STUDIES ON THE PHYSICAL STATE OF WATER IN LIVING CELLS AND MODEL SYSTEMS. I. THE QUANTITATIVE RELATIONSHIP BETWEEN THE CONCENTRATION OF GELATIN AND CERTAIN OXYGEN-CONTAINING POLYMERS AND THEIR INFLUENCE UPON THE SOLUBILITY OF WATER FOR NA' SALTS

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• *The quantitative relationships between the concentrations of solutions of gelatin, polyvinylpyrrolidone, poly(ethylene oxide), polyvinylmethylether, and poly(ethylene glycol), and their ability to reduce the solubility of water for Na citrate arepresented. The data in general are in harmony with the polarized multilayer theory ofprotein (and polymer) dominated water in vitro and in living cells.*

INTRODUCTION

According to the membrane-pump theory, cell water and ions are largely free as in a dilute aqueous salt solution. To explain the asymmetrical distribution of Na' and other permeant solutes, membrane pumps were postulated. Extensive evidence now exists against the membrane-pump theory. For example, (i) pumps require an amount of energy greater than that available;[5,14] (ii) closed membrane sacs without cytoplasm do not generate and maintain asymmetrical Na' or K' gradients,[9,15] while cells without functional cell membranes do;[11] (iii) in the membrane-pump theory, the major intracellular cation, K^+, must exist in a free state in order to explain the maintenance of osmotic balance and the generation of a "membrane potential"; recent evidence shows that cell K^+ does not exist in a free state but is adsorbed on specific sites on intracellular proteins.[1,2,3,9,19] While contradicting the membrane-pump theory, these findings are either in harmony with or directly support an alternative theory of the living cell, the association-induction hypothesis.[4,5,6,7,10,12] According to this hypothesis, the high level of cell K' is due to specific adsorption of this cation on proteinaceous anionic sites (e.g., β- and γ-carboxyl groups) and the low levels of Na', sugars, and free amino acids reflect a unitary cause: reduced solubility in the cell water of these and other "large" and complex molecules and hydrated ions. Due to interaction with a matrix of protein chains, called the matrix proteins — which are postulated to exist throughout the cell interior of all cells — the bulk of cell water exists in the state of polarized multilayers. The postulated matrix. proteins in this theory must exist in an extended conformation with their polypeptide chain NH and CO groups directly exposed to the bulk phase water, providing anchoring and polarizing sites for multiple layers of water molecules.

Recently reported studies of several model systems lend support to this view. Proteins which, for structural reasons (e.g., gelatin) or in response to secondary-structure breakers (e.g., urea, guanidine HCl), exist in an extended conformation, reduce the solvency of the bulk phase water for Na^+ salts, sucrose, and glycine. In contrast, many native globular proteins with their NH and CO groups locked in a-helical or other intramacromolecular H-bonds are ineffective. Several synthetic polymers resemble the postulated

matrix proteins and also carry oxygen atoms at a distance roughly equal to two water diameters from the nearest neighboring oxygen atoms and are unable to form intra-macromolecular or intermacromolecular H-bonds. These polymers, including polyvinyl-pyrrolidone (PVP), poly(ethylene oxide) (PEO), polyvinylmethylether (PVME), and a number of native gums, polysaccharides, etc., also reduce water solvency for Na^+ salts, sucrose and glycine.[16,17] In the terminology of the association-induction hypothesis, a matrix of extended protein chains with alternatingly negative (N) CO and positive (P) NH sites is called an NP-NP-NP system, while a matrix of polymer chains like PVP, and PEO with only negative (N) oxygen separated from each other by vacant (O) sites is called an NO-NO-NO system.

These water-polarizing polymers provide a way to produce experimental models of water in a physical state (the state of polarized multilayers) hitherto known only in theory. The reduced solvency of water in this state is highly relevant to the physiological role of cell water in the maintenance of cell solutes at physiological levels; it also makes it possible to measure quantitatively the minimal amount of water affected by the polymer present. To date, our published data on this subject have been presented only in a piecemeal manner. The present communication presents in a systematic way the quantitative relationship between the degree of solvency change and the concentration of the water-polarizing polymer present. Results of studies of a new polymer, poly(ethylene glycol) are also presented.

