STUDIES ON THE PHYSICAL STATE OF WATER IN LIVING CELLS AND MODEL SYSTEMS. VIII. WATER VAPOR SORPTION ON PROTEINS AND OXYGEN-CONTAINING POLYMERS AT PHYSIOLOGICAL VAPOR PRESSURES: PRESENTING A NEW METHOD FOR THE STUDY OF VAPOR SORPTION AT CLOSE TO AND INCLUDING SATURATION

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The three major components of all living cells are water, proteins, and ions. When mature, healthy and at rest, each type of living cell maintains an essentially constant quantitative relationship among these components. There is an essentially constant ratio between cell K' content and cell water content; there is an essentially constant ratio between cell K^+ content and cell protein content; and there is an essentially constant ratio between the cell protein content and cell water content.

According to the membrane-pump theory, there are no intimate associations between the bulk of any two of these three major components. Both K' and water exist in the free state as in a dilute salt solution. The constant level of K' (and Na^+) in the cell water is due to the continual activities of postulated pumps in the cell membrane. An outward water pump regulating the amount of water in living cells was also proposed at one time (Robinson, 1950), disputed (Conway and McCormack, 1953, Leaf, 1956), and then withdrawn (Robinson, 1956). Left unexplained is the question. ***why in each cell type is there a quantitative relationship between its water andprotein content?***

In 1923, Heinrich Walter suggested that water sorption on proteins and other biological materials might be the cause of water accumulation in living cells. Walter based his argument on the historically well-recognized strong interaction between biological materials and water, often referred to then as "Schwellungswasser," and more specifically on the experimental studies of the water vapor sorption of proteins and various other quellbaren Korpern by J. R. Katz (1919). With the decline of colloidal chemistry in the thirties and early forties — for reasons seemingly compelling at the times but completely reversed very recently (Ling, 1984, 1988) — the deserving idea of Walter was all but forgotten.

Departing radically from the membrane pump theory including its free K^+-free water tenets, Ling introduced the association-induction (AI) hypothesis, in which the major components of the living cells, water, proteins, and ions are closely associated (Ling, 1952, 1962).

In 1965 Ling presented his polarized multilayer theory of cell water (Ling, 1965, 1972). Originally introduced to explain the low level of Na' and other solutes in living cells, the theory also offers a molecular mechanism as to how the protein-water association in living cells is achieved: Certain proteins exist in the fully extended conformation with their backbone NHCO groups directly exposed to, and polarizing (all) the bulk phase cell water in multilayers. An underlying assumption is that for each specific cell type, under a specific set of conditions, the concentration of "water-polarizing protein(s)" makes up a constant

proportion of the total cell proteins.

In the preceding papers of this series of studies on the physical state of water in living cells and model systems, Ling and coworkers have provided extensive evidence that water in the presence of proteins, which for one reason or another exist in the fully extended state, do indeed exclude Na^+, sugars, and free amino acids (Ling et al., 1980, 1980a, Ling and Ochsenfeld, 1983).

To explain the constant water contents of normal resting living cells in terms of a multilayer adsorption phenomenon, one implies that the cell proteins existing in the correct conformation (in an environment containing water vapor at a partial vapor pressure equal to that of a normal Ringer solution) has the inherent capability of sorbing enough water to match that of the normal living cell. In fact, Ling and Negendank (1970) have already confirmed this expectation in the study of vapor sorption of isolated frog muscle. Thus, in a vapor phase kept at a vapor pressure equal to that of a normal Ringer solution ($p/p_o = 0.9966$), the isolated frog muscle fibers maintain an essentially normal water content while other specimens kept at constant lower vapor pressure maintained a lower water content.

However, one might argue (as A. V. Hill did in 1930) that this maintenance of normal water content in the vapor phase at equilibrium with a Ringer solution is expected because the cell contains just enough (free) K^+ (and anions) to match the vapor pressure (osmotic activity) produced by the normal Ringer solution. Withholding a full answer to this argument until Discussion, we find that it would be, nevertheless, enlightening to see if those proteins and polymers, completely free of K^+ or other solutes, but which exist in the fully extended state and which reduce the solvency of bulk phase water to Na', sucrose, and glycine, may sorb enough water to match in quantity the water content seen in normal living cells. It would be also enlightening to see if other proteins which do not exist in the fully extended conformation, nor reduce solvency for Na^+, etc. are unable to achieve the same. This communication describes our results, to achieve which a new method for the study of water sorption at extremely high vapor pressure was introduced and described.

MATERIALS AND METHODS

Materials. The sources, catalogue and lot numbers of the proteins, polymers, and other chemicals and materials used are as follows: polyethylene oxide (PEO) (Union Carbide, 270 Park Avenue, New York, N. Y. 10017; Catalogue No. UCC2498C, Lot 2204); polyethylene glycol (PEG) (Fisher Scientific, King of Prussia, Pa.; Catalogue No. P156, Lot 735570); polyvinylpyrrolidone (PVP-360) (Sigma Chemical Co., St. Louis, Mo., Lot 57C-0071); polyvinylmethyl ether (PVME) (Gantrez-M-094, Lot 1003 GAF, 140 W. 51st St., New York, N. Y. 10020); bovine hemoglobin (mixture of appr. 25% oxyhemoglobin, 75% methemoglobin) (Sigma Chemical Co., Catalogue No. A4378-H2500, Lot 74F9395); gelatin (swine skin), Sigma Chemical Co., Catalogue No. A-4378, H2500, Lot 74F9395; bovine serum albumin (Sigma Chemical Co., Catalogue No. A-4378, Lot 70F-9350); bovine γ-globulin (Sigma Chemical Co., Catalogue No. G-5009, Lot GOF-9316); sodium chlor-

TABLE I. The molal concentration of NaCl used to make up solutions of the seven relative vapor pressures studied at 25°C. Data from Fraser (1927).

Concentration (molal)	p/p_o
0.000	1.00000
0.0422	0.99858
0.0709	0.99775
0.1015	0.99667
0.1976	0.99354
0.6095	0.980156
0.9084	0.970125

ide, C. P. grade (J. T. Baker Chemical Co., Phillipsburg, N. J. 08865, Catalogue No. 2624-S, Lot 418712), Filter paper, Thomas Scientific, Swedesboro, N. J., Catalogue No. 4704-H21.

The Basic Experimental Setup. The basic equipment used was extremely simple. Screw-cap "self-sealing" Kerr mason jars (15 ounce or 450 ml capacity) were held fully immersed on the shaker platforms of a constant temperature bath (25°C ± 0.1°C), gently shaken at the rate of 88 excursions per minute each full excursion covering 2 cm. The wall of the canning jars were lined with a double layer of filter paper kept wet by 70 ml of distilled and deionized water or NaCl solution of precisely known molal concentrations as given by Fraser (1927) (Table I). These solutions provided the five partial vapor pressures used in this study.

The double layer filter paper measuring 24 cm by 9.5 cm, was held together and kept from collapsing by pieces of scotch tape, 3M, St. Paul, Minn., Scotch Removable Transparent Tape 810 (3/4 in.)). The total length of tape used for each jar was 24 cm. The soluble ashe of both the filter paper and tape used in one jar were determined from the evaporated acid extracts of the combined filter paper and tape (boiling 1 hr. in 0.5 M HCl) and found to equal 0.00398 ± 0.0006 g. per set. Assuming the soluble ashe to be NaCl, the NaCl content of the 70 ml of vapor pressure controlling solution in each jar would have increased by 0.974 mM. This amount of NaCl would reduce the partial vapor pressure of pure distilled water from 1.00000 to 0.99997. This difference is quantitatively insignificant.

