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How Much Water Is Made "Non-free" by 36% Native Hemoglobin?

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Abstract: At equilibrium, the concentration ratio of poly(ethylene gycol) (PEG-4000) in a dialysis sac containing a 35.1% solution of *native* bovine hemoglobin over that in the external solution is 0.196 ± 0.028 (mean ± SD). This *apparent equilibrium distribution constant* or ρ-value of 0.196, when viewed side-by-side with the near-equal distribution of sucrose and raffinose in similar native-hemoglobin dominated water suggests *all* (rather than 80%) of the water in this solution has been altered by the native hemoglobin and is no longer free liquid water. Based on Ling's equation for solute exclusion, we found that an excess of water-to-water interaction energy of a mere 4.25 cal/mole could account for both the observed exclusion of PEG-4000 and non-exclusion of sucrose and raffinose. Finally, the long-range action of (even this relatively inactive) native hemoglobin on the dynamic water structure was compared with the exclusion of coated latex microspheres from the altered water 100 μm from the surface of polyvinylalcohol gel (Zheng and Pollack) — in the light of Ling's new theory of *ad infinitum* water polarization-orientation (under idealized conditions) first publicized at the Gordon Research Conference on "Interfacial Water in Cell Biology" on the campus of the Mount Holyoke College in June 2004.

WATER, PROTEINS and K^+ are the primary ingredients of the basic unit of all life — the living cell. As a rule, each living cell spends its life in an aqueous environment rich in Na^+ but poor in K^+. In the traditional membrane-pump theory, this asymmetric distribution of K^+ and Na^+ is maintained by the ceaseless activity of postulated pumps located in the cell membrane. This membrane (Na^+) pump theory is still widely taught as truth at all levels of education, although it has been thoroughly disproved long ago (Ling 2001). Among many contradictory evidence, the simplest and the most unequivocal is that the minimum energy needed to operate the sodium pump far exceeds the total energy available (Ling 1962; 1997).

In an alternative (unifying) theory of the living cell, known as the *association-induction hypothesis* (AI Hypothesis), the segregation of the two ions is the combined consequences

of two causes: (1) low solvency of the bulk of cell water for Na^+ (and K^+); (2) preferential adsorption of K^+ (over Na^+) on the β-, and γ-carboxyl groups of intracellular proteins (Ling 1951; 1952; 1962; 1965; 1969; 1977; 1988; 1992, pp. 160–201; see also Troshin 1966).

The Polarized-Oriented Multilayer Theory of Cell Water, or PM theory, is a subsidiary of the AI Hypothesis (Ling 1965; 1972 pp. 663–700; 1984, pp. 163–181; 1992 pp. 69–110; 2004; 2005). In the PM theory, all or virtually all cell water assumes the dynamic structure of polarized-oriented multilayers — in consequence of direct or indirect interaction with parallel arrays of exposed NHCO groups of fully-extended (segments of) intracellular protein(s) (Ling 1965; 1972).

Instantly, the PM theory has offered a theoretical mechanism for one of the most basic manifestation of life phenomenon. That is, as a rule, a large amount of water exists in living cells. As of today, the PM theory is the only known theory that can explain and/or obeys — indeed, fits like hand and glove (i) the specificity of water content to each cell type, (ii) the law of conservation of energy and (iii) the inextractibility of cell water by centrifugation at 1000 g. after surgical ablation of a part of the cell membrane — which does not regenerate (Ling 2005).

Historically speaking, however, the PM theory was introduced in 1965 with its focus on explaining the low concentration of Na^+ in cell water (Ling 1965). Twenty eight years later, a general and quantitative theory of solute distribution in cell water and model systems was introduced (Ling 1993). An important principle that came from this theory is the "size rule." That is, other things being the same, the larger the solute size, the lower its (true) equilibrium distribution coefficient or q-value.

Aiding greatly in the testing and developing the PM theory of cell water were two classes of experimental models, called respectively **extroverts** and **introverts** (Ling 1992, p. 107). Extroverts include gelatin and various denatured proteins, each containing a major part of its polypeptide chains in the **fully extended conformation.** As a result, their backbone NHCO groups are directly exposed to the bulk-phase water**.** Other extroverts include linear polymers carrying properly spaced oxygen or nitrogen atoms (bearing unshared lone-pair electrons). Examples are poly(ethylene oxide) (PEO), polyvinylpyrrolidone (PVP) and polyvinylmethylether (PVME) (Ling *et al*. 1980; 1980a).

In theory, water under the influence of suitable concentrations of extrovert agents should exhibit properties akin to those of water in living cells. Worldwide testing proved that, within the confines of what has been tested so far, they do (for summary see Ling 1992, Table 5.5, p. 108–109). Introvert models include most of what are conventionally called **native** proteins — usually obtainable from biochemical supply stores in a bottle. The majority of (but not all, see below) the peptide CO and NH groups in these so-called native proteins are locked in α-helical and other macromolecular H-bonds. In these folded or otherwise tethered conformations, these backbone NHCO groups are, in theory, less able or unable to alter the physical properties of the bulk-phase water. Experimental studies support this expectation (Ling *et al*. 1980a; 1980b).

The research presented in this article centers on what is known as the *apparent equilibrium distribution* of the polymer, poly(ethylene glycol). With a molecular weight of 4000 daltons, this large polymer is also known by its short name PEG-4000 (for definition of the ρ-value, see below). Historically, this study began as a part of our broader investigation on the size-dependent solvency of various solutes in the cell water and in model systems (Ling and Hu 1988). However, we did not fully complete our PEG-4000

study until very late. Once completed, we realized that the insight the PEG-4000 study revealed went beyond our original aim. It was then that we decided to write a separate paper. This communication is the result.

