AD-A136 294 MOLECULAR MECHANISMS INVOLVED IN TISSUE SWELLING DUE TO 1/1
INJURY AND DUE TO..(U) PENNSYLVANIA HOSPITAL
PHILADELPHIA DEPT OF MOLECULAR BIOLOGY.. G N LING
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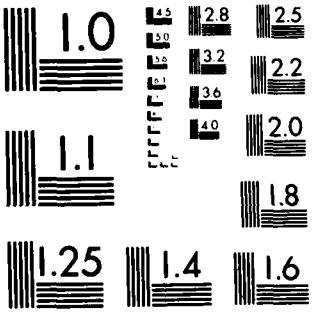

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS 1963-A

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(2)

Annual Progress Report
November 1981 - October 1, 1982

"Molecular Mechanisms Involved in Tissue Swelling Due to Injury and Due to Exposure to Low Temperature and Massive Water and Electrolyte Loss in Diarrheal Disorders"

N00014-79-C-0126

This annual progress report will follow past tradition and will be given in three sections: (Section I) a brief Background Information including much work we accomplished under ONR contract; (Section II) a summary of laboratory work; and (Section III) a summary of non-laboratory work.

I. Background Information

As soon as biologists realized that living cells are the basic units of all life forms from single amoeba to dinosaurs, they immediately were faced with the most fundamental question, "What is a living cell?" One may well say that the degree of our knowledge of the living cell will determine how well we understand all living phenomenum.

According to the conventional view, the living cell is primarily a tiny droplet of water containing proteins and K^+ salts kept apart from the body of similar aqueous solution of Na salts by a microscopic lipid membrane which contains pores or channels and a wealth of specific devices called pumps. It is the size of the pores as well as the continuous activities of the pumps at the expense of energy derived from metabolism that keeps the inside of the cell different from that of its environment.

There has been serious reason to doubt the validity of this theory of the living cell, i.e., the membrane-pump theory, for well over the last 30 years. But it was primarily in the course of the last 20 years in work consistently and without interruption supported by the Office of Naval Research, that there is now no longer any question that this theory has been disproven; and that a new theory, called the association-induction (AI) hypothesis offers a new foundation of cell physiology that is compatible with by far the greatest majority of the results of experimental testing. These efforts and their results will be presented in a comprehensive treatise to be more fully described under Section III. The key finding, that has settled the long controversy concerns the physical state of the major cation in all living cells, K^+. The membrane theory depends absolutely on the existence of K^+ in a free state. Failing this, the theory will not be able to explain any one of the four classic phenomena of the living cell; but particularly the theory of cell volume regulation and osmotic balance, and the cellular electric potentials.

In the last 4-5 years major experimental evidence, besides those already mentioned, included the successful demonstration that proteins with its polypeptide existing in an extended conformation as well as model polymer containing oxygen atoms (as do proteins) at regular intervals with distances between nearest oxygen atoms equal to that of two water diameters polarize water in multilayers. Water so polarized shows decreased solubility properties strongly resembling that seen in living cells, for Na^+, sugars, and free amino acids (Ling et al, 1980).

Just as important or perhaps even more so, were the confirmation of the predicted localized adsorption of cell K^+ (or its surrogates Cs^+ and Tl^+) at the edges of the A band and at Z-lines. The A band and Z-lines in frog muscle cells are the sites where β- and γ-carboxyl groups are concentrated as shown by protein amino acid residue data of myosin which is found only in the A band and from the staining pattern of uranium ion which also binds the β- and γ-carboxyl groups in glutaraldehyde fixed, uranium stained muscle cells (Edelmann, 1980, 1981a, 1981b; Trombitas and Tigyi-Sebes, 1979). These findings were established unanimously from three different laboratories, using a total of four different methods (i.e., autoradiography of air dried and of frozen dried single muscle cells, transmission electron microscopy, dispersive x-ray microprobe analysis, and laser mass spectrometer microprobe analysis (LAMMA)).