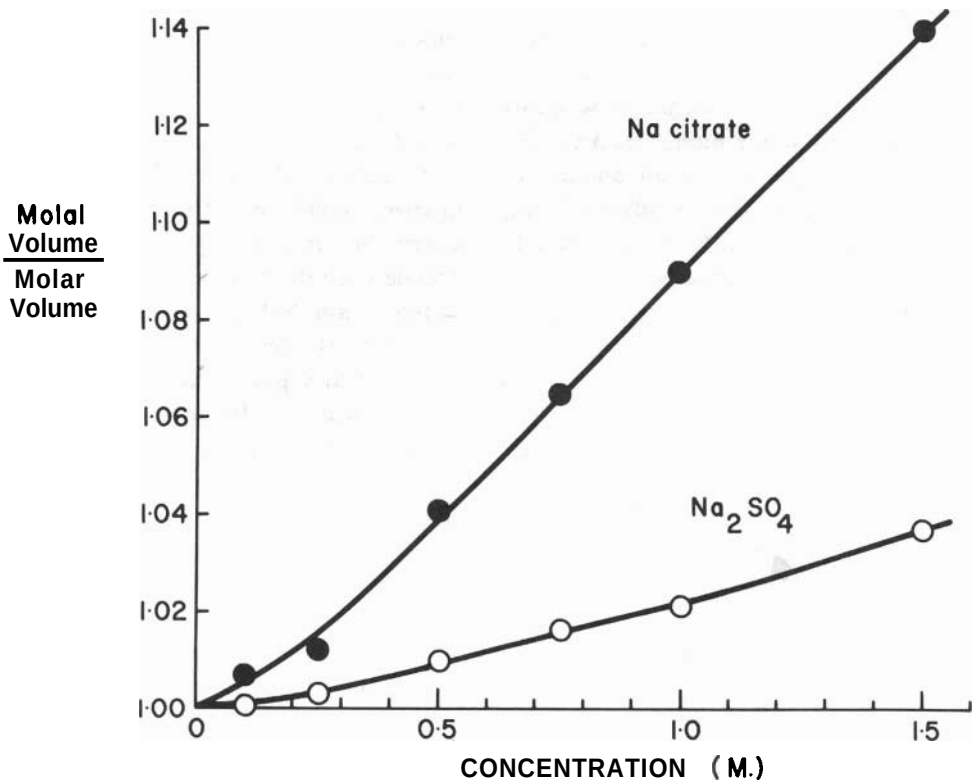


FIGURE I. The molal-molar conversion factor for solutions of Na citrate and Na sulfate.

MATERIALS AND METHODS

The basic method used was equilibrium dialysis by procedures described in earlier work.[16,17] Polymer solutions (20 to 40%) were projected into ¼ inch dialysis tubing and incubated with gentle shaking in solutions of various concentration of Na citrate labelled with ^{22}Na. Incubation was, in most cases, at 25° ± 1°C in a constant temperature room for a length of time long enough to insure equilibrium (2 to 3 days). At the conclusion of the experiment, the labelled Na^+ content was assayed with the aid of a y-scintillation counter and expressed as a ratio to the labelled Na' concentration in the external bathing solution, called the apparent equilibrium distribution coefficient or p-value.

$$\rho = \frac{\text{Na' concentration in the sac}}{\text{Na' concentration in the bathing solution}}$$

The p-value of Na^+ (as citrate) equals the true equilibrium distribution coefficient (or q-value) of Na' when there is no adsorption on or complexing of this ion with macromolecules and when the Na' is entirely dissolved in the water in the sac. The water content within the sac and in the external bathing solution were determined by weighing before and after drying under vacuum in a 100°C oven for PVME, PVP, and gelatin and in a 60°C oven for PEO.