Materials and Methods

Bovine hemoglobin (about 75% methemoglobin and 25% oxyhemoglobin) (Lot 112F-9300) was purchased from Sigma Chemical Corp, St. Louis, Mo. So was poly(ethylene glycol) with a molecular weight of 3300 (Catalog No. P-3640, Lot 16F-0477). Labeled polyethylene glycol-4000, ^{3}H-PEG-4000, Lot 2109-295 was obtained from Du Pont (formerly New England Nuclear, Boston). Its average molecular weight given was 4000 daltons.

The basic procedure used was the equilibrium dialysis method earlier described (Ling and Hu 1988). However, instead of following the standard procedure of measuring only the uptake of a radioactively labeled solute by the protein solution inside a dialysis sac, in the present study we did it two ways. In one, the radioactively labeled PEG was added initially to the solution outside the sac; in the other set, the labeled PEG was added to the solution inside the sac. The purpose of this dual approach was to remove any uncertainty on the time it took for diffusion equilibrium of the labeled PEG to be attained. The exact knowledge on the time for the attainment of diffusion equilibrium in turn assures the accuracy of the *apparent equilibrium distribution coefficient* (or ρ-value) of the probe molecule obtained. For the ρ-value of an extraordinarily large and thus slow-moving probe molecule like PEG-4000, this extra effort was essential.

It should be made clear here that the ρ-value of a solute could only be equal to or larger than the true equilibrium distribution coefficient (or q-value). While the q-value refers exclusively to *free* PEG (or other probe solute) in the water of the living cell or the water in the dialysis-sac content, the ρ-value may include PEG bound in one way or another to the macromolecules in the cell or the dialysis sac.

Solution A was a 40% (w/v) solution of bovine hemoglobin dissolved in a solution called Solution B. Solution B contained 0.4 M NaCl; 0.01% penicillin G, sodium; 0.01% streptomycin sulfate; 0.01% thymol and 5% (w/v) polyethylene glycol (approximately 12.5 mM, calculated on the basis of the nominal molecular weight of 4000 for the labeled PEG-4000, see below). In a preceding paper, Ling and Hu (1988) have shown that this combination of antibiotics and thymol was able to check completely the growth of bacteria even at 25°C. The low temperature (0°C) of this study further insured the sterility of the system under study.

To each of four 100-ml screw-cap tubes was added 50-ml aliquots of Solution B and four small sacs of Spectra Por 2 dialysis tubing (diameter 6.4 mm, with molecular cut-off at 12,000 to 14,000) containing 1.2 ml.of Solution A (for details of procedure used in filling and tying these sacs, see Ling and Hu 1988, p. 294). A 0.005-ml aliquot of radioactively labeled H^{3}-poly(ethylene glycol)-4000, containing 500 micro-curie of radioactivity per ml, was added to the solution *outside* the sacs in two of the four screw-cap tubes. Similar 0.005-ml aliquots of the same radioactive solution were added to the *inside* of the dialysis sacs of two other screw-cap tubes. Each capped tube was covered completely in at least one layer of thin paraffin film (Parafilm, Fisher Scientific). The tubes were immersed horizontally in a constant temperature bath kept at 0°C and shaken at the rate of 20 excursions per minute, each excursion covering a distance of one inch.

At various time intervals, ranging from one to four day(s), one dialysis sac from each of the four screw-cap tubes was taken out, and aliquots of its content assayed for their water contents and radioactivity. Briefly, each sample removed was divided into two portions. One portion was weighed, dried in an oven at 105°C for 48 hours and weighted again to determine the water and solid content. The other portion was transferred to a preweighed 15 ml-graduated centrifuge tube and the assembly weighed to determine the sample weight. 2.0 ml of 0.3 M LiOH solution was then added to each of these centrifuge tubes. In this alkaline medium, the dried samples were fully dissolved after overnight incubation. 2.0 ml. of 0.6 M trichloroacetic acid (TCA) was then added to each tube, mixed thoroughly with the LiOH extract before centrifugation. Aliquots of the clear supernatant solution (0.50 ml) were mixed with Bray's scintillation fluid before counting on a Packard γ-Scintillation Counter (Bray 1960). Samples of the initial and final bathing solutions were similarly diluted with LiOH-TCA mixture and Bray's scintillation fluid before assaying for radioactivity.

To achieve the highest accuracy possible, we counted all samples three times before and after the addition with a Hamilton micro-pipet of the same volume of a radioactive ^{3}H-bearing solution — containing at least five times more radioactivity than the experimental sample. The large increments in counts allowed us to determine precisely the counting efficiency of each individual sample and its bathing solution, and to apply the *individual* counting efficiency corrections to each sample set.

Results

Somewhere between the 8th day and 12th day of incubation at 0°C, diffusion equilibrium was reached between labeled PEG-4000 inside and outside the dialysis sacs. This is indicated in Figure 1 and Table I by the attainment of the same levels of radioactivity in the sacs where the radioactivity was initially added to the inside and to the outside of the dialysis sac. The numbers, 0.637, 0.632 etc. in parentheses refer to percentage water content of the sac content in each specific sample.

Now, the ordinate of Figure 1 represents the ratio of the concentration of PEG-4000 in the dialysis sac over that in the bathing fluid. This ratio becomes *the apparent equilibrium distribution coefficient* or ρ-*value* toward the end of the time-course study when diffusion equilibrium of the labeled PEG-4000 has been reached. From the ρ-values obtained on the 12th day from both sets of values, we obtained an average ρ-value for the labeled PEG-4000 of 0.196 ± 0.028 (mean ± S.D).

The water content of the solutions inside the sacs on the 12th day was 63.9% ± 0.36%. By difference, the final concentration of native hemoglobin in the sacs was 36.1%. This percentage is close to the concentration (34%) of hemoglobin in most mammalian red blood cells (Ponder 1971, p. 117; Ling 2005).