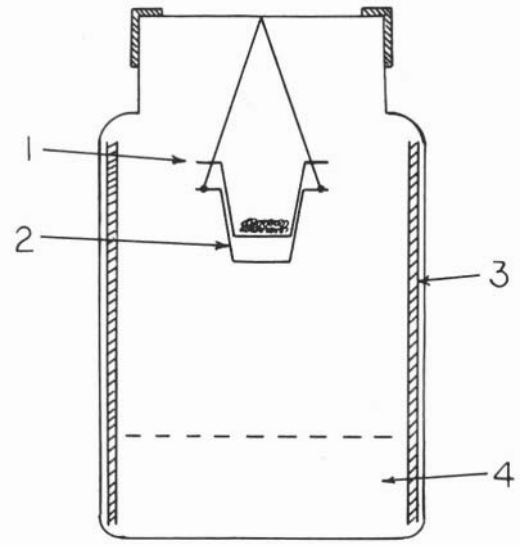

FIGURE 1. A diagram of the experimental set-up. 1, weighing cup; 2, support cup; 3, filter paper; 4, H_2O or NaCl solution.

As shown in Figure 1, the key components of the set up include a set of two thin 5 ml polystyrene cups with flanges (Fisher Scientific, Disposable polystyrene microbeakers, Cat. No. 2-544-30), the lower one, called the "support cup" has four needle holes. Surgical threads (Deknatel, No. 6) are tied to these holes on one end and the other end attached to the center of the inside of the screw cap of the canning jar. Thus suspended, the cup assembly moved back and forth in the mason jar when the jar (and its contents) was shaken in the constant temperature bath. The back and forth motion of the suspended cups served to keep the vapor phase stirred during incubation.

The second polystyrene cup which holds the sample (called the "weighing cup") sat snugly in the support cup during incubation but could be easily removed for sample introduction and weighing. Since the weighing cup was weighed with the sample, it was a matter of great importance that the weighing cup itself did not sorb water to any significant degree. The thin polystyrene cups served this need perfectly.

Table II shows that the weights of cups after being taken out of their commercial packaging, demonstrated no significant change of weight after 24 hours of heating at 90°C (A), or 48 hours of heating at 75°C in vacuo (B). Nor did soaking for two hours in distilled water affect the cup weights after

adhering drops of water had been wiped off with filter paper (C).

Sterility Precaution. To greatly reduce bacterial or fungal growth, all jars, filter paper, NaCl were dry sterilized by heating at 110° C for at least 48 hours prior to use.

Sample Drying. The water contents of polymers and protein solutions were determined from the difference in weight before and after drying for 48 hours in a vacuum oven kept at 75° C at 20 mm Hg vacuum.

The New Method (Null Point Method). The new method relies on the fact that the

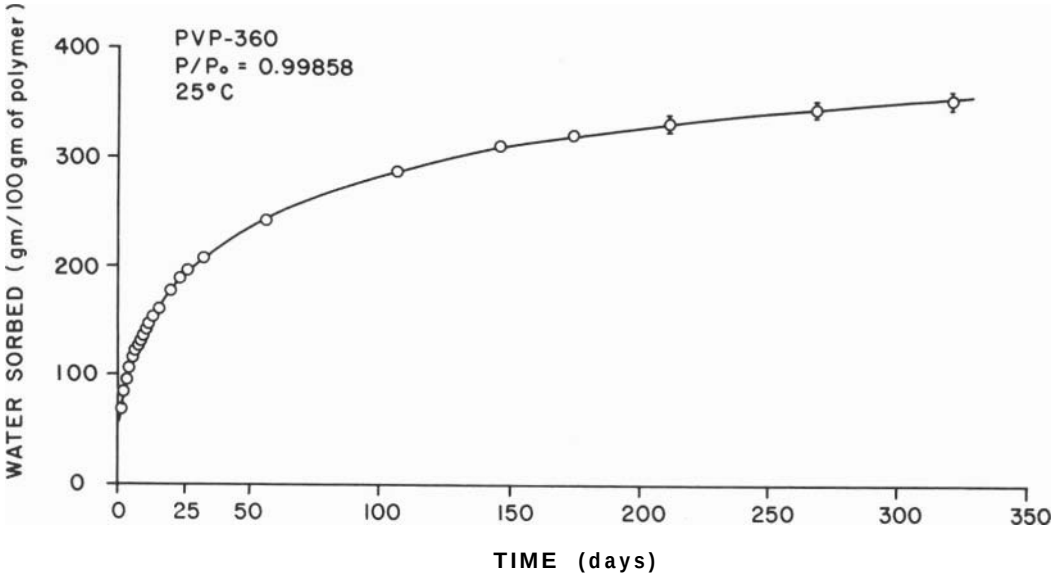

FIGURE 2. The time course of water vapor uptake by polyvinylpyrrolidone (PVP-360) in an atmosphere containing water vapor at the relative vapor pressure of 0.99858 at 25° C. For most experimental points the standard deviations were smaller than the diameters of the circles and are not shown in this figure but are given in Table III.

TABLE II. Weight changes of polyethylene weighing cups after heating at 90° (24 hrs.) and 75° (in vacuo) 48 hrs.) and after soaking in distilled water (2 hours).

		Weight (gram)							
	Cup Number	1	2	3	4	5	6	7	8
A	Before heating	0.2370	0.2333	0.2211	0.2283	0.2541	0.2093	0.2570	
	After heating (90° C, 24 hrs.)	0.2370	0.2333	0.2211	0.2283	0.2541	0.2097	0.2572	
B	Before heating	0.2555	0.2786	0.2495	0.2879	0.2809	0.2765	0.2567	0.2737
	After heating (75° C, in vacuo, 20 mm Hg, 48 hrs.)	0.2558	0.2786	0.2497	0.2879	0.2810	0.2765	0.2565	0.2735
C	Before soaking							0.2565	0.2735
	After soaking in distilled water (2 hrs.) and wiped dry							0.2565	0.2735

initial rate of loss or gain of water by a polymer or protein solution varies with the difference between the vapor pressure of a specific sample and the vapor pressure of the environment controlled by the 70 ml of distilled-deionized water in each jar with or without NaCl added (see Table I). Note that we are dealing only with fully dissolved protein or polymer solutions, not involving water sorption on dry proteins or polymers or the phenomenon of hysteresis associated with it. By preparing a series of sample solutions of different water contents per unit weight of the dry polymer (or protein) and exposing all of them to an atmosphere of the same relative vapor pressure for five days, the percentage weight gain or loss of water in each sample is determined and plotted against the water contents of each sample. The best fitting straight (or curved) line is then drawn. The water content corresponding to zero gain (or loss) of water is the water content at equilibrium with the relative humidity of that specific environment (see Figure 3 below).

TABLE III. **Detailed data on the time course of weight changes illustrated in Figure 2 of polyvinylpyrrolidone (PVP-360) in an atmosphere at the relative vapor pressure of 0.99858 (25° C).**

Time Course: PVP-360, p/p_o 0.99858, 25° ± 0.1°C, Weight of sample 0.3 ~ 0.4 g.