By definition, both the q-value and the ρ-value are ratios of *molar* concentrations. The molar concentration of the probe molecules or ions in the bathing solution is easily determined by dividing the measured probe content in moles by the volume of the solution. The molar concentration of the probe molecule inside the sac is less simple since the sac contains a high content of the polymer under study. The method chosen to obtain the molar concentration is to determine first the *molal* concentration of the probe by dividing the quantity of probe in moles in a sample by its water content (in liters). The

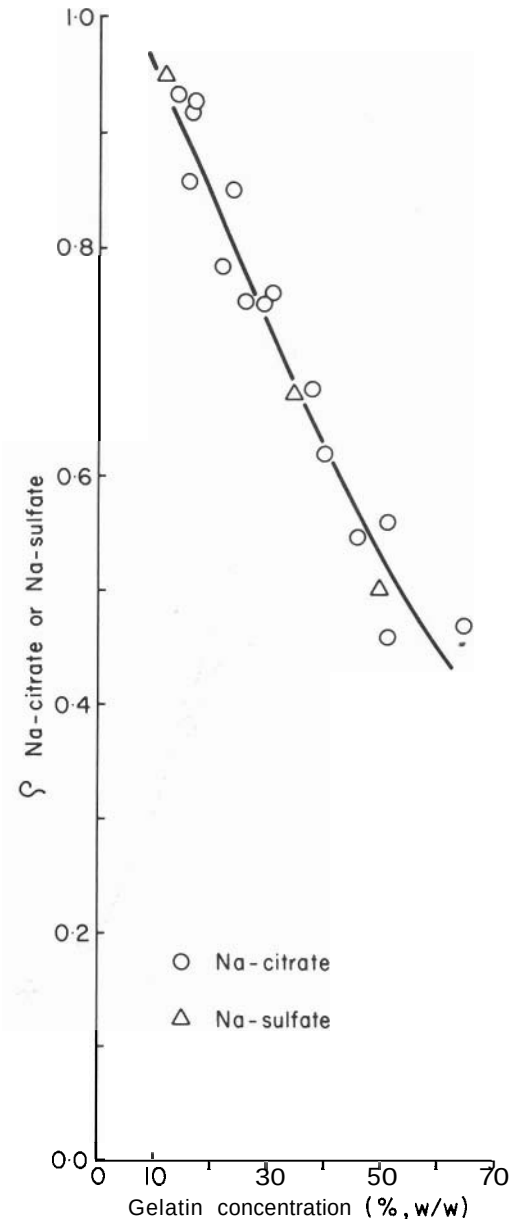


FIGURE 2. The apparent equilibrium distribution coefficient (p-value) of Na citrate between various concentrations of gelatin solutions in the bags and in the external solution (37°C). The higher temperature was to keep the gelatin in the same fluid state as in other polymers. For data at 25°C and 0°C see Ling *et al.*[17]

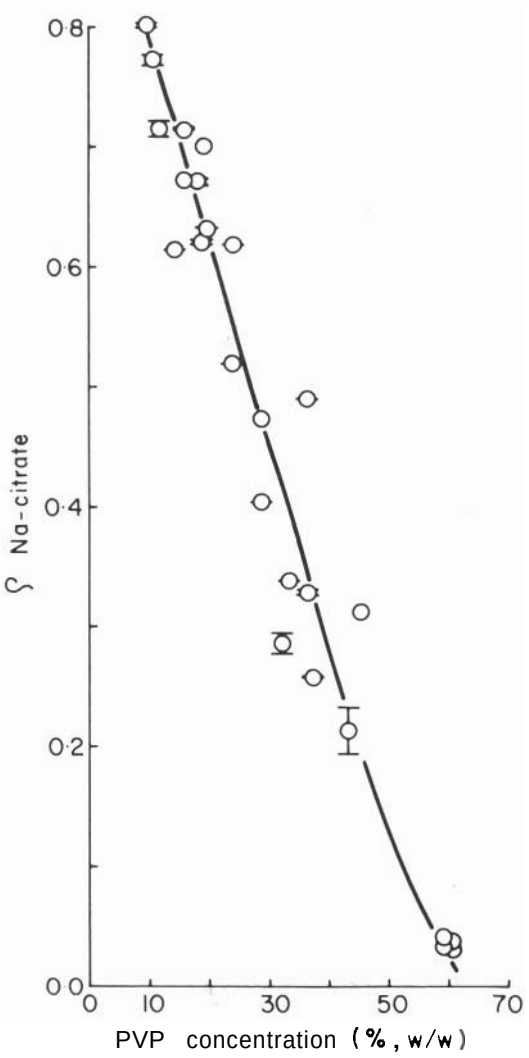


FIGURE 3. The apparent equilibrium distribution coefficient (p-value) of Na citrate between various concentration of *PVP* solutes in the bag and in the external solution at various concentrations of *PVP* (25°C).