Discussion and Conclusion

1. How much bulk-phase water was altered by the 36.1% native hemoglobin?

The study described above shows that the ρ-value of PEG-4000 in the 36.1% hemoglobin solution is 0.196. Since 1.000 – 0.196 = 0.804, 80.4% of the water in the dialysis

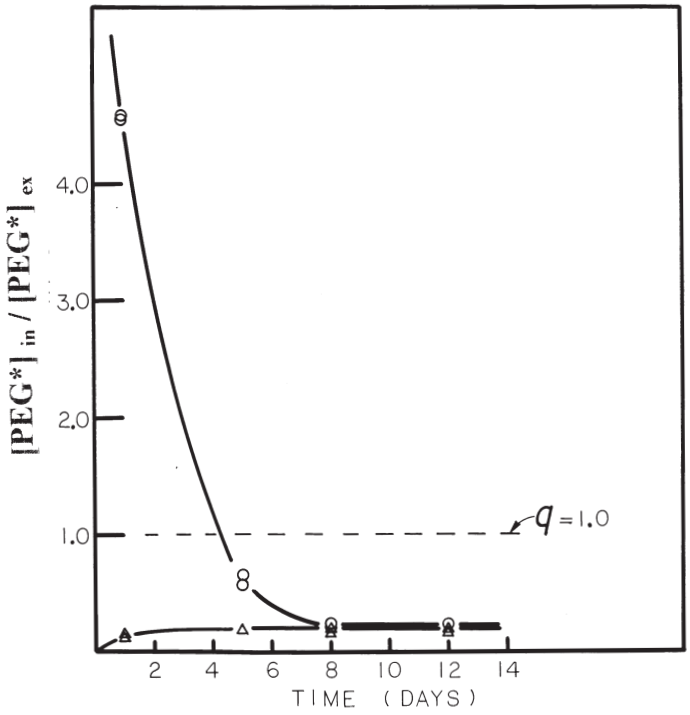

FIGURE 1. The time course of change of the concentration of radioactively labeled poly(ethylene glycol) or PEG-4000 inside a dialysis bag initially containing 40% (w/v) native bovine hemoglobin. The set of data represented by circles came from an experiment in which the radioactively labeled PEG-4000 was added to the inside of the dialysis sac containing the protein. The set of data represented by triangles came from experiments in which the labeled PEG-4000 was added to the outside bathing solution. Dotted line indicates equal concentration of labeled PEG-4000 inside and outside the dialysis bag. Full diffusion equilibrium was attained when the radioactivity of labeled PEG-4000 represented by the circles and that by the triangles reached the same level. The ratio of the labeled PEG-4000 concentration at and after that time represents the apparent equilibrium distribution coefficient or ρ-value of PEG-4000 in the bulk-phase water dominated by the native bovine hemoglobin at 0°C. Numerical data are given in Table I.

TABLE I. Time course of equilibration of labeled PEG-4000 in solution of native hemoglobin.

Incubation time (days)	PEG-Distribution Ratio (Conc. in Sac/External Conc.) 1	5	8	12
Tracer added outside	0.161 (0.637) 0.130 (0.632)	0.202 (0.630)	0.185 (0.628 0.170 (0.629)	0.185 (0.642) 0.176 (0.641)
Tracer added inside	4.599 (0.636) 4.553 (0.632)	0.562 (0.636) 0.661 (0.628)	0.240 (0.634) 0.221 (0.633)	0.238 (0.640) 0.186 (0.634)
Mean ± S.D.			0.196 ± 0.028 (0.639 ± 0.0036)	

Number in parenthesis refers to the water content of the content of the sac yielding the PEG distribution ratio in front of it. Mean and S.D. in the last row is the final ρ-value of PEG-4000 in the hemoglobin solution.

sac appears to have lost its normal solvency for PEG-4000 in consequence of the presence of the hemoglobin. However, this seemingly reasonable quantitative assessment rests upon a rather unlikely assumption. That is, hemoglobin has separated the bulk phase water into two drastically different fractions. One fraction, 80% in quantity, has zero solvency for PEG-4000; the other 20% has 100% full solvency for PEG-4000.

Now, if the 36.1% native hemoglobin has indeed transformed the bulk phase water into these dramatically different fractions, one should be able to confirm their existence with other probe molecules. That, however, is not true.

Ling and Hu have shown earlier that in a solution containing native hemoglobin at an even higher concentration (39%), sucrose (molar volume, 342 cc) and raffinose (molar volume, 504 cc) show q-values of 0.976 and 0. 971 respectively (Ling and Hu 1988). These near-unity q-values indicate a basic homogeneity of the bulk-phase water in the native hemoglobin solution studied. The presence of 36–39% native hemoglobin does not separate the bulk phase water into two diametrically different fractions.

That being the case, the challenging question has shifted. It is now "Why does the same bulk-phase water of a 36 to 39% native hemoglobin solution behave like perfectly normal liquid water to sucrose and raffinose but keeps out PEG-4000 from most of it? To give a meaningful answer, we need a quantitative theory. Ling's PM theory of solute exclusion is the one of choice as we know of no other alternatives.

Very succinctly, the answer lies in the "size rule" mentioned above. Namely, the q-value decreases with increasing molecular size. Sucrose and raffinose exhibit much higher q-values while PEG-4000 exhibits a much lower one, because sucrose and raffinose are roughly ten times smaller than PEG-4000. In the next section, we will go a little deeper into the how's and why's.

2. Ling's quantitative theory of solute distribution — as summarized in his equation of the q-value

In 1993 Ling presented an equation of the q-values of solutes of different size in water polarized-oriented to different levels of intensity (Ling 1993; Ling *et al*. 1993). This equation is reproduced in full in Appendix 1 at the end of this article. In this Equation of solute distribution, the q-value of a solute in (polarized-oriented) water is largely characterized by two parameters: ΔE_v and ΔE_s. For the sake of simplicity, we have omitted from separate consideration the entropy contribution to the q-value. Instead, we consider that the entropy contribution has been incorporated into ΔE_v. (See Equation 26 on p. 157 in Ling 1993).

