operative energy consuming pumping (e.g., the sodium pump);

MECHANISM 3, a different physiocochemical environment in one space, which at equilibrium accommodates a lower concentration of substance than in the adjoining space.

Let us now examine how well each of these theoretical mechanisms has survived experimental testings.

Radioactive tracer studies in the late 1930's and early 1940's showed that there is no insurmountable barrier to the traffic of Na^+ across the cell surface, thereby disproving Mechanism 1. Profound energy difficulties described above ruled out Mechanism 2. The only remaining viable mechanism is Mechanism 3. The polarized multilayer theory of cell water and its subsidiary solute exclusion theory belong to this category and they are both part of a more comprehensive theory of the living cell, called the association-induction (AI) hypothesis (Ling, 1962, 1969, 1984).

What kind of physicochemical environment can there be within the living cells so that it will, without spending energy continually, keep intracellular solutes like Na^+ at a much lower concentration than in the surrounding medium? It seems that we have not many alternative choices: such a physicochemical environment can only be provided by the premier solvent of all living cells: water.

In most living cells, water is by far the largest component, making up, very frequently, 80% of the cell weight. A standing concentration of Na^+ in the cell water at 15% of that in the external medium — a very common phenomenon — means that at least 100% − 15% = 85% of the cell water must be quite different from normal liquid water in terms of its solubility for solutes like Na^+. However, if indeed only 85% of the cell water is different from normal liquid water, the remaining 15% being normal liquid water, then this 85% of cell water must have absolutely no solubility for Nd, not a likely conjuncture. More likely this 85% of cell water has some solvency for Na^+. In this case, more than 85% or virtually all of the cell water must be different from normal liquid water.

At ambient temperature and atmospheric pressure, cell water cannot, of its own accord, assume different physicochemical properties describing all other water in this universe under the same conditions. That the water in living cells is, nevertheless, quite different can only be the consequence of its being under the influence of some other component present in the living cells.

Furthermore, the cell's ability to partially exclude Na^+ and other solutes is not a transient phenomenon but a long lasting one. This permanence, in turn, requires that virtually all the cell water must be under the *lasting* influence of some other components of the cell. What can that other component be?

To search for this water-influencing component, let us consider the mature human red blood cells, which like many other cell types contain also a low level (16 mM) of Na^+ in its cell water. The blood plasma, in which the red blood cells spend their lives, contains Na^+ at a concentration nearly 10 times higher (150 mM). (Ponder, 1948, p. 120). The mature human red blood cells contain virtually no RNA or DNA. Therefore, the only non-water component that is found in the red blood cells at a high enough concentration to control and modify the properties of the enormous number of intercellular water molecules can be nothing else than the proteins. Or more specifically, hemoglobin, which makes

up 97% of the total intracellular proteins of the mature human red blood cells.

Yet it is known that water in a solution of native hemoglobin does not exclude Na^+ at all (Ling et al., 1980a). One is thus forced to conclude that the intracellular hemoglobin that alters the solubility of the bulk of cell water must be profoundly different from the native hemoglobin studied. How can one hemoglobin molecule be profoundly different from another hemoglobin molecule? It seems that there are again not too many alternatives. The only choice is that the hemoglobin in the whole red blood cells must assume a different *conformation* than those in the (native) hemoglobin studied. To determine whether a non-native conformation confers on a protein the ability of changing the solvency property of water for Na^+, we return to some early work Liig and hi coworkers reported in 1980 (Ling, et al., 1980a).

These authors found that at an initial concentration of 20%, 12 native proteins and one polysaccharide (chondroitin sulfate) exercise no influence on the solubility of water for Na^+ (sulfate). In contrast, gelatin exercises strong influence on the water, reducing its solubility for Na_2SO_4. Indeed our observation reported in 1980 was by no means the first time this observation had been made. In 1934, Hollemann, Bungenberg de Jong and Moddermann observed and reported the same phenomenon.

What then is the fundamental difference between the native proteins and gelatin?

First of all, gelatin is not a native protein, it is *denatured* collagen. This fact already indicates that being *denatured* sets gelatin apart from the other 12 proteins studied. But this is not the only difference. Gelatin, unlike many other denatured proteins, *remains* in the same denatured conformation long after the agent that caused the denaturation has been removed and the gelatin restored to a normal environment. What then causes gelatin to stay denatured in this normal environment which by itself does not cause denaturation?