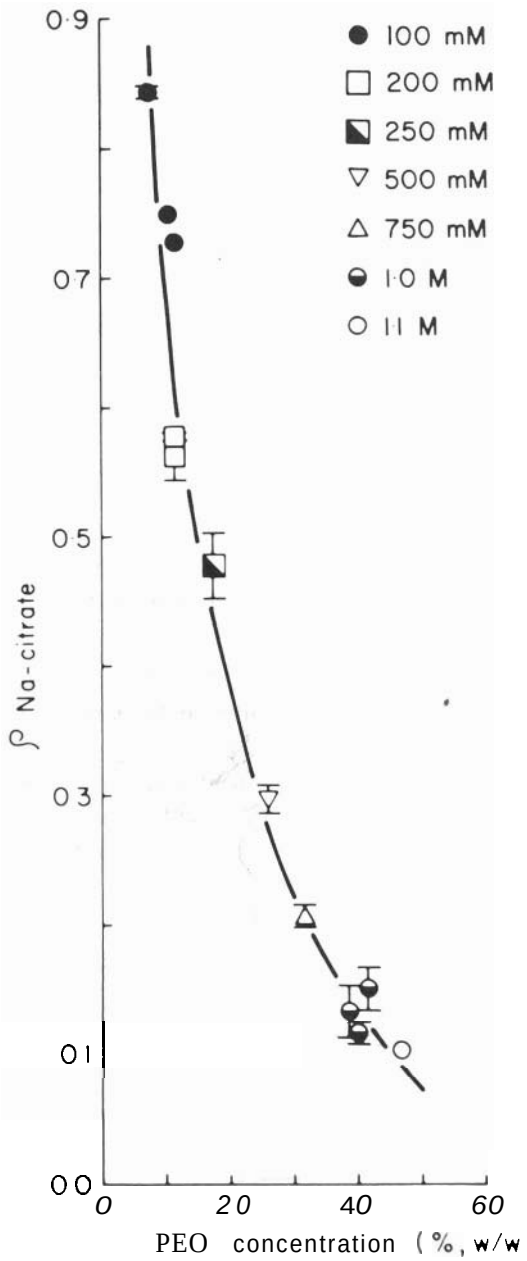


FIGURE 4. The apparent equilibrium distribution coefficient (p-value) of Na citrate between water in the presence of varying concentrations of poly(ethylene oxide) in the dialysis sacs and the water in the external solutions containing no polymers (25°C). Combined results of 4 sets of experiments. Distances between bars represent twice the standard error.