Time (hrs.)	Number of Samples	Amount of sorption w. %
0		0.0
24	1	67.0
48	2	82.6; 87.0
72	3	97.0 ± 3.0
96	3	106.0 ± 3.7
120	4	113.0 ± 4.2
144	3	118.7 ± 4.9
168	3	125.0 ± 5.7
192	4	131.3 ± 5.7
216	4	133.6 ± 5.3
240	2	143.7 ± 3.4
264	6	145.4 ± 3.0
312	6	152.1 ± 3.6
360	6	159.7 ± 5.0
456	6	175.7 ± 6.6
528	4	187.6 ± 3.5
622	4	196.3 ± 4.1
766	4	205.8 ± 4.1
1342	4	241.2 ± 5.3
2542	4	285.5 ± 5.7
3478	4	309.0 ± 6.3
4150	7	320.8 ± 5.8
5038	7	327.5 ± 12.0
6406	6	344.9 ± 6.6
7660	6	355.3 ± 9.0

RESULTS

Unsuccessful Determination of the Equilibrium Water Vapor Sorption of Polyvinylpyrollidone (PVP-360) at Very High Relative Vapor Pressure by the Conventional Method (25° C). Figure 2 shows the time course of water vapor uptake of dried polyvinylpyrrolidone powder (PVP-360) (average molecular weight 360,000) in an atmosphere with a relative vapor pressure equal to 0.99858. The details of the data presented in Figure 2 are given in Table III. From these data one concludes that it would be impractical to obtain the vapor sorption data at vapor pressure equal to 0.99858 or higher. For the study of vapor sorption equilibrium at very high humidity, a new method requiring much less time would be essential and was developed. Note that even after 319 days, there was no indication that the uptake was approaching equilibrium.

Successful Determination of Equilibrium Water Sorption of Polyethylene Oxide (PEG-8000) at Very High Relative Humidity with the New Null Point Method (25° C). Following the procedures for the Null Point Method given under Materials and Methods, we determined the vapor sorption of polyethylene oxide (PEG-8000) at a relative vapor pressure of 0.99775. Table IV records the details of one experiment from a set of four. The final percentage weight gain of water was plotted against the (initial) water contents in gram of

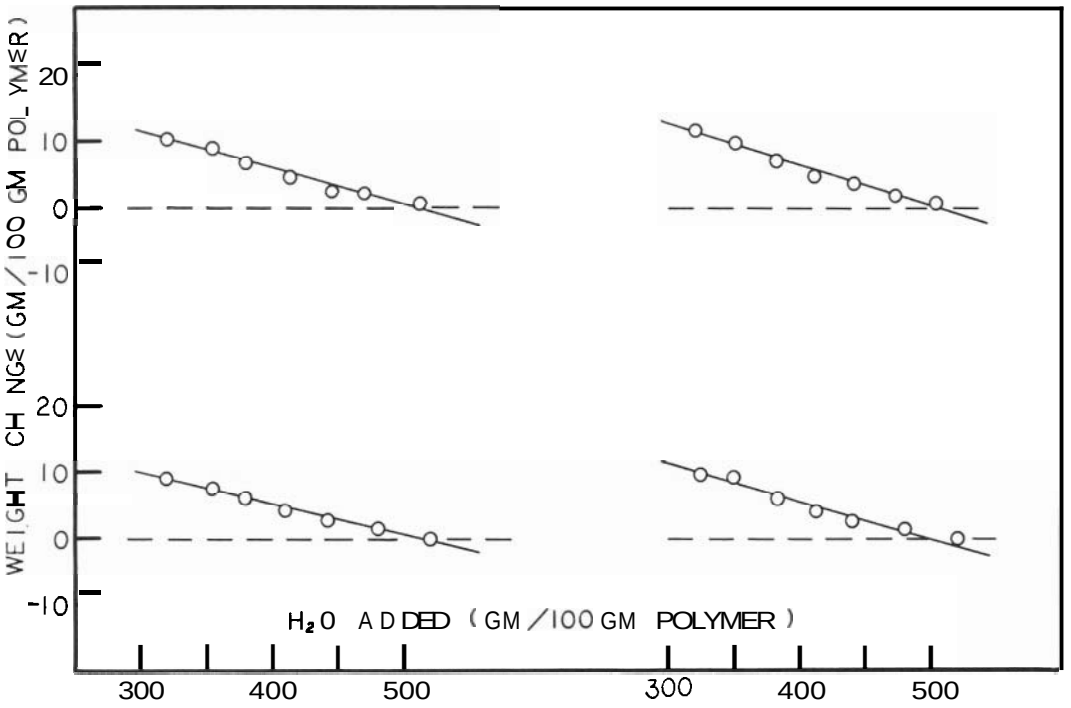

FIGURE 3. Quadruplicate determinations of the equilibrium water sorption in an atmosphere containing water vapor at a relative vapor pressure equal to 0.99775, of polyethyleneglycol (PEG-8000) by the new Null Point Method. The ordinates represent the percentage gains (or losses) of water in the different PEG samples after 5 days of incubation at 25°C following the addition of different amounts of water to each sample (in grams of water added per 100 grams of dry sample weight) shown as abscissa.

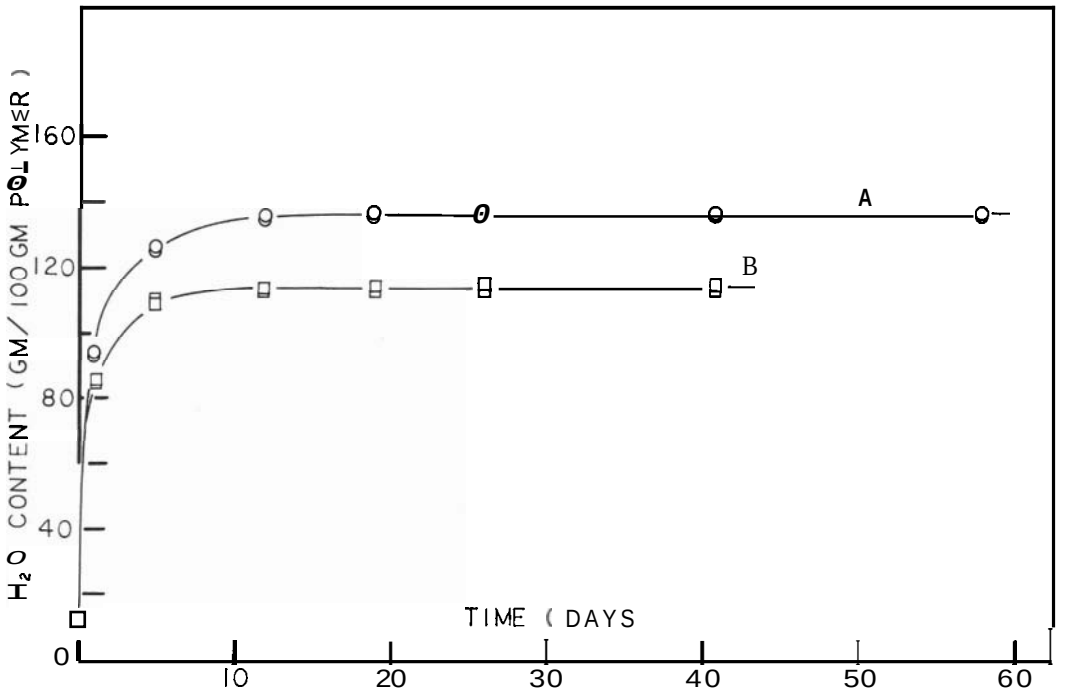

FIGURE 4. The course of water vapor uptake at 25°C by polyvinylpyrrolidone (PVP-360) in an atmosphere containing water vapor at the relative vapor pressures of 0.980156 (A) and 0.970125 (B).