Now, ΔE_v, the *specific solvent polarization energy* is in units of cal per mole per cm^3; it measures the *difference* in the energy spent in excavating a hole in the polarized-oriented water (to accommodate the probe solute) and the energy recovered in filling up the hole left behind (by the probe solute) in the normal liquid water of the source solution. On the other hand, ΔE_s, the *specific surface polarization energy* is in units of cal per mole per cm^2; it measures the difference between the interaction energy of the surface of the solute with the surrounding polarized-oriented water and that with the normal liquid water of the source solution. At a specific temperature, it is the algebraic sum of the product of molar volume v of the solute and ΔE_v and the product of the surface area of the solute (expressed as a function of v) and ΔE_s that determines the q-value of each solute. For simplicity, Figure 2 shows the q-value due to the action of the volume factor only.

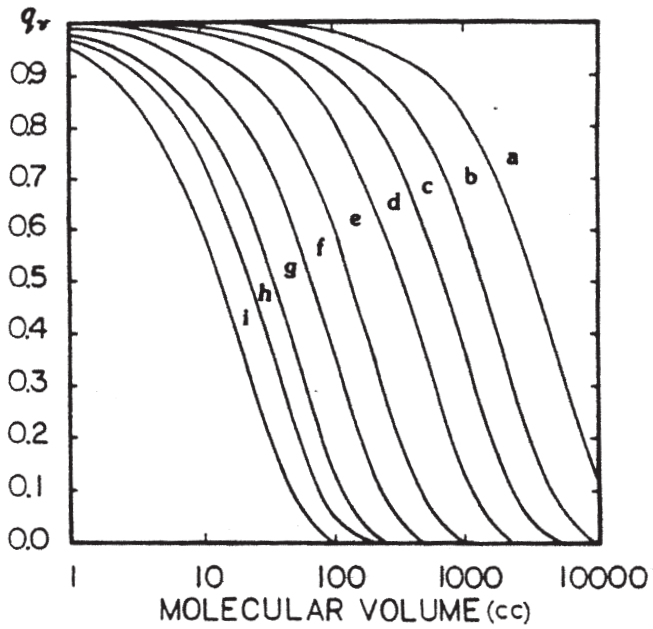

FIGURE 2. The theoretical volume (or solvent) component of the equilibrium distribution coefficient (q_v) for solutes of different molecular volume in water polarized at different intensity (See Equation A3 in Appendix 1.) The intensity of water polarization due to the volume component of the polarization energy is given as the specific solvent polarization energy, ΔE_v. The specific value of ΔE_v in units of RT per cm^3 is indicated by the letter near each curve, where a represents 0.0002; b, 0.0005; c, 0.001; d, 0.002; e, 0.005; f, 0.01; g, 0.02; h, 0.03; i, 0.05. R is the gas constant and T the absolute temperature. At room temperature (25°C), RT is equal to 592 cal./mole. (From Ling 1993)

The ordinate of Figure 2 represents the logarithm of the q-value; the abscissa represents the logarithm of the molecular volumes in units of cm^3 or cc. As examples, the molecular volume of water is 18.02 cc, while that of PEG 4000 is 4000 cc.

The small letters a, b, c etc near each of the curves in Figure 2 indicate the value of ΔE_v used in computing that specific curve and the ΔE_v is given in units of RT, where R is the gas constant and T, the absolute temperature. At room temperature, RT equals 592 cal/mole. Thus, the curve marked with the letter standing for 0.0002 indicates a ΔE_v of $0.0002 \times 592 = 0.118$ cal/mole/cal.

Assuming that the ρ-value of PEG-4000 obtained (0.196) is equal to its q-value, we can draw a horizontal straight line across Figure 2 at that q-value and a vertical straight line at a molecular volume of 4000cc. The point of intersection of these two straight lines gives us an approximate estimate of the ΔE_v of the bulk phase water in the 36.1% hemoglobin solution studied, namely, 0.0004 RT or 0.236 cal/mole/cc. Multiply this figure by the molar volume of water, 18.02 we obtain a volume contribution of the *exclusion intensity*, $\mathcal{U}_{vp}$ (which includes an entropy contribution assumed to be included in the ΔE_v adopted and not explicitly mentioned) of 4.25 cal/mole. The predicted q-value of sucrose and raffinose on the basis of the ΔE_v of 0.236 value and their respective molar volumes are roughly 0.9 and 0.85 to be compared with the experimentally determined values of

0.976 and 0.971 respectively. Because these estimates were made on the basis of the volume contribution only, the agreement must be considered satisfactory.

Thus, Ling's equation of solute distribution in polarized-oriented water can adequately explain at once the low ρ-value of PEG-4000 and high q-values of sucrose and raffinose in an aqueous solution dominated by 36.1% native hemoglobin. And, despite the fact that native hemoglobin like other native proteins in general, is a weak introvert model.

Now, according to the PM theory, (store-bought) native proteins in general fall into the category of weak introverts. (Store-bought) native hemoglobin is no exception. On the other hand, when proteins are made to unfold into a more fully-extended conformation such as by exposure to denaturants like NaOH, they become extroverts. Experimental studies have fully confirmed this.

A parallel study on the q-values of similar family of probe molecules including sucrose and raffinose yielded a $\mathcal{U}_{vp}$ of 16.5 cal/mole of water, which is almost four times higher than the $\mathcal{U}_{vp}$ of 4.25 cal/mole of native hemoglobin (Ling 1993, Table 1 on p. 164) even though the hemoglobin concentration of the NaOH-denatured preparation used was only 20% rather than 36.1% as in the native hemoglobin solution studied here.