While these methods established the localized distribution of K^+ in frog muscle cells, other experiments with intact and EMOC preparation of frog muscle showed that the K^+ localization is due to one site-one ion adsorption and not due to existence of K^+ as free counterions, since different univalent cations (K^+, Cs^+, Tl^+) compete against radioactive K^+ (K^{42}) accumulation and have profoundly different effects; were the cell K^+ free as counterions, the displacing should be equally effective (Ling, 1977).

The clear establishment of the adsorbed and hence osmotically inactive state of the major intracellular cation, K^+, leaves the balance of intracellular osmotic activity against external isotonic NaCl to the only possible agent remaining, i.e., (matrix) proteins. Because after all, osmotic activity is only a measurement of a lowering of water activity. That multilayer polarization of water lowers water activity is established by the measurement of osmotic activity of the different model polymers which polarize water, including PVP and PEO. Due to the large molecular weight (600,000) a 30% PEO solution is only 0.5 mM. Yet this electrically uncharged polymer is able to reduce water activity to equal that brought about by 1 molar sucrose solution (Ling, 1981).

In summary, disproof of the membrane-pump model is about as complete as Phlogiston theory was at the end of the 18th century. There is also now considerable evidence in support of the AI hypothesis according to which neither cell K^+ nor cell water are free as in the membrane-pump theory. Rather both are adsorbed in cells under normal resting state, K^+ singly on β- and γ-carboxyl groups belonging to a large extent to myosin, and water in multilayers, on matrix proteins existing in an extended state. The precise nature of matrix proteins is as yet undetermined though some evidence suggests that actin perhaps may play a significant role.

We now turn our attention to the question of the significance of NMR relaxation times T_1 and T_2 measured in living, deteriorated, and dead tissues.

As water molecules become more and more immobilized the rotational correlation time of the water proton τ_c increases. Highly immobilized water has very short T_2 but much longer T_1. Therefore rapid exchange with a very minute amount of highly immobilized water is able to reduce the overall T_2 of living cells to much shorter values than the T_2 of the bulk phase water. For this reason, T_2 is not likely to tell us much about the physical state of water in living cells. Nor, of course, would the ratio of T_1/T_2 do any better.

The difficulty with T_2 does not apply to T_1, for the reason that T_1 of

highly immobilized water is not very short. Therefore a minute fraction of highly immobilized water would not completely mask the true T_1 of the bulk phase water.

Other major reasons that have obstructed progress in NMR studies of water in living cells include: (1) no known model of water that can represent water in the state of polarized multilayers as suggested in the association-induction hypothesis, and (2) a premature rejection of a role of paramagnetic ions in living cells in determining the T_1. Both of these shortcomings have been overcome by work supported by ONR in this laboratory; the first more or less completely, the second having made a good start and will be described in the Progress Report. The T1, T_2, and τ_c of water existing in the state of polarized multilayers have been successfully measured in water interacting with pure synthetic polymers, satisfying the requirements for long range polarization of water as stated in the AI hypothesis and possessing the property of excluding Na^+, glycine, and sucrose (solutes as a rule excluded from the water in living cells). The main finding is that water possessing solute-exclusion properties has reduced T_1 as well as T_2. However, the T_1/T_2 ratio is not very different and is in fact close to unity as normal liquid water is. These traits allow us to estimate the τ_c value of water in the state of polarized multilayers: longer than that of normal liquid water by a factor not more than 10.

II. Laboratory Work

(A.) The masking effect of paramagnetic impurities on the anticipated T_1 lengthening with cell inury and death.

The establishment in vitro that water interacting with extended protein chains and with polymers having similar attributes, can indeed yield an osmotic activity far beyond that due to the number of polymer molecules, makes it all but certain that this is the basis for the osmotic balance in cells. The fact that water in this state excludes Na^+, sucrose, and glycine as they are excluded from most living cells also permits a quantitative estimate of the number of water molecules involved - this estimate confirms the multilayer nature of the water adsorbed. With this background information, the measurements of equally shortened T_1 and T_2 of water oriented by PEP, PVP, and PVME furnish the basis for estimating a τ_c value (3×10^{-11} sec.) no more than one order of magnitude longer than that of pure liquid water (Ling and Murphy, in print). Let us know see how these data can help understand the NMR studies thus far recorded.