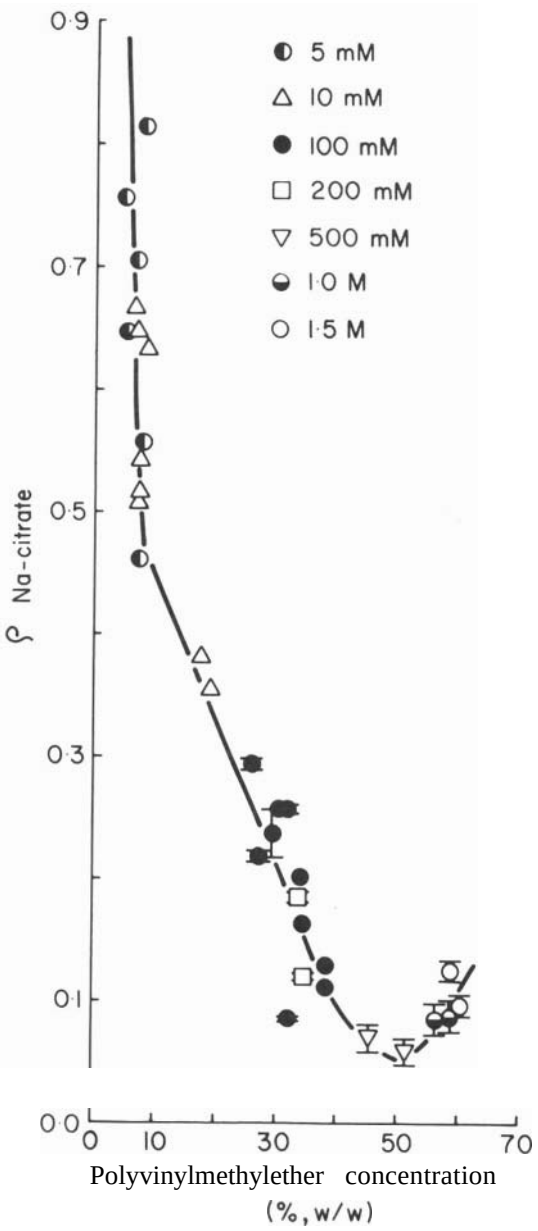


FIGURE 5. The apparent equilibrium distribution coefficient (p-value) of Na citrate between varying concentration of polyvinylmethylether solutions in the dialysis sacs and the external solutions (25°C). The initial Na citrate (or sulfate) concentrations in the bathing solutions were from 5 mM to 1.5 M and are indicated in the figure. Combined data from 7 sets of experiments. Distances between bars represents twice the standard error. Points at low PVME concentration were given as single determinations; the great variability of the water and polymer contents made averaging of these data undesirable.

molal concentration of the probe in the sac thus obtained is then converted to the molar concentration with the aid of the data given in Figure 1 which presents the molal to molar conversion ratios for the Na citrate and Na sulfate at 25°C.

The p-value determined was used to calculate the amount of Na citrate in the sac, which was subtracted from the total dry weight of the sac content to yield by difference the final polymer concentration in percentage (w/w) in the sac.

To determine the molar concentrations of Na citrate and Na sulfate prepared in molal concentrations, calibrated Babcock "milk bottles" with thin and long graduated necks were used (A. H. Thomas, Phila., PA).

Gelatin manufactured from pig and calf skin was obtained from Eastman and from Fisher Scientific Co., Phila., PA. PVP was from Sigma Chemical Co., St. Louis, MO., (Mol. wt. 360,000). PVME (Gantrez M-154) was in part a gift and purchased from GAF Corp., New York, NY. PEO (Polyox 205®) was a gift from Union Carbide, New York, NY. ^{22}Na was obtained from ICN, Irvine, CA (Lots 39 and 40) and from New England Nuclear, Boston, MA (Lots 4771CG10 and 5812RG6). Poly(ethylene glycol) (Carbowax, PEG 20,000) was from Fisher Scientific Co., Phila., PA (Lot 714-714).

RESULTS AND DISCUSSION

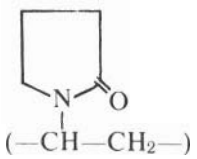
Figures 2, 3, 4, 5, and 6 present the *p*-values for Na citrate in the presence of different concentrations of gelatin, PVP, PEO, PVME, and PEG respectively. The data on gelatin, PVP, PEO, and PVME are those accumulated between 1976 and 1982 and include previously published data.[16,17] The data on PEG are new.

When compared on equal weight percentage basis, gelatin was the least effective in reducing the water solvency for Na citrate. The most effective are PEG and

PVME with PEO and PVP falling in between PVME and gelatin.

Figures 2 to 6 provide the data to calculate the minimum number of water molecules that have been affected by interaction with the polymer at a specific concentration. Thus, if at a polymer concentration of, say, 20% the p-value for Na citrate is 0.5, then $\frac{(1-0.2)\times 0.5)}{0.2}$ = 2 grams of water per gram of dry polymer has appeared to have completely lost its solvency for Na citrate. This water has been referred to as the apparent minimal "non-solvent" water (AMINOW).[16]

Since each of the polymers studied contain only one kind of monomeric unit and since there are good reasons to believe that the sites of polymer-water interaction are the oxygen atoms present in each monomeric unit, the minimal amount of affected water can be more meaningfully expressed in terms of the number of water molecules per oxygen atom or per monomer unit of the polymer. These monomers are



$(-CH-CH_2-)$ for PVP, $(-\underset{OH}{\underset{|}{C}}H-)$ for PEG, $(-CH_2-O-CH_2-)$ for PEO, and $(-\underset{OCH_3}{\underset{|}{C}}H-CH_2-)$ for PVME, with monomer weights of 112.15, 32.04, 44.05, and 58.08 respectively. As an example, for the calculation of the molar AMINOW, we chose a 20% PEO solution with a ρ-value equal to 0.5. In this solution, there are 200144.05 = 4.54 moles of monomers each carrying one oxygen site. The minimum number of water molecules affected by each oxygen site is then