3. The much larger amount of water altered than literature values and reconciliation

Hemoglobin makes up 97% of mammalian red cell proteins (Ling *et al*. 1984). There is no known reason to question that this protein plays a correspondingly dominant role in causing the water in red blood cells to exhibit properties different from those of normal liquid water. The fact that even in its folded introvert conformation, it still possess the power to alter all the bulk phase water shows that the difference between the influence of a relatively weak *introvert* model (native hemoglobin) and a much more powerful *extrovert* model (e.g., NaOH denatured hemoglobin) does not lie in the *reach* of the influence of the bulk phase water but in the *intensity* of water-to-water interaction energy of all the water present. This, in fact, is a facet of the PM theory not clearly expressed until 1993 when the quantitative theory of solute exclusion was presented (Ling 1993). Its confirmation coming from the study of the *ineffective* introvert model is at once exciting and intriguing.

However, the present finding that all the water in a 36.1% native hemoglobin solution is altered also tells us that each gram of native hemoglobin has altered the property of all $(1 - 0.361) = 0.639$ grams of water equivalent to 1.77 grams of water altered by one gram of native hemoglobin. How does this compare with figures from other studies on similar native hemoglobin in the literature?

In a review Ling (1972) showed that using a total of six different methods, experts in this field had provided data yielding an average of only 0.25 grams of hydration water per gram of dry native hemoglobin. This is seven times lower than the 1.77 g/g value from the present study. Nonetheless, this divergence does not indicate real conflict.

The explanation we offer for this sharp contrast is this. To begin, we are not dealing with a fixed amount of altered water in a unit volume of total water and accordingly every competent investigator using a competent method would arrive at the same percentage figure of protein-altered water. Rather, each method could have measured a different portion of the protein-altered water. Indeed, as pointed out by Ling recently (Ling 2003) it is possible that a majority of the older methods used to measure hemoglobin hydration in fact measures the fraction of water tightly bound to polar side chains. In contrast, solvency

probes like PEG-4000 measure all, or close to all, water in the protein solution (Ling 2004).

4. What part(s) of the native hemoglobin molecules polarize water weakly but still extensively*?*

The polypeptide chains of store-bought "native" hemoglobin exist largely in the folded α-helical conformation. Yet data shown in Figure 1 and Table I clearly demonstrate that even with most of its backbone NHCO groups locked in intra-macromolecular H bonds, this native hemoglobin at a concentration of 36% still can exercise a long-range water-polarizing power on all the bulk phase water present. What part(s) of the "native" hemoglobin molecule participate in this long-range alteration of the bulk of surrounding water?

The part of a protein molecule that potentially could influence the surrounding water can be put into three categories. They are respectively (i) the α-helically-folded polypeptide backbone; (ii) polar side chains and (iii) segments of fully-extended polypeptide chains with their NHCO groups directly exposed to the bulk phase water. Our best guess at this time is that in the order of decreasing effectivess, the categories fall in the order: (iii) > (ii) > (i).

But before entering into the details, it would be helpful to take a backward glance at a figure that Ling presented in his 1972 review and is reproduced here as Figure 3. The different figures labeled a to f in Figure 3 illustrate how the spatial distribution of negatively charged N sites, positively charged P sites and sites bearing no charges (called O sites) determine the depth of layers of water or other polar molecules. A checkerboard of alternating N and P sites at proper distance apart is called an NP systems (c,d). They and their equivalent checkerboard of P and O sites or N and O sites respectively called PO and NO systems (e,f) are theoretically capable of polarizing and orienting deep layers of water molecules. So are parallel arrays of linear chains carrying alternatingly N and P sites etc. with similar power. In contrast, a checkerboard of all P sites or all N sites (a,b) is not able in theory to achieve multilayer polarization and orientation of water molecules because water dipoles oriented in the same direction strongly repel each other. With these guidelines in mind, we now can proceed to take a deeper look into each of the three types of potential polarizing-orienting sites mentioned above under (i), (ii) and (iii).

Let us begin with a piece of evidence that argues against the idea that α-helically-folded polypeptide chains could polarize and orient multlayers of bulk-phase water. Doty and Gratzer (1962) demonstrated long ago that a block polymer of poly-L-alanine (130 residues) when sandwiched between two water soluble-block polymers of D- and L-glutamic acids (each with 200 residues), remain in the fully folded and water insoluble form even at a temperature as high as 95°C or in the presence of 8 M urea. That total insolubility in water demonstrates that the potentially water-interacting polar NH and CO groups have lost their ability to interact with bulk-phase water when they form α-helical folds.

There are three main types of polar side chains. They are the (1) anionic α-, β- and γ-carboxylate groups, (2) the cationic ε-amino and guanidyl groups and (3) the hydroxyl groups of serine, threonine and histidine. No doubt that some polar side chains can exercise some influences on some surrounding water especially the hydroxyl groups. But there are overwhelming evidence that every α-, β- and γ-carboxylate group as well as every ε-amino and guanidyl group in native hemoglobin are locked in what is known as salt