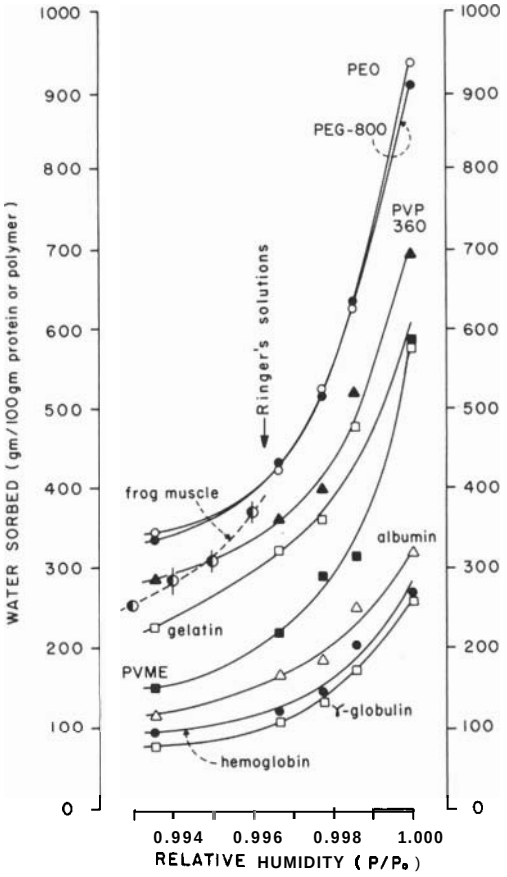

FIGURE 5. Equilibrium water vapor sorption obtained with the Null Point Method at relative vapor pressures from 0.99354 to 0.99997 of polyethylene oxide (PEO), polyethylene glycol (PEG-8000), polyvinylpyrrolidone (PVP-360), polyvinylmethyl ether (PVME), gelatin, bovine serum albumin, bovine hemoglobin, and γ-globulin at 25° C.

water for 100 grams of dry polymer and shown in Figure 3. The intercepts of the best fitting curves with the line of zero gain yielded the following equilibrium water contents: 513.7; 505.0; 510.8; and 508.9 averaging 509.6 ± 3.65 (S.D.). These data indicate that the new method is capable of generating reproducible results.

A Comparison of Results Obtained by the Standard Method and the Null Point Method for Vapor Sorption at Lower Vapor Pressures Where Both Conventional and New Method Worked. Figure *4* shows that at the lower vapor pressure of 0.98016 and 0.970125, vapor sorption of PVP-360 reached equilibrium after about 15 and 12 days respectively. Equilibrium water uptake by PVP-360 at the same relative humidities were also determined by the new Null Point Method. The two sets of data obtained were compared in Table V. A 5% or 7% difference was obtained, with the conventional method yielding somewhat lower figure.

Vapor Sorption Isotherm at Very High Vapor Pressure of Polyvinylpyrrolidone (PVP-360), Polyethylene Glycol (PEG-8000), Polyethylene Oxide (PEO), Polyvinylmethyl Ether (PVME) and Gelatin. Using the Null Point Method, we determine the equilibrium

TABLE IV. Detailed data of an equilibrium water sorption assay at relative vapor pressure of 0.99775 by the Null Point Method corresponding to one of the four sets of data shown in Figure 3.

Expt. No.	4-1	4-2	4-3	4-4	4-5	4-6	4-7
tare (g.)	0.2683	0.2843	0.2900	0.2750	0.2935	0.2766	0.2876
tare + sample (g.)*	0.5072	0.4991	0.5147	0.4938	0.5219	0.5182	0.5173
dried sample (g.)	0.2381	0.2141	0.2240	0.2181	0.2277	0.2408	0.2290
(tare + sample + $H_2O_{init.}$ (g)	1.2670	1.2555	1.654	1.3945	1.5364	1.6532	1.6900
initial H_2O wt. (g.)	0.7606						
$(H_2O/sample)_{init.}$ X 100	319.4	353.6	380.1	413.3	445.8	471.7	512.4
(tare + sample + $H_2O)_{final}$ (g.)	1.2920	1.2747	1.3806	1.4049	1.5421	1.6588	1.6918
final H_2O wt. (g.)	0.7856						
$(H_2O/sample)_{final}$ X 100	329.9	362.6	386.9	418.1	448.4	474.0	513.2
A H20 (%)	+10.5	+9.0	+6.8	+4.8	+2.6	+2.3	+0.8

*initially the PEG sample contained 0.32% water.

water sorption of the four oxygen-containing polymers, PVP-360, PEG-8000, PEO, and PVME in addition to gelatin. The data are shown in Figure 5 and numerical data given in Table. VI. As a group, all four polymers and gelatin exhibit very high water uptake. At the vapor pressure of 1.0000, the water sorbed ranged from 577 (gelatin) to 945 (PEO) per 100 grams of dry polymer or protein. At the vapor pressure corresponding to that of amphibian (0.9964) or mammalian (0.9966) Ringer solution the water sorption ranged from 235 to 434 grams of water per 100 grams of dry polymer or protein. Note that the equilibrium vapor sorption isotherm of isolated frog muscle (also reproduced in Figure 5) falls within this range.

Vapor Sorption Isotherm at Very High Vapor Pressure of Three Native Proteins: Bovine Serum Albumin, Bovine Hemoglobin, and y-Globulin. Using the null point method, we also determined the equilibrium vapor sorption of three native proteins:

TABLE V. Comparisons of the equilibrium water vapor sorption of polyvinylpyrrolidone obtained by the conventional method and the Null Point Method.

Date of Experiment	p/p_o	Methods Used	Equilibrium water content (gms. of H_2O per 100 gm. polymer)
10-8-86	0.970125	Null Point Method	123
11-5-86			120
	0.970125	Conventional Method	114*
11-12-86	0.980156	Null Point Method	143
11-19-86			144
	0.980156	Conventional Method	137*

*Details of data presented in Figure 4.

TABLE VI. Detailed data of vapor sorption of PEO, PEG-8000, PVP-360, PVME, gelatin, bovine hemoglobin, bovine serum albumin and y-globulin illustrated in Figure 5.

Vapor Absorption of Some Polymers and Proteins Under High Relative Vapor Pressure

p/p_o	0.99354			0.99667			0.99775			0.99858			0.99997		
Polymer or Protein	n	r	H_2O%	n	r	H_2O%	n	r	H_2O%	n	r	H_2O%	n	r	H_2O%
PEO	6	0.993	340.7	7	0.991	424.3	7	0.952	523.3	7	0.983	629.7	7	0.876	944.4
PEG-8000	6	0.987	333.8	7	0.960	433.3	7	0.996	516.9	8	0.921	632.5	6	0.839	913.2
PVP-360	7	0.983	285.4	6	0.933	360.9	9	0.971	399.6	8	0.937	518.3	6	0.950	693.7
PVME	7	0.972	148.2	7	0.954	217.4	6	0.842	292.5	7	0.746	314.2	7	0.855	586.4
Gelatin (swine skin)	5	0.964	223.6	7	0.961	323.3	7	0.985	360.6	5	0.995	478.5	6	0.961	577.7
Hemoglobin (bovine)	6	*	95	7	*	120	7	*	145	6	0.919	203.5	7	0.949	270.8
BSA	6	*	115	7	*	165	7	*	185	6	0.958	251.4	6	0.949	322.0
y-globulin	6	0.966	74.1	5	0.929	108.4	6	0.972	132.6	6	0.978	174.6	6	0.949	268.6

n — number of samples

r — correlation coefficient between percentage weight changes and initial water contents

* — curves were non-linear

bovine serum albumin, bovine hemoglobin, and y-globulin. The data are also presented in Figure 5 and Table VI. In general, water óósorption is much lower in this group. Thus at the relative vapor pressure of 1.000, the equilibrium vapor uptake ranged from 320 (bovine serum albumin) to 262 (y-globulin). At the vapor pressure corresponding to that of Ringer solution, the equilibrium water uptake was in the range of from 165 to 109. The detailed numerical data are also presented in Table VI.