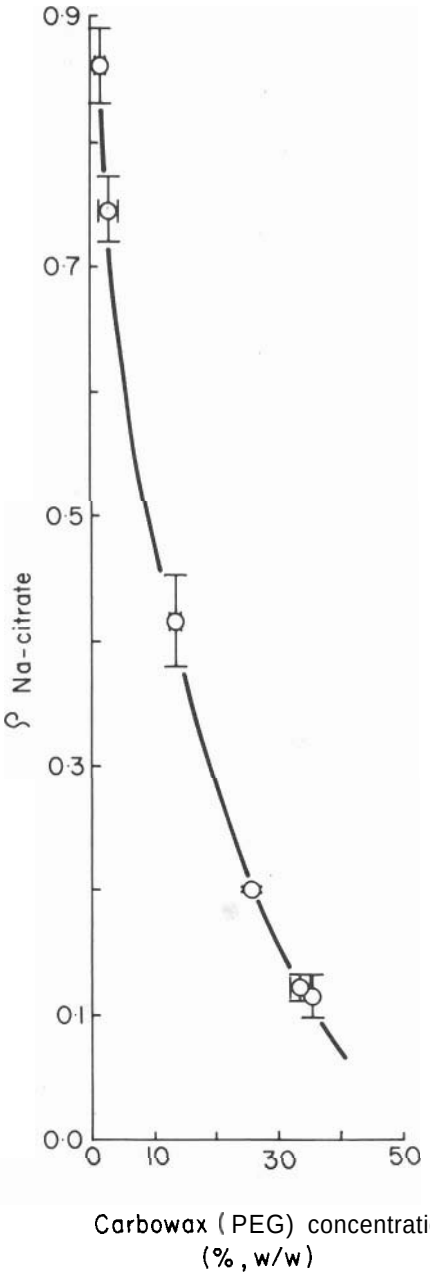


FIGURE 6. The apparent equilibrium distribution coefficient (p-value) of Na citrate between various concentrations of PEG solution and the external solutions (25°C).

$\frac{55.5 \times (1\text{-}0.2) \times 0.5}{4.54} = 4.89$. From the smoothed curves of Figures 2 to 6 the *p*-values and AMINOWS for all concentrations of PVP, PEO, PVME, and PEG are tabulated in Table 1. We have no idea how many of the NHCO groups in gelatin are free are not locked in "collagen folds"[19] and thus made unavailable in reacting with bulk phase water. Therefore we could not make a similar estimate of AMINOW for gelatin.

Figure 7 plots the molar AMINOW at different concentrations of the polymer. Note that as the concentrations of PEO and PVME increase there is first a rapid rise of AMINOW, followed by a decline. The rise and decline are absent or less conspicuous for PEG and PVP in the concentration ranges studied.

The low AMINOW at very low PEO and PVME content suggests that the existence of oxygen atoms on the polymer chain at right distances apart is, by itself, an insufficient condition for the maximum reduction of water solvency toward Na citrate. It seems that the polymer to polymer relation may play a role in the enhancement of the water-solvency reducing effect. In the polarized multilayer theory, the role of the chain to chain interaction is to reinforce water polarization as the chain-to-chain distance decreases, diagrammatically illustrated in Figure 8. However, when the chain-to-chain distance becomes too close (i.e., polymer concentration too high), the number of water molecules with "non-solvent" properties will decrease as the water molecules polarized by one chain overlap those polarized by neighboring chains. This decreased efficiency may not only reflect duplication of the polarizing effects but may also involve mutual cancellation since in these cases all polarizing sites carry electrical charges of the same sign.