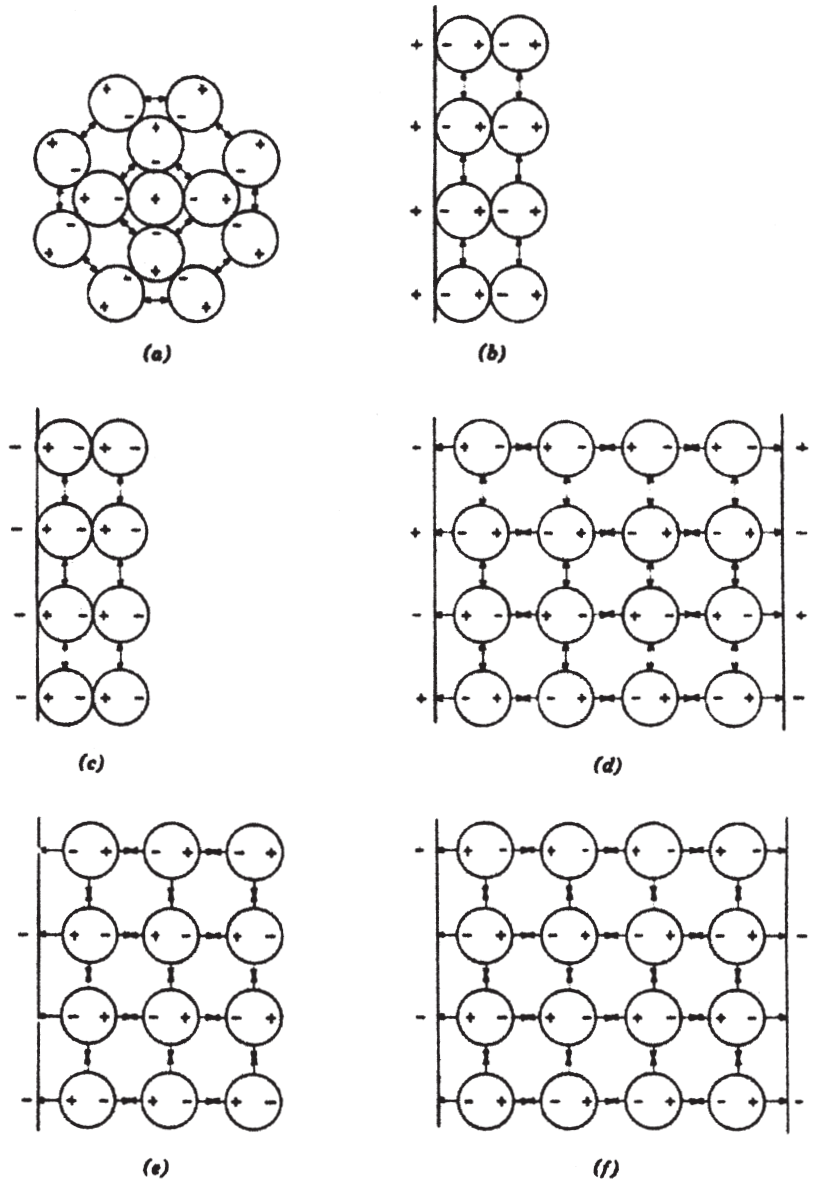

FIGURE. 3. Diagrammatic illustration of the way that individual ions (a) and checkerboards of evenly distributed positively charged P sites alone (b) or negatively charged N sites alone (c) polarize and orient water molecules in immediate contact and farther away. Emphasis was, however, on uniformly distanced bipolar surfaces containing alternatingly positive (P) and negative (N) sites called an NP surface (d). When two juxtaposed NP surfaces are facing one another, the system is called an NP-NP system. If one type of charged sites is replaced with vacant sites, the system would be referred to as PO or NO surface (e). Juxtaposed NO or PO surfaces constitutes respectively an PO-PO system or NO-NO system. Not shown here is the NP-NP-NP system comprising parallel arrays of linear chains carrying properly distanced alternating N and P sites. (From Ling 1972 reprinted with permission of John Wiley & Sons, Inc.)

linkages (See Steinhardt and Zaiser 1955; Edsall and Wyman 1958, p. 539; Ling and Zhang 1984). Being as a whole electrically neutral, polar groups locked in salt linkages are less prone to interact with surrounding water.

Going back to Figure 3, we may say that if these positively charged and negatively charged groups are arranged in an order like that shown in Figure 3c and d, they would end up neutralizing each other by forming salt linkage pairs. Like the NHCO groups locked in α-helices, they are neutral and ineffective. On the other hand, if they stay in groups of all negative or all positive, they would fall into the category of Figure 3a and b, and thus also be ineffective. However, this set of prohibiting rules does not apply to the hydroxyl groups. A hydroxyl group has both a proton accepting site and a proton-donating site. An array of stretches of all OH groups may find itself functioning like chains of the fully extended protein backbone NHCO. And, as such, achieve long-range water polarization and orientation. However, an examination of known primary structure of proteins does not reveal large stretches of hydroxyl bearing serine, threonine and tyrosine in close proximity, so the greatest potentially effective NPNPNP system remains parallel arrays of fully-extended NHCO groups of the polypeptide chains.

Now, many workers in this field of study believe that about 75% of the polypeptide chains of native hemoglobin exist in the α-helical conformation (See Weissbluth 1974, p. 20). To this must be added the other non-water polarizing segments of the hemoglobin chains engaged in the β-pleated sheet conformation. All in all, they set an upper limit to the percentage of fully-extended segments at less than 25%. Next, we would like to take into consideration factors that would raise the percentage of fully extended polypeptide linkages in native hemoglobin to higher than below 25%.

Accurate determination of the fine structures of proteins relies heavily on X-ray studies and X-ray studies can only be carried out on proteins when their constituent atoms remain sufficiently stationary to be photographed, i.e., in a crystalline state. To induce a dissolved hemoglobin (and other proteins) to crystallize, a common procedure is to add a great deal of innocuous salts to a concentrated hemoglobin solution in order to reduce the activity of surrounding water. By the Le Chatelier Principle the protein is driven to adopt the crystalline conformation that X-ray crystallography can investigate with precision. One must remember that it was this type of X-ray investigation that gave us the 75% figure for the percentage in the α-helical conformation.

All this points out that in the 36% native hemoglobin solution, the percentage of the hemoglobin's peptide bonds engaged in α-helical conformation may be less than the widely-accepted 75% figure. And, as a result, the total percentage of coils, turns and other segments of exposed NHCO groups may be equal to or even higher than 25%.

5. Are multilayers of water polarized and oriented in a 36% solution of native hemoglobin*?*

We know that all the bulk-phase water in a 36% native hemoglobin solution has lost on the average 80% of its normal solvency for PEG-4000. We now want to find out if this observed fact is in harmony with the notion that here also multilayers of water had been altered by the *native* hemoglobin.