The answer lies in the highly unusual amino acid composition of this protein, including proline (13.6%), hydroxyproline (8.5%), and glycine (32.6%). (Veis, 1964, p. 142). Each of these three amino acids conspires with the other two in keeping the gelatin molecule permanently in the denatured state. Both proline and hydroxyproline lack the hydrogen atom on their peptide nitrogen atoms to form H-bonds in a-helical or β-pleated — sheet conformation. Glycine, on the other hand, is a strong "helix-breaker" (Chou and Fasman, 1974). With a large percentage of its amino acid residues unable or indisposed to form a-helix and other secondary structure typical of all native proteins, a major part of the gelatin molecule must remain irreversibly in the *fully extended conformation* with its backbone carbonyl groups (and some imino groups) directly exposed to the bulk phase water.

The general conclusion drawn from the comparison of the Na^+ exclusion behaviors of solutions of the 12 native proteins and that of gelatin is that in order for a protein to exercise a strong influence on the solvency of the bulk phase water, that protein must exist in the fully extended conformation. We shall next examine some other experimental findings that offer insights linking the fully extended conformation to water.

In trying to understand the seats of hydration of proteins, Ling discovered in 1972 an intriguing dichotomy: scientists working in biochemical laboratories as a rule ascribe to the theory (the Pauling theory) that only polar side chains of a protein sorb water, whereas scientists working in industrial laboratories tend to believe in the alternative theory in which the backbone NHCO groups also sorb water (Jordan-Lloyd theory) (Ling, 1972, 1984).

A careful reading of their published papers

on the subject led to the revelation that those subscribing to the Pauling theory as a rule worked with pure globular, native proteins. Those subscribing to the Jordan-Lloyd theory worked primarily with fibrous proteins and synthetic polypeptides. Among the polypeptides studied is benzoylated polyglycine which does not contain any polar side chains but also sorbs water. Since fibrous proteins and synthetic polypeptides studied contain at least a major portion of their polypeptide chains in the fully extended conformation, Ling concluded that *polypeptide NHCO groups sorb water only when they exist in the fully extended conformation and ore thus directly exposed to the bulk phase water.* However, these data do not tell us whether a single layer or multilayers of water are adsorbed on the exposed NHCO groups. To find the answer to that question will he the objective of the next section.

*The natural tendency of water to exist as polarized multilayers in a suitable environ*ment. It is well known that due to its asymmetric geometry, a water molecule possesses a large permanent dipole moment (1.86 debye). In addition, it also has a high polarizability (1.44 X 10^{-24} cm), which endows the water molecule with the propensity to acquire an induced dipole when the molecule is found in the vicinity of an electric field. Such electric fields exist at the surface of a salt crystal (e.g., titanium dioxide) with its checkerboard of alternately positive (titanium) and negative (oxygen) sites on the crystal surface.

It was de Boer and Zwikker (1929) who first pointed out the significance of having such a checkerboard of altematingly positive and negative sites in producing stable polarized multilayers of gas molecules on the surface of the salt crystals. On theoretical grounds, Brunauer, Emmett and Teller (1938) then pointed out that only gaseous molecules (like water) possessing a permanent dipole moment can form deep and stable polarized multilayers on the positive (P) and negatively charged (N) sites on the surface of the crystals. It was Harkins who provided experimental conformations of these theories.

Harkins (1945) showed experimentally that no less than 5 layers of water molecules are polarized and adsorbed on the surface of titanium dioxide, thus leaving no question that water, with its large permanent dipole moment and strong polarizability, does indeed form deep polarized multilayers on suitable surfaces. A surface presenting a checkerboard of alternatingly positive P sites and negative N sites at suitable distances apart is referred to as a NP system (Ling, 1972). Two NP surfaces in close juxtaposition is called an NP-NP system.

Ling then pointed out that a matrix of fully extended linear chains bearing altematingly N and P sites at proper distances apart are fundamentally similar to an NP-NP system in its effect in polarizing multilayers of water and is given the name of an NP-NP-NP system. A solution of polyethylene oxide or PEO where each negatively charged oxygen site is flanked by two neutral(O) sites represents an NO-NO-NO system. It was also pointed out that only an NP-NP-NP system (or an equivalent NO-NO-NO or PO-PO-PO system) can influence the property of a large body of water, while the amount of water polarized by an NP-NP or NP systems is minute.

In the next section, we shall review the recent confirmation of the predictions of the existence of multilayers of water on the exposed NH and CO sites of gelatin and other model systems.

Experimental evidence of the adsorption of multilayers of water on the backbone NH and CO sites of gelatin. Using a newly developed method, which has made it possible for the first time to accurately study water sorption

at relative vapor pressure near saturation, Ling and Hu (1987) showed that at the relative vapor pressure of that of a normal frog Ringer solution (0.9966), gelatin sorbs about 300 grams of water per 100 grams of dry gelatin. Now an average amino acid residue weight in proteins is 112 (Ling, 1962, p. 48). 100 grams of dry gelatin therefore corresponds to 0.89 of an amino acid residue. The weight of water sorbed by one NHCO group in gelatin is therefore 300 X (112/100) = 336 grams, or 336/ 18 = 18.7 water molecules.