At a 7.5% concentration, the molar AMINOW of PVME reaches a figure of about **20.** There are reasons to believe that under favorable conditions the actual number of water molecules that an oxygen atom of PVME can polarize may exceed **20** considerably: (1) the polymer chains in our samples must be highly random in distribution. Many oxygen sites are therefore too close to other oxygen sites, and overlap of their polarization realms reduces the average number of water polarized by these sites. In support of this contention, Ling *et. al.*[16,17] already showed that stirring, which tends to

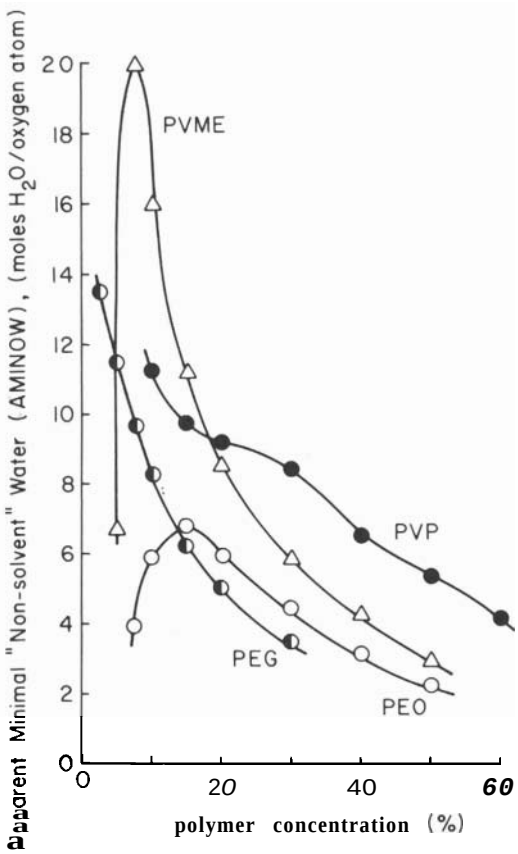


FIGURE 7. The apparent minimal "non-solvent" water (AMINOW) to Na citrate in solutions of PVP, PEO, PVME, and PEG solutions of different concentrations. The molar AMINOW is given in average number of H_2O molecules per oxygen atom in the polymer.

line up linear polymer chains, decreases *p*-values. (2) The AMINOW is an estimate of the minimal number of water molecules polarized. Since it is highly unlikely that there is absolute exclusion of Na' from this water, the actual number of water molecules under the influence of each polymer oxygen must also, for this reason, be higher. With these considerations in mind, we are inclined to believe that under optimal conditions a PVME oxygen atom polarizes considerably more than 20 water molecules. For a conservative estimate, let us assume that it is 30. Regardless of exactly how many more than 20 are adsorbed, there seems little question that multilayers of water are involved.

Let us next raise the question: If the optimal spatial configuration of the PVME molecules as seen in a 7.5% solution can be uniformly maintained at a higher concentration, what PVME concentration would be required to yield a q-value of 0 for Na citrate? Let us represent this concentration as n%. The total number of non-solvent water would be $30 \times \frac{10\ n}{58.08}$ while the total molar concentration of water is $(1 - \frac{n}{100})\ 55.5$. All this water is nonsolvent when $(I - \frac{n}{100})\ 55.5 = \frac{10\ n}{30 \times 58.08}$ One calculates that n is equal to 9.7. Thus, ideally, a 9.7% PVME should be able to have a q-value of 0 for Na citrate. In reality, the q-value of a 9.7% PVME is not zero but about 0.45. There are no doubts that the random distribution of the chains play a role in this loss of efficiency.

As mentioned earlier, PVP, PEO, and PVME are all what are called NO-NO-NO systems. Theoretically NO-NO-NO systems are less effective than one like the NP-NP-NP system of the extended polypeptide chains. Here, the alternatingly positive and negative sites of CO and NH group further stabilize the multilayer water structures.[8,12] It may well be expected that the postulated matrix proteins in living cells may be so ordered as to avoid any wasteful overlap of water polarization as has been shown for the solutions of synthetic polymers. In this case, perhaps less than 9.7% of the matrix protein would be needed to polarize all the cell water, even if one completely ignores (i) the space-filling and water-content reducing effect of other non-matrix proteins and macromolecules in the cell; and (ii) the weak but significant water polarizing effects of some globular proteins.[13] That only a small concentration of