With the conventional method, we also measured the water vapor sorption of bovine hemoglobin and bovine serum albumin at the vapor pressure of 1.0000 (Figure 6). Like the data displayed in Figure 2 for water sorption of PVP-360 at 0.99858, equilibrium was also far from being reached here. Nevertheless, the vapor sorption had already exceeded 100 gm/100 gm for hemoglobin, and 120 gm/100 gm of serum albumin. The significance of these findings will be made clear under Discussion.

DISCUSSION

Early History of Water Sorption Studies at Physiological Vapor Pressure. Carl Ludwig, sometimes called the father of modern physiology, studied the water update of dried pigs' bladder. In discussing his findings, he mentioned that "the smallest components of the (bladder) membrane, have a pronounced affinity for water — whether it is chemical or adhesive will one day be made clear when chemistry lifts itself out of its (present) theoretical misery" (Ludwig, 1849). Seventy years later, J. R. Katz (1919) quoted this passage of Ludwig under the title of "Carl Ludwig's Problem" in Katz's publication entitled "The Role of Swelling." Katz then commented that chemistry in the meanwhile had indeed lifted itself out of its theoretical misery with the

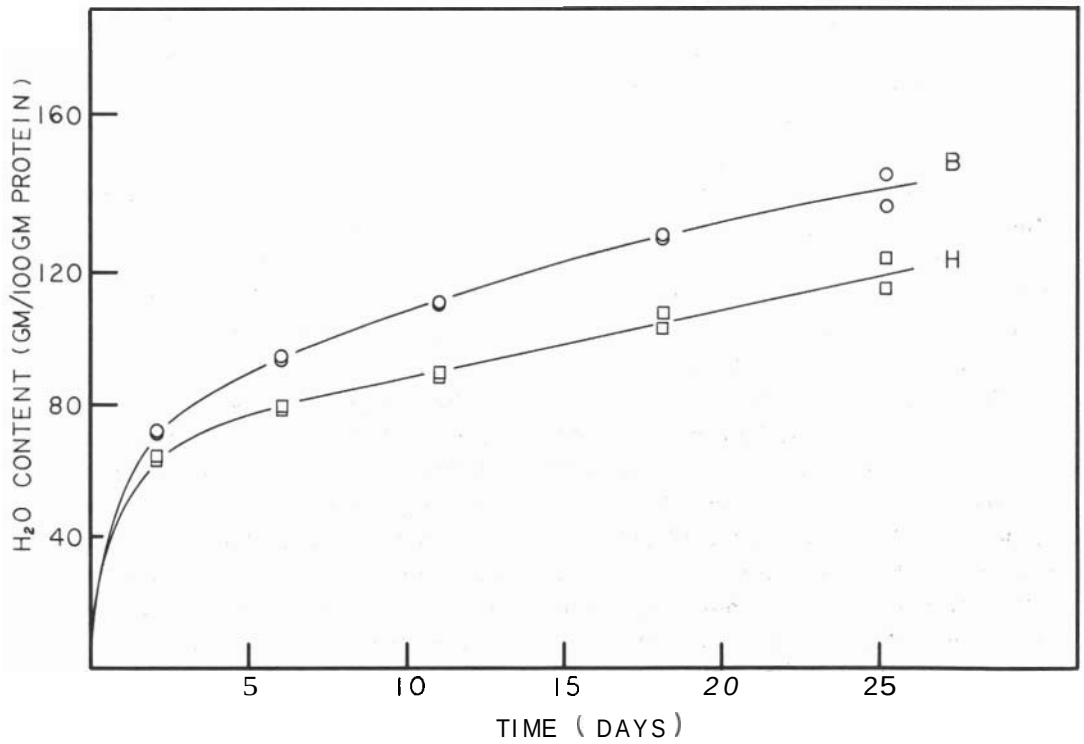

FIGURE *6*. The time course of water vapor uptake by bovine serum albumin (B) and bovine hemoglobin (H) in an atmosphere at a relative vapor pressure of 0.99997 (25°C).

work of van't Hoff, Nernst, Ostwald, Arrhenius, Gibbs, . . . The main purpose of Katz's work was to study extensively the sorption of water from near zero all the way to physiological vapor pressures (i.e., near saturation) and saturation.

Approximately another 70 years have passed since Katz's publication. Within this period, theoretical physics has advanced to incredible heights; man landed on the moon and the genetic code was successfully deciphered. How much progress has been made on the pronounced affinity of the "smallest components" of living matter for water in this 70 years? Some progress has been made but not nearly as much as what could and should have been achieved (see Ling, 1988).

Nevertheless, early in this period, physicists de Boer and Zwikker (1929) and Bradley (1936) published their quantitative theories of polarized multilayer adsorption. Subsequently it was found that gas molecules without permanent dipole moments (e.g., argon) cannot be adsorbed as polarized multilayers and that their adsorption beyond the first layer was due to capillary condensation (Brunnauer et al., 1938). On the other hand, gaseous molecules like water with a large permanent dipole do adsorb as polarized multilayers. Multilayer water sorption on proteins was shown to be independent of state of subdivision, and hence the total available surface area or capillary spaces, but only on the chemical nature of the proteins (Mellon et al., 1948; Benson et al., 1950).

As mentioned earlier, Ling proposed the polarized multilayer theory of (all) cell water in 1965 (Ling, 1965, 1972); this theory was supported by Ling and Negendank (1970) in a study of vapor sorption of surviving frog muscle. In this study it was shown that about 5% of cell water was adsorbed tightly and apparently on isolated sites as the sorption of this fraction of water follows a Langmuir adsorption isotherm; the remaining 95% of the cell water in frog muscle follows Bradley's polarized multilayer adsorption isotherm. The possibility that vapor sorption reflects the presence of approximately isotonic solution of K^+ salts in the cell water, as once widely believed and mentioned above (Hill, 1930), was ruled out by the extensive evidence now on hand that the bulk of K^+ in muscle cell exists in an adsorbed state on β- and y-carboxyl groups located on proteins at the edges of the A bands and Z-lines (Edelmann, 1977, 1980, 1984; Ling, 1977, 1984, 1988). With the bulk of cell K^+ adsorbed and hence osmotically inactive and no other intracellular solutes to match it in concentration, one is left with cell protein as the main source of the osmotic activity (i.e., a reduction of water activity). In agreement, Ling (1983) has already shown that those proteins and polymers which have the power to reduce the solvency for Na salts, free amino acids, and sugars do indeed possess enormous power to generate osmotic activity far beyond that expected on the basis of their molar concentrations.

With all these in the background, it is most reassuring that the data presented in this paper have directly established that the intensive power of the fully extended polypeptide chains or the oxygen-containing polymers can indeed sorb water in quantity to match that of living cells and that native proteins with their backbone NHCO groups locked in a-helical and other inter- and intramacromolecular H-bonds cannot.

While the present series of studies of water sorption of gelatin and oxygen-containing polymers at near physiological vapor pressure have not provided sufficiently extensive data to warrant a similar analysis, Katz's data (Katz, 1919) of water sorption on gelatin over the entire vapor pressure range (despite some uncertainty of one point at the highest vapor pressure) do indeed (like frog muscle) follow Bradley's polarized multilayer adsorption isotherm as analyzed and shown by Ling (Ling,

1984, p. 288).

Comparison of Present Data with Prior Results of Katz. In Katz's (1919) vapor sorption studies, the highest relative vapor pressure was full saturation (for some samples) and the next highest relative vapor pressure was 0.965. To the best of our knowledge, until our own work began, Katz's paper was the only one in which vapor sorption in isolated proteins and other biomacromolecules were studied at vapor pressure at saturation. Most of the well known studies of vapor sorption did not reach beyond a relative vapor pressure of 0.95 (Bull, 1944; Benson et al., 1950; Hnojewyj and Ryerson, 1961).