First, we must find out how many moles of water there are in a 36% hemoglobin solution. To do that, we need to know what is the volume of the hemoglobin in a 36% solution. Most proteins have a *partial specific volume* of 0.70 to 0.75 cc per gram (Edsall

1953, p. 563). For safety, we adopt the larger figure of 0.75 and find out that in a liter of the 36% hemoglobin solution, $360 \times 0.75 = 270$ milliliters are taken up by the protein. The remaining volume of 730 ml is occupied by water. Assuming a specific gravity of unity for the water, 730 ml of water is equivalent to 730 / 18.02 = 40.5 moles of water molecules. Since the ρ-value of PEG-4000 is 0.196, of the 40.5 moles of water in one liter of the protein solution, at a minimum $40.5 \times (1 - 0.196) = 32.6$ moles is altered by the presence of the native hemoglobin.

The average amino acid residue weight in many proteins is estimated to be 112 (Ling 1962, p. 48). Dividing this number into the weight of hemoglobin in a liter of 36% hemoglobin solution would yield the maximum number of amino-acid residues and hence also the approximate number of polypeptide NHCO groups in the 36% hemoglobin solution. And, this equals 360/ 112 = 3.21 moles of NHCO groups per liter of the protein solution.

Now, each free NH group forms a H-bond with one water molecule; each CO groups can form H-bonds with two water molecules. Thus the first layer of water molecules acted on by each NHCO group is three. Each of these three water molecules can in turn form H-bonds with three water molecules, thereby providing a total of nine water-adsorbing "sites."

Let us start out from an exaggerated estimate and assume that 50% of the polypeptide chains exist in the fully-extended conformation. The number of water molecules affected by each of these exposed NHCO groups would be $32.6 / (0.5 \times 3.21) = 20.2$ water molecules. This would be enough to cover the first layer (3), the second layer (9) and still have $20.2 - (3 + 9) = 8.2$ water molecules to fill up a third layer of affected water molecules. There is then no question that our data of PEG-4000 exclusion by a 36% bovine hemoglobin solution has demonstrated that at this concentration, multiple layers of water have lost their normal solvency for PEG-4000.

However, the assumption that 50% of the polypeptide NHCO groups of native hemoglobin exist in the fully-extended conformation is far too high. Half of that, or 25% may be closer to what one may say to be reasonble. With the 25% figure, the number of water molecules affected by each NHCO group would rise to $32.6/ (0.25 \times 3.21) = 40.4$ water molecules. Subtracting 3 molecules in the first layer, 9 in the second layer, and 27 in the third layer, we still have a few left to begin yet another layer.

With fully three or even more layers of water affected by each exposed NHCO groups, the number of layers of affected water molecules between adjacent protein chains would be six or more layers deep.

6. The recent theoretical breakthrough in the long-range dynamical water structuring and its wide significance for both the study of the inanimate world and the living

In 1965 Ling first introduced what is now called the Polarized-Oriented Multilayer (PM) Theory of Cell Water. It is a subsidiary of his unifying theory of cell and subcellular physiology, the association-induction (AI) hypothesis introduced three years earlier (Ling 1962; 1965). Although worldwide experimental confirmation of the AI Hypothesis in general and the PM theory in particular has been uniformly affirmative, its broad acceptance has been unbelievably slow to say the least. The major cause of this is political (See Ling 1997). Nonetheless, there are also some legitimate reasons for the slowness in response. That is, the long range dynamical water structuring, until very recently, lacked a solid theoretical foundation.

That gas and water vapor can gather in deep layers has been known for a long time (See McBain 1932; Henniker 1949). Within a ten year period between 1929 and 1938, no less than four sets of rigorously derived theoretical treatments were presented — by some of the most outstanding physicists of the time (deBoer and Zwikker 1929; Bradley 1936, 1936a; Brunauer, Emmett and Teller 1938). Yet in different ways, each of these theories failed. The overall comment is that the admirable sophisticated methods developed by physicists in studying simple systems encounter great trouble when used to explain natural phenomena like life, which is inextricably complex.

As pointed out by Brunauer *et al*., de Boer and Zwikker's polarization theory as well as Bradley's theory of non-polar gases could not predict more than one layer of adsorbed gas. Brunauer, Emmett and Teller's own theory (known as the BET theory from the first letters of their names) also cannot explain the large quantity of water taken up at near-saturation humidity, nor the altered physiochemical attributes of this water. That leaves only Bradley's theory for gas molecules with permanent dipole moments. And, until 2003, that was the best theoretical foundation for the PM theory. Then there was a lucky break. Ling found a simplying shortcut.

As a result, Ling was able to introduce a new theory with far-reaching consequences. Briefly, under ideal conditions, an idealized checkerboard of alterrningly positively-, and negatively-charged sites of just the right distance apart as illustrated in Figure 4 will polarize and orient multiple layers of water. As one moves farther and farther away from the idealized NP-surface, the water-to-water interaction energy (E_n) does not taper off to zero