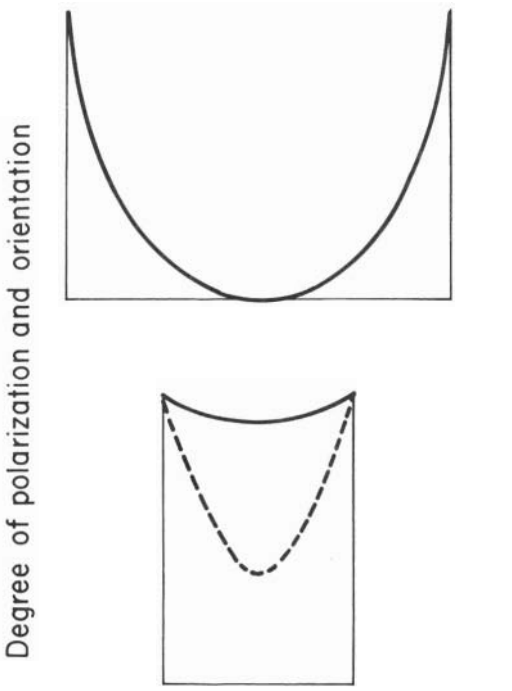


FIGURE 8. Diagrammatic illustration of the effect of decreasing distance between extended protein chains or model polished glass surfaces. N represents negatively charged sites and P positively charged sites. NP-NP system represents two juxtaposed surfaces (e.g., polished glass) containing N and P sites with regular spacing like a checkerboard. NP-NP-NP system represents an equivalent matrix of linear chains carrying N and P sites at regular intervals separated from each other by distances roughly that of one water diameter. N and P may represent the CO and NH groups of an extended protein chain.

matrix proteins can polarize all the cell water is necessary in the polarized multilayer theory of cell water.

Finally one would like to comment on the ability of PEG to reduce water solvency. Note that in contrast to PVP, PEO, and PVME, each of the oxygen atoms of the alcoholic group of PEG is placed between two other similar oxygen atoms on adjacent carbon atoms in the (—CH—CH—), backbone. This is in apparent contradiction to the conclusion that to achieve a solubility reducing effect, the oxygen atoms must be separated by distances roughly equal to twice the water diameters (as in the case of PVME where the oxygen atoms are attached to every other carbon atom on the hydrocarbon backbone). A possible solution to this apparent contradiction may lie in the unusual nature of the oxygen carrying groups, the alcoholic —OH. Thus, we suggest that PEG, instead of representing an NO-NO-NO system, actually represents an NP-NP-NP system. Here the lone pair of electrons on the oxygen atom acts as an N site very much in the same way as do the oxygen atoms in the —OCH_3 groups in PVME. The immediately neighboring OH groups may then act as P sites analogous to the peptide NH group. It is also well known that hydroxyl groups behave as strong proton donors as well as strong proton acceptors.[18] Other evidence supports the idea that PEG represents an NP-NP-NP system: (i) the high effectiveness of PEG in reducing the solvency for Na citrate; and (ii) the failure of the AMINOW of PEG to undergo a sharp drop even after PEG concentration has reached as low as 2.5%.

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TABLE I. The p-value and the molar apparent minimal "non-solvent" water for Na citrate in solutions of PVP, PEO, PVME, and PEG of different concentrations.

	Polymer Content (%, w/w)	2.5	5	7.5	10	15	20	30	40	50	60
	Water Content (%, w/w)	97.5	95	92.5	90	85	80	70	60	50	40
PVP	$\rho_{Na\text{-}Cit.}$	—	—	—	0.80	0.72	0.63	0.42	0.30	0.13	0
	AMINOW (average number of H_2O molecules per oxygen atom)	—	—	—	11.2	9.77	9.21	8.43	6.52	5.40	4.15
PEO	$\rho_{Na\text{-}Cit.}$	—	—	0.87	0.73	0.51	0.39	0.22	0.13	0.08	—
	AMINOW (average number of H_2O molecules per oxygen atom)	—	—	3.92	5.90	6.80	5.96	4.45	3.19	2.25	
PVME	$\rho_{Na\text{-}Cit.}$	—	0.89	0.50	0.45	0.39	0.34	0.22	0.11	0.05	—
	AMINOW (average number of H_2O molecules per oxygen atom)	—	6.70	19.9	16.0	11.2	8.52	5.87	4.30	2.96	
PEG	$\rho_{Na\text{-}Cit.}$	0.75	0.65	0.56	0.49	0.38	0.29	0.16			
	AMINOW (average number of H_2O molecules per oxygen atom)	13.5	11.8	9.66	8.17	6.26	5.05	3.49			

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