Katz used what we refer to as the conventional method: continued exposure to vapor pressure until constant weight was reached. Katz described his experiment as lasting from "one to many months" but gave no time course to illustrate that in his setup equilibrium had in fact been reached at the vapor pressure of 1.000. In our case, even at the somewhat lower vapor pressure of 0.99858, the trend of the uptake curve shown in Figure 2 gave no indication that equilibrium was approaching even after 10.6 months. This observation of extremely slow attainment of equilibrium was confirmed by comparing the equilibrium water sorption data eventually reached after 319 days (355 gm H_2O/100 gm polymer) with that obtained by the new Null Point Method of the same polymer (PVP-360) at the same vapor pressure (ca. 500 gm H_2O/100 gm polymer). Clearly even after 10.6 months, the water sorption had reached only 71% of its final equilibrium value.

The water sorbed by gelatin at full saturation given by Katz was (equivalent to) 460 gm. of H_2O per 100 gm of protein, compared to our own (higher) value of 577 gm H_2O/100 gm protein. Katz's data of water sorption of horse and dog CO-hemoglobin at full saturation was 71 to 80 H_2O/100 gm of protein respectively and thus very much lower than our own data of 262 gm H_2O/100 gm of bovine oxyhemoglobin (25%) and methemoglobin (75%). It was for this reason that we undertook the experiments described in Figure 6, where long before equilibrium had been reached, the water uptake of hemoglobin (as well as bovine serum albumin) had already exceeded Katz's equilibrium values. Thus barring some unknown differences in our setups and samples studied, there was a distinct possibility that equilibrium might not have been reached in those studies of Katz at full saturation. If true, then our present set of data would be the first of its kind ever successfully achieved.

Comparison with Water Sorption by Living Cells. As mentioned earlier, the equilibrium water sorption of isolated frog muscle at the higher end of vapor pressure reported by Ling and Negendank (1970) falls in the range of vapor sorption by PEO, PEG, gelatin, and PVME. This general agreement supports the prediction that the full amount of water in resting living cells is there not by accident, or merely water-logging because they are found in an aqueous medium, rather, the water is in the cells because, in terms of free energy, it is more favorable for water to be there. These findings therefore answer the question: why living cells have a more or less constant amount of water per unit of weight of cell protein.

Does all the protein in the cell polarize and sorb water in multilayers? As of this moment, we have no clear-cut data to answer this question. One then asks, "If only part of the cell protein polarize water, why should there be proportionality between the amount of cell water and the ***total*** cell protein?" One possibility is that, even if only a small fraction of the cell protein is involved in water polarization, one still may anticipate a constant ratio between total cell water and total cell protein if the percentage of water-polarizing proteins stays a more or less constant fraction

of the cell's total protein.

Why Do Different Types of Cells Have Different Water Contents? Now that there is extensive evidence that water in living cells exists as polarized multilayers, one may attempt to answer the question. Why do different types of living cells have different water contents? We suggest that there are three mutually dependent factors that determine the cell's equilibrium water content:

(1) ***Variation in the percentage of protein backbones in the entire cell that exist in the fully extended, water polarizing conformation.*** If the cell contains a significant quantity of non-protein solid matter (e.g., DNA, glycogen) that might or might not polarize water, that too must be taken into account.

(2) ***Variation in the q-value of the (polarized) cell water for the major osmotically active substance in the external medium (e.g., NaCl).*** The relation between water content and q-value has been elucidated and a qualitative theory presented and experimentally confirmed (Ling, 1987). The q-value in turn is postulated to reflect the degree of water polarization. Strongly polarized water has low q-values (and hence low water content); weakly polarized water has high q-value (and hence high water content).

(3) Variation in the density and effectiveness of volume-restraining salt linkages (and H-bonds among adjacent water polarizing protein chains). The concept of a key role of the salt linkages in preventing cell expansion has been introduced long ago (Ling, 1962, p. 249). Other studies (Ling and Peterson, 1977) indicated that the density of volume-restricting salt linkages depends on the concentration and nature of salt ions in the cell's environment that can dissociate the salt linkages thereby causing cell swelling. NaCl provides the major ions of the ***cell's environment;*** it is ineffective in causing expansion of normal

TABLE VII. A comparison of the various physico-chemical properties of water in the presence of different proteins (native and denatured) as well as oxygen-containing polymers. WEX stands for the presence of an exothermic peak during warming or "warming exothermic peak" in the warming thermogram (see Ling and Zhang, 1983, p. 396).

			Freezing and Thawing Properties				
	Reduced Solvency for Na^+ Salts, Sucrose and Glycine	Osmotic Activity	Freezing Point Depression	Freezing and Thawing Peak Widening and Disappearance	WEX	Swelling and Shrinkage in Hypo- and Hypertonic Solution	Water Sorption at Physiological Vapor Pressure
Native globular proteins	no or little	somewhat higher than ideal	no or little	no or little	no or little	little or none	low
Gelatin	yes	much higher than ideal	yes	yes	yes	pronounced shrinkage	high
Denatured globular proteins urea	yes	—	yes	yes	yes	pronounced swelling and shrinkage	—
SDS	no or little	—	no or little	no or little	no or little	—	—
Synthetic polymers (PEO, PVME, PVP)	yes	verymuch higher than ideal	yes	yes	yes	pronounced swelling and shrinkage	high

cells by dissociating key salt linkages but becomes so in response to cell injury and ATP depletion (Ling and Kwon, 1983). Isotonic KCl, on the other hand, causes extensive cell swelling and gain of water in healthy cells (Ling and Peterson, 1977) (for explanation see Ling and Kwon, 1983).

The Segregation of High Water Sorbing Gelatin, PEO, PEG, PVP, and PVME and Low Water Sorbing Native Proteins and Relationships to Other Properties of Water Studied in These Systems. Results of the present investigation have added another important physicochemical property of polarized water that offers insights into the understanding of yet another physiological property of living cells: the maintenance of a constant water content. Once more, proteins and polymers which satisfy the theoretical requirements of providing an NP-NP-NP (gelatin) or NO-NO-NO system (PEO, PEG, PVP, PVME) sorb water in amounts comparable to those seen in living cells while native globular proteins, with their NHCO group locked in a-helical and other intra- and inter-macromolecular H bonds, sorb much less water (Ling, 1984, p. 169). With the new addition, a table compiled in 1983 by Ling and Zhang is now revised and shown here as Table VII including solute exclusion, osmotic activity, freezing and thawing properties, swelling and shrinkage and now vapor sorption.

Not included in the table are the important findings that water, made nonsolvent for Na citrate in consequence of interaction with PEO, PEG, PVP, and/or PVME, demonstrates reduction of rotational (and translational) motional freedom which was the prediction of the polarized multilayer theory of cell water and model systems. NMR (Ling and Murphy, 1983), high frequency dielectric dispersion (Kaatze et al., 1978), and in particular quasielastic neutron scattering (Rorschach, 1984) provided the evidence. Much of these findings compliment the direct demonstration of the reduction of rotational motional freedom of water in living cells by dielectric dispersion studies (Clegg et al., 1984) and above all by quasielastic neutron scattering (Trantham et al., 1984; Heidorn et al., 1986).

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The following presents the comments of one of the reviewers of this paper and the author's answers. Since both the questions and answers are of possible interest to the readers, the editors have decided to reproduce them as part of the effort toward making scientific publications in this journal interesting and more alive than the usual format permits.