Part of this water sorbed is on polar side chains. In order to estimate the amount of water sorbed at the polypeptide NHCO groups only, one must know how much of the total water sorbed is on the polar side chains and then subtract it from the total water sorbed. Fortunately, we have the data to make this calculation; they are discussed below.

(1) Native hemoglobin adsorbs at physiological vapor pressure about 100 grams of water per 100 grams of dry protein (Ling and Hu, 1987, p. 257), equivalent to 100 X (112/100)/18 = 6.2 water molecules per amino acid residue.

(2) The total percentage of the polar side chains (asp, glu, lys, arg, ser, thr) in hemoglobin is 34% (Haurowitz and Hardin, 1954, p. 290) and that in gelatin is 25% (Veis, 1964, p. 142). Thus there are more polar side chains in hemoglobin than in gelatin.

(3) If one assumes that all the backbone NHCO groups of native hemoglobin are locked in a-helical and other inter- and intramacromolecular H-bonds and thus unavailable for water sorption and if one further assumes that the same number of water molecules are adsorbed on the polar side chains in gelatin as in hemoglobin, one can then make a safe estimate of the water adsorbed on the backbone NHCO groups (only) in gelatin by subtracting from the total number of water sorbed per amino acid residue the number of water molecules sorbed on one amino acid residue by native hemoglobin at the same physiological vapor pressure: 18.7–6.2 = 12.5. On assuming that equal numbers of water molecules are adsorbed at the backbone NH groups and on the backbone CO groups, the average number of water molecules sorbed at each one of the backbone P and N sites is then 12.5/2 = 6.25. The number of water molecules between each pair of N and P sites on adjacent protein chains is twice as many, or 12.5 molecules. A 12.5 thick layer of polarized water between adjacent chains constitutes what one calls polarized multilayers.

Having demonstrated that at physiological vapor pressure, gelatin does indeed sorb and polarize multilayers of water molecules on the exposed NH and CO groups of its fully extended portion of polypeptide chains and recalling that water in a gelatin solution (or gel) does indeed exclude Na^+ (Ling et al., 1980a, Ling and Ochsenfeld, 1983), our next task is to establish that it is indeed the presence of the exposed NHCO groups of the fully extended part of the gelatin molecule that change the property of the bulk phase water so that it now excludes Na^+.

To achieve this goal, we need to demonstrate in some other non-gelatin model systems which show, as does gelatin, the possession of fully extended protein chains or the equivalents — similar adsorption of multilayers of water and similar exclusion of Na^+. In fact, both of these objectives have already been accomplished. Some have been reported elsewhere; others are, of course, reported here in the present paper.

(1) Multilayer adsorption of water on other model systems with fully extended NP-NP-NP and NO-NO-NO chains.

Linear oxygen-containing polymers like polyethylene oxide (PEO), polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) all containing properly spaced sequences of oxygen atoms (with their negatively charged lone pair electrons) are, like gelatin, also unable to form intramacromolecular H-bonds due to

the absence of proton-donating P sites on the absence of proton-donating P sites on the chains. Instead, the sites separating each pair of adjacent oxygen atoms are vacant or neutral sites. Ling and Hu (1987) have shown that these polymers adsorb even more water at physiological vapor pressure than gelatin (*300* grams of water per 100 grams of dry gelatin). Both PEO and PEG adsorb about 400 grams of water and PVP, 350 grams of water per 100 grams of dry polymer. There are, therefore, deeper layers of water between adjacent chains of these models than in the case of gelatin.

(2) Solute exclusion from water dominated by non-gelatin fully extended protein chains and other models systems.

Elsewhere we have demonstrated how PEO and PVP, like gelatin, exclude Na^+ (Ling, et al., 1980, Ling and Ochsenfeld, 1983). We have also shown that gelatin, PEO and NaOH-denatured hemoglobin exclude large molecules like sucrose (Ling and Hu, 1988). But it is in the present paper that we have left no doubt that proteins in general can be made to acquire the ability of reducing the solvency of water for large solutes like hydrated Na^+, sucrose and glycine, when the proteins are made to exist in the fully extended conformation.