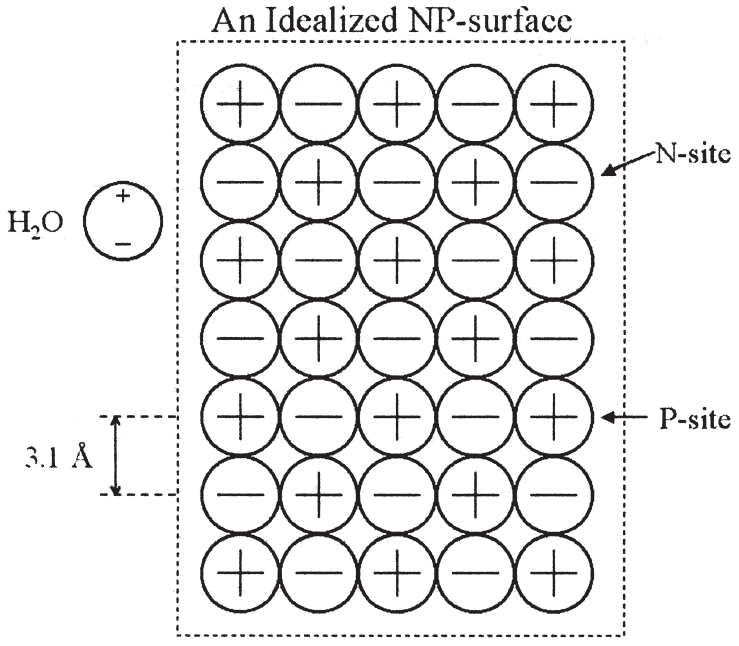

FIGURE 4. An idealized NP surface. The distance between a pair of the nearest-neighboring N and P site is equal to the distance, r, between neighboring water molecules in the normal liquid state and approximately 3.1 Å. (From Ling 2003)

but stays at a constant value *ad infinitum* described by the following equation and illustrated in Figure 5;

$$E_n = (4\mu^2 r^3) / (r^3 - 8\alpha)^2. \quad (1)$$

Based on this finding, three predictions could be made and each had been affirmed retroactively:

1. Thin layer of water held between nearly ideal NP surfaces like that of polished AgCl prisms would not freeze at any attainable temperature. In fact, this was what Canadian chemists, Giguère and Harvey discovered by accident and reported fifty years ago (Giguère and Harvey 1956).
2. Thin layer of water held between near ideal polished NP surfaces would not boil at temperature as high as 400°C. This too was observed and reported fifty years ago by Hori (Hori 1956).
3. Truly long-range (ideally *ad infinitum*) water polarization and orientation under proper conditions (e.g., exposure to NP-, NO-, PO surfaces) was predicted by the new theory and it too was retroactively confirmed but more recently.

   Thus, in 2003 Zheng and Pollack demonstrated exclusion of 10 μm wide, coated latex microspheres at a distance of 100 μm or still farther from the surface of polyvinylalcohol gel (Zheng and Pollack 2003).

Leaving a detailed analysis of Zheng and Pollack's spectacular finding to a later paper, we conclude this communication with a backward look at history, armed with the simple "size rule" of the PM theory.

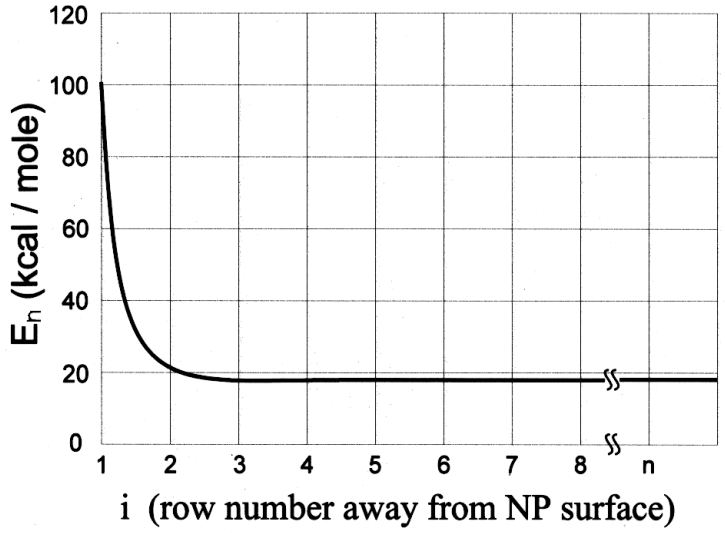

FIGURE 5. The theoretically computed adsorption energy of a water molecule, E_n, in one of successive layers of water molecules away from an idealized NP surface at a temperature very near absolute zero. Note that as the distance between the water molecule and idealized NP surface increases, the adsorption energy does not taper off to zero. Rather, it continues at a constant value described by Equation 1. For details on the makeup of an idealized NP surface, see Figure 4 above. (From Ling 2003)

That is, how the exclusion of PEG 4000, the subject matter of this communication, offers a link between the exclusion from the polarized-oriented water of small particles like hydrated Na^+ and sucrose on one end and the gigantic particles like the coated latex microspheres at the other extreme. In-between are the multitudes of similar exciting findings of deep layers of water altered by special surfaces (or parallel arrays of linear chains) (Henniker 1949) that have been relegated to the ranks of the curious and the forgotten — for the lack of a supportive physical theory. That has finally changed.

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Appendix 1

$$q = \exp\left\{\frac{1.23v\Delta E_s\left[1-(1-b)\dfrac{(kv)^n}{1+(kv)^n}\right]-(\Delta E_v = 1.23\Delta e^*)v}{RT}\right\} \tag{A3}$$

where q is the equilibrium distribution coefficient of the solute in question — v is the molecular volume (molar volume) of the solute and it is in cm^3. b is a small fractional number describing the probability of (very large) molecules in finding adsorbing sites on the water lattice. k and n are parameters describing the steepness of the declining probability of finding adsorbing sites with increase of molecular volume. ΔE_s is the *specific surface (or solute) polarization energy* per cm^2 in units of $cal.mol^{-1}$ $(cm^2)^{-1}$, when the solute is moved from normal liquid water to the polarized cell water. ΔE_v is the *specific solvent polarization energy*, equal to the difference between the energy spent in excavating a hole 1 cm^3 in size in the polarized (cell) water and the energy recovered in filling up a 1 cm^3 hole left behind in the surrounding normal liquid water; it is in units of $cal.mole^{-1}(cm^3)^{-1}$.Δe^* is the *increment of the activation energy* for overcoming the greater rotational restriction per unit surface area in units of $cal.mole^{-1}(cm^2)^{-1}$, when a solute is transferred from normal liquid water phase to the polarized water phase. R and T are the gas constant and absolute temperature respectively.

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