Editor

Question #1. Globular vs. Extended Macromolecules.

It is self-evident that globular forms have less surface area per unit mass than extended ones for the same mass. Question: Much importance is assigned in the AIH to the repeating peptide linkage groups of extended forms, but it is also true that surface area *per* se also increases as the globular to extended transition occurs (or, comparing these two "forms" for different macromolecules). But there is a very large body of evidence that surface area per se is critical to water properties "near" the surface. This evidence comes from Ninham, Pashley, Israelachville, Parsegian and others (I cite their work and consider it in Amer. J. Physiol. 246:R133, 1984; and in the enclosed reprint from "Organization of Cell Metabolism"). Some of these surfaces are devoid of peptide bonds (mica sheets). Also, Bradley's original work used (as I vaguely recall), very finely divided, "non-protein powders". Thus, how can you distinguish the effect of surface area *per* se from strictly spaced and repeating peptide bonds? (I should also mention: the angle of curvature of the surface is important — reason why globular proteins differ in their effects on water.)

Answer

To help understand the question raised and the answer to be given, I shall begin with a sketch of the polarized multilayer theory of cell water (Ling, 1965, 1972). In this theory a checker-board, or better a pair of closely juxtaposed checker-boards, of properly spaced positively charged (P) and negatively charged (N) sites are called an N-P system and NP-

NP system respectively. A matrix of linear chains carrying properly spaced N and P sites at regular intervals is called an NP-NP-NP system. If one type of charged site, say P sites, is replaced by a neutral site, they will be referred to as NO system, NO-NO system or NO-NO-NO system. In the near vicinity of any one of these systems, water tends to assume the dynamic structure of polarized multilayers. Such a dynamic structure is ***vast***ly less organized than in crystalline solids but exhibit physical properties often different from that of normal water. In living cells, the number of "layers" of water molecules between adjacent fully extended protein chains are rather few (e.g., $<$ 10).

In the general theory of polarized multilayers described above, ***the specific nature of the N and P sites*** are not defined and may include any type of positive or negative sites. Therefore the fact that surfaces devoid of peptide bonds like mica or other finely divided "nonprotein" solid powers (i.e., titanium dioxide) present no problem. Indeed polished glass surfaces (Ling, 1970, 1984, p. 279) AgI crystals (Ling, 1984, p. 280) have been my favorite examples to demonstrate the influence of these NP-NP systems to polarize water in multilayers.

There are other reasons to believe that in a protein-water system, it is not ***surface*** of the protein per se, but specific hydrophilic groups on the protein that determine the amount of water sorbed, the most important of these protein hydrophilic groups being the peptide NHCO groups.

The surface of a protein or polymer molecule is in dimensions of Angstrom units. At this ***microscopic*** dimension, the surface of proteins cannot be compared directly with properties derived from the examinations of ***macroscopic*** objects, like the surface, say, of a glass plate. As an example, consider the microscopic objects of Na^+ and K' ions. The crystal radius of a Na^+ ion is 0.95 Å, that of K, 1.33 Å. Their respective surface areas are thus $4\pi \times (0.95)^2 = 11.3$ Å^2 for Na^+ and $4\pi \times (1.33)^2 = 22.2$ Å^2 for K^+ which is 1.96 times larger than that of Na'. Yet it is Na^+ that has much greater influence on the surrounding water molecules than K' (i.e., Na^+ is more intensely hydrated) (see Ling, 1962, p. 548). This example shows that in the microscopic world of molecules and atoms, the effect of surface-area differences, while not meaningless, are overshadowed by other more powerful influences, in particular electrostatic forces. Na^+ exerts more influences on surrounding water molecules ***because*** of its smaller size, and hence stronger electrostatic attraction for the surrounding water dipoles. If it were only a matter of surface area, K' should be more hydrated than Na^+, contrary to facts.

When one examines a protein molecule, one encounters chemical groupings of the same dimension as Na^+ and K. Thus positively charged ϵ-amino group and negatively charged β- and y-carboxyl group resemble Na^+ and K^+ in dimension and in the net electronic charges they carry. As such, they also have powerful influence on water. It is therefore not surprising that proteins differ widely in the extent of water sorption. Indeed, both Pauling (1945) and Bull and Breese (1968) presented convincing evidence that in native proteins, the extent of hydration varies directly with the number of their hydrophilic groups, including the ϵ-amino group, β- and y-carboxyl groups carrying net charges, as well as other polar groups that do not carry net charges (e.g., OH groups of serine and threonine residue).

By far the most abundant polar group of all proteins are the carbonyl (CO) and imido (NH) groups of the backbone, which were not taken into account in Pauling's nor Bull and Breese's calculations, which, nevertheless, fit the data of water sorption on many native proteins. Yet there are also strong evidence that the backbone CONH groups are major seats of hydration for other proteins and

models studied. In 1972, I saw a simple way of resolving this apparent paradox: in all native globular proteins where the CONH groups are locked in a-helical and other macromolecular H bonds, the backbone NHCO groups do not interact with water. But if for one reason or another, the protein exists in the "fully extended conformation" exposing these NHCO directly to the bulk phase water, then these NHCO groups become the major seats of hydration (Ling, 1972; 1984, p. 166). These considerations led once more to the conclusion that it is specific hydrophilic groups, in particular the backbone NHCO groups, that determine protein hydration.

Finally, I would like to cite two more specific sets of experimental data that have bearing on the problem of surface per se vs. specific hydrophilic groups in determining the degree of hydration.

(1) A solution of polyethylene oxide $(-CH_2-CH_2-O-)_n$ (PEO) is a bona fide NO-NO-NO system; PEO is highly soluble in water. As shown in the text of the present communication, it sorbs a large quantity of water from an environment containing water vapor at physiological vapor pressure. Stone and Stratta (1967) in their review on this polymer pointed out that "Although polyethylene oxide is highly soluble in water, closely related polymers are insoluble in water. The related water-insoluble species include the polymer of formaldehyde $(-CH_3-O-)_n$, trimethylene oxide $(-CH_2-CH_2-CH_2-O-)_n$ . . ." Thus both adding or subtracting one CH_2 group from PEO drastically alters the water solubility, demonstrating that the surface provided by a CH_2 group is totally different from that of an oxygen atom with its lone pair of electrons. However, that is not all. The oxygen atoms must also be at the proper distance apart. In this, the fact agrees with the polarized multilayer theory of cell water mentioned earlier on two accounts: specific hydrophilic groups and their proper distances apart.

(2) The total surface areas of several dry, lyophilized proteins have been measured by the technique of nitrogen gas adsorption, with the aid of the Brunauer-Emmett-Teller theory (Benson and Ellis, 1948; Shaw, 1944). The surface area values obtained turned out to be several orders of magnitude lower than the surface area estimated on the basis of water sorption data (Bull, 1944) and analyzed in the same way. The profound difference suggests that water and the N_2 gas interact with the protein differently, and that water is adsorbed on sites hidden or otherwise non-available to N_2 gas.

Using a variety of surface probes including N_2, O_2, CH_4, A, n-butane, and the BET Theory, Benson, Ellis and Zwanzig (1950) were able to obtain consistent surface area data on similar sample of dry, lyophilized proteins. From their studies they concluded that (1) the nonpolar gas adsorption is independent of the specific protein but depends principally on the state of subdivision (Benson and Ellis, 1950) and that (2) water sorption is independent of surface area and . . . probably takes place at specific sites on the protein molecule (Benson et al., 1950). These findings once more show that it is not the surface area per se that determines water sorption, although the nonpolar gases adsorption does depend on the total surface area regardless of its chemical nature.