In all these cases, Na^+, sucrose and glycine, all of which are found in low concentrations in the water of most living cells, were used as probes. Their exclusion from water containing urea- and GuHCl-denatured proteins, and their lack of exclusion from water containing either native proteins or proetins denatured by SDS and n-propanol complete the story which began with the difference observed between the solubility of Na salt in solutions of gelatin and of native proteins. This affirms once more that it is the fully extended protein chains that polarize water in multilayers and that in this water solutes are excluded in a sizedependent manner.

That the same protein solution can at once exhibit strongly reduced solubility for sucrose or glycine but no exclusion for urea points out clearly and unequivocally that there is no categorically "non-solvent" water unable to dissolve *any* solute. With this understanding, it is also clear that urea, ethylene glycol and even D-glucose may have near normal solubility, yet the water in which they are dissolved nonetheless exists in the fully polarized state. The experimental finding of MacLeod and Ponder, and of Hill, while offering convincing evidence against the "non-solvent" water concept, offer at the same time strong evidence not against, but for the polarized multilayer theory for solute exclusion. We shall now turn to the sister concept of "non-freezing" water.

On the question of freezing-point depression, Ling and Zhang found that native proteins in concentrations as high as 50% have no effect on either the freezing point, nor the rate of freezing of water in native protein solutions. This observed indifference of freezing point and freezing rate to such high concentrations of native protein has refuted the conclusion of Blanchard that the presence of proteins *mechanically* block the formation of ice and delay (indefinitely) the onset of freezing. Ling and Zhang's companion finding that in the presence of gelatin, PEO, PVP and PVME, there is profound, concentration-dependent lowering of freezing as well as the slowing in the rate of freezing point demonstrates that, by and large, the original findings of Rubner and Thoenes are not wrong. They deserve to be remembered as valuable contributors in the continued effort to understand the living phenomena.

The finding of Ling and Zhang also confirmed the essence of Moran's finding in 1924: at high concentration, gelatin cannot be frozen at extremely low temperature (i.e., −190° C).

Concluding remarks and redefining colloids. As mentioned earlier, the rejection of

the protoplasmic theory of the living cell and colloid chemistry has more than one cause. One major cause could be the failure to present a coherent theory of colloids. The polarized multilayer theory of cell water and model systems offers just such a theory.

Once we realized the profound difference between water in the presence of native proteins and water in the presence of proteins in the fully extended conformation, we began to undertake a systematic study on a variety of physicochemical attributes of water thus differently affected. From these studies it has become apparent that water existing in the state of polarized multilayers on one hand, exhibits a gamut of physicochemical properties strikingly different from that of normal liquid water, and on the other hand, bear strong resemblance to properties of the living cell. As examples, the following may be mentioned:

(1) The maintenance of low levels of Nd, sucrose and glycine in living cells is not due to metabolic pumps but due to the existence of the bulk of cell water in the state of polarized multilayers and the property of this water partially to exclude these solutes.

(2) The maintenance of a steady cell volume reflects the balance between two opposing tendencies: the tendency to collect and acquire more cell water (in order to build up deeper layers of water) and the tendency to lose water (in consequence of the lower level of solutes dissolved in the polarized water than in the surrounding medium).

(3) The osmotic activity of the living cells is primarily due to the reduction of the activity of the bulk phase water (since the major intracellular ions are in an adsorbed and hence osmotically inactive state).

From these examples, one sees clearly that living systems are indeed gelatin-like and, hence, by definition, colloidal. What colloidal chemists did not clearly see is that this fundamental similarity between gelatin and living systems reflects not so much the properties of the large colloidal materials themselves but the properties of water that the gelatin-like proteins in living cells have acted upon, restructured and altered. The tight and efficient control of water in a single living cell by certain protein molecules in the cell then set the stage for the ***switching*** of the cell water between the two sharply different physical states*, one having the properties of normal liquid water (or something similar to it) and the other having properties of water in the state of polarized multilayers.

The ability of water to exist in two states with profoundly different properties and its switching between these two alternative states reflects the different degrees of interaction with intracellular proteins, when these proteins themselves undergo all or no autocooperative changes of their conformation. This hierarchial relationship in turn permits the entire protoplasm to be controlled as a functional whole when the switching of the key proteins between its two alternative states is in turn directed by the adsorption or adsorption on key cardinal sites on the protein of small molecules, including ATP, Ca^{++}, drugs, hormones, transmitters, collectively called the ***cardinal adsorbents***. For a detailed description of the general theory on the subject the reader may consult a 1984 monograph (Ling, 1984), or another more up-to-date one in the process of being published (Ling, 1990). For more specific developments, see Ling and Fu, 1987, 1988.

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*The first suggestions that water in living cells might exist in two states: one free and the other bound, were by Balcar, Sansum and Woodyatt in 1919. These authors also suggested that there is an equilibrium between the free and bound state in the cell.

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