TABLE A. Molar Energy of Desorption of Molecular Layers of Water From The Surface Of Titanium Dioxide (Anatase) Minus The Energy Of Vaporization Of Water (E_a-E_l) (Calories per mole) [from Harkins, 1945].

Layer	Heat of Desorption	E_a-E_l
1	16,450	6,550
2	11,280	1,380
3	10,120	220
4	9,971	71
5	9,951	51
6	9,932	32

***Question #2. On** the matter of polarized multilayers.*

Here we may best "agree to disagree"! In my opinion, having read carefully most or all of your papers (including this one) I sincerely do not believe you have presented any compelling evidence for that *specific* case of molecular arrangement. Altered properties of water (cells and model systems) — yes, but polarized multilayers — I do not think so.

Answer

The skepticism expressed about the polarized multilayer theory of cell water can be separated into two components: (1) skepticism about the existence of water in the form of polarized multilayers anywhere; (2) the skepticism about the existence of water existing as polarized multilayers in living cells. I shall deal with the first component first.

Professor William D. Harkins measured the energy of desorption (E,) in excess of the energy of vaporization of water (El) of multilayers of water sorbed on the (polar) surface of titanium dioxide powder. His data reproduced here in Table A led Harkins to the conclusion that, "While the effect of the attraction of the solid dies off rapidly, it extends to somewhat more than 5 molecular layers . . ." (Harkin, 1945, p. 295).

The next question is, Why are water molecules held more tightly than in normal liquid water even when they are several layers of water away from the solid surface?

A review of the physics texts reveals laws of physics which describe the interaction between an electric charge and an electric dipole and between one electric dipole and another electric dipole. In molecules, these electric dipoles can come in two forms: permanent dipoles and induced dipoles. Based on these fundamental principles, physicist Bradley published an equation describing the formation of polarized multilayers on a polar solid surface for gaseous molecules with permanent dipoles and high polarizability. Bradley's theory of polarized multilayers of gaseous molecules with permanent dipole moment was affirmed theoretically by Brunauer, Emmett and Teller (1938) (this is the world renowned physicist Edward Teller of H bomb fame). Since water molecules have a large permanent dipole moment (1.86 debyes) as well as a good polarizability (1.444×10^{-24} cm), there seems to be no reason to doubt that there is a good theoretical explanation of the data of Harkins. To wit, the formation of polarized multilayers of water on the titanium dioxide surfaces. That the excess energy (E, $-E_1$) should be highest in the first layer, tapering off gradually is also in full harmony with this theory of polarized multilayers. Thus one may say that the formation of polarized multilayers of water is not only a feasible event experimentally demonstratable and demonstrated but an inevitable event given the presence of a suitable polar surface and the properties of water molecules as they are.

Having established theoretically and experimentally the existence of polarized multilayers of water on polar solid surfaces, we proceed to answer the skepticism about the existence of cell water in the form of polarized multilayers. It seems that this skepticism can be in turn resolved into two components: the first components express doubts that a matrix of fully extended protein chains and similar polymer chains can achieve what a polar surface like that presented by titanium dioxide can achieve on polarizing multilayers of water. In fact, the experimental demonstration of water sorption by gelatin and other oxygen containing polymers described in this present communication has already answered the question fully. These data show that like titanium dioxide, these fully extended proteins and polymer chains also adsorb multiple layers of water molecules. The first question may be raised, how many layers of water are sorbed?

Some years ago I had shown that if one stretches out and splices all the proteins in a

frog muscle cell into a single, continuous chain, and then distributes the chain evenly throughout the cell, the average distance between the adjacent chains is about 20° Å, a distance which will take about 7 to 8 layers of water molecules to fill (Ling, 1962).

In the present communication on water sorption on proteins and polymers, I have shown that in an environment maintained also at the physiological vapor pressure ($p/p_o = 0.9966$), the total amount of water sorbed by the polymers PEO, PEG, and PVP are similar to, or even higher than the water content of frog muscle at the same relative vapor pressure. In other words, in these model systems there are also 7 to 8 layers of water molecules between the nearest neighboring chains if they are distributed uniformly. If not, there can be more than 7 to 8 layers between the chains.

A survey of the literature reveals that there are two kinds of theories explaining the condensation of multilayers of gaseous molecules. The first is the polarized multilayer theory of de Boer-Zwikker and Bradley already discussed. The other is the capillary condensation theory of which the most modern version is the theory of Brunauer, Emmett and Teller (BET Theory) also cited above. In this theory, only the first layer of gas molecule is adsorbed by a Langmuir type of adsorption mechanism; succeeding layers are condensed as normal liquids. However, as pointed out by these authors themselves, BET applies primarily to gas like argon with no permanent dipole moment. For gaseous molecules like water which has a large permanent dipole moment, the adsorption follows the Bradley's polarized multilayer theory. Thus theory alone already makes it virtually inescapable that the 7-8 layers of water collected between the extended protein and polymer chains are held there through multilayer polarization. Indeed the 7 to 8 layers number jibes well with Harkin's polarized layers on titanium dioxide (i.e., $>$ 5 layers), when one takes into account that here we are dealing with layers between chains and not just on a single surface. However, one asks, Are there some new prediction of the polarized multilayer theory that can be experimentally tested independently? The answer is Yes.

de Boer and Zwikker (1929) in their original theory already alluded to the importance of a checkboard of negatively and positively charged sites in generating a polarized multilayer of adsorbed gas molecules. However, it is in the polarized multilayer theory that I further elaborated that the greatest attention must be placed on the essential geometry of the polar sites in the adsorption of polarized multilayers of polar molecules like water. Certainly the experimental observation of Stone and Stratta (1967) cited above has strongly confirmed this view.

The required geometry is that each fixed charge site must be surrounded by either oppositely charged fixed sites or by neutral sites at distance approximately that of a water molecule. It is only under these conditions that water molecules in each row is surrounded by water dipoles oriented in the opposite direction. Were it otherwise (i.e., neighboring sites are of the same electric charge) the water molecules will have similar orientation and they will repulse one another, making the formation of multiple layers highly unlikely.

That successfully polarized multilayers of water have neighboring rows of oppositely oriented water molecules signifies strong interaction not only with neighboring water molecules in the radial direction but laterally with neighboring water molecules in the same layer. The result of this multiple interaction is that individual water molecules, like a horse being tied to six more or less fixed poles, suffer motional restriction, in particular rotational motional restriction. With this knowledge on hand, one seeks experimental methods to determine if the multilayer layers

of water in the presence of the various polymers that sorb large quantity of water, indeed suffer rotational restriction as predicted.

In fact there are at least three different nondestructive, sophisticated methods to make this test, of which the most satisfactory is quasielastic neutron scattering (QENS). Using QENS method one can determine the rotational diffusion coefficient of the bulk phase water. Indeed the QENS study of Rorschach of a 35% solution of PEO led to the unequivocal conclusion that water in this polymer solution is indeed rotationally restricted (Rorschach, 1984).

Two other methods of studies, ultra high frequency dielectric relaxation studies (Kaatze, et. al., 1978) and NMR relaxation time studies of water in PEO, and other similar polymers (Ling and Murphy, 1983) gave results entirely in harmony with the QENS studies.

To answer the last component of the skepticisms, all one needs to recall is that QENS studies of two kinds of living cells have also been successfully accomplished. The bulk of water in the less hydrated Artemia cysts and the highly hydrated frog muscle cells, like that in PEO solution, suffer rotational motional restriction (Trantham et. al., 1984; Heidorn et. al., 1986). Like water in the PEO solution, water in living cells must therefore also exist in the state of polarized multilayers.

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