First, the large difference between T_1 and T_2 seen in living cells (and in serum albumin) indicates that some part of the water must have a long τ_c. The corresponding low T_2 value cannot be that of bulk phase water, or else the diffusion coefficient would be much more reduced than by a factor of 2 as measured (Cleveland et al, 1976; Hazlewood, 1979). Therefore the low T_2 must originate from a small fraction of water in rapid exchange with the bulk phase water. One realizes that in fact only a minute amount of water with a τ_c of, say, 10^{-6} sec. will all but overwhelm the contribution of the bulk phase water regardless of whether it has a τ_c of 3×10^{-11} sec. (as in water in the PEO, PVP, PVME systems) or 3×10^{-12} sec. (as in normal water). Therefore, the T_2 value recorded from a complex system like the living cell is virtually completely masked by this minute, rapidly-relaxing fraction of water (Ling, 1979). The same may not, however, apply to T_1, because with $\tau_c = 10^{-6}$ sec., T_1 measured at high field frequency may be as large as T_1 of water with τ_c equal to 3×10^{-11} or 3×10^{-12} sec.

In Cooke and Kuntz's three fraction model of protein solutions, which was extended to living cells, they used for the bulk phase water the τ_c of that of pure liquid water, 3×10^{-12} sec. This would have predicted a T_1 much larger than recorded. To explain the lower T_1, they assigned 10% of the cell water to hydration water with a longer τ_c. This was necessary because in 1974, they did not have the data of Ling and Murphy yet to appear in print, on the T_1 and T_2 of water polarized by PEO and other polymers. I would like to suggest that a major share of the spin-lattice relaxation (T_1) is due to bulk-phase water in the state of polarized multilayers with a τ_c somewhere near 3×10^{-11} sec.

If this is the case, T_1 of the water proton should reflect the multilayer state of normal cell water and it would be expected to increase with cell death and deterioration. Yet the data of Neville, Civan, Shporer, and others show the opposite: T_1 decreased with cell deterioration and death. Future research is needed to answer this question fully, but the following suggestions can be made. Our investigations suggest that water associated with proteins, or perhaps protein-paramagnetic ion complexes, in living cells may undergo changes in T_1 in such a way as to counter the effect of a lengthening of T_1 due to depolarization of bulk-phase water expected to occur as a result of deterioration. In support of this, we found that fresh sartorius muscle yields a T_1 of 620 msec. After heating for 10 minutes at 60° C, T_1 lengthened to 700 msec. Its pair, which was exposed to a Ringer solution containing 5×10^{-4} M Mn^{++}, had a T_1 of only 260 msec. After similar heating, T_1 of the Mn^{++}-treated muscle did not lengthen. Instead it went down to 100 msec.

Normal frog gravid oviduct showed a T_1 of 98 msec; heating for 10 min. at 60° C. caused a shortening to 34 msec. Another normal oviduct gave a T_1 of 10 msec. After exposure to a Ringer solution containing 5 mM iodoacetate and 1 mM NaCN for 30 min. T_1 increased to 140 m sec. Three hours later it rose still higher to 165 msec. but then it began to decline, reaching 108 msec after 18 hours and 31 msec after 2 days.

It is well known that heating the frog tissues to 60° causes the loss of their ability to exclude sucrose, which according to the AI hypothesis denotes depolarization of cell water. A consequent lengthening of T_1 would be expected. Heating of frog sartorius muscle does indeed produce a T_1-lengthening but nowhere nearly as much as anticipated. Heating frog gravid oviduct, however, brought about shortening of T_1. Since inclusion of Mn^{++} converts a heat-induced T_1-lengthening to T_1-shortening in frog muscle, one may suppose the weak T_1-lengthening in muscle and the T_1-shortening in oviduct to be consequences of an enhancement of the T_1-shortening effect of the Mn^{++}-protein complex as a result of heat denaturation.

That IAA and CN also brought about a T_1-lengthening of frog oviduct suggests that IAA-CN topples the living state by lowering the cell ATP content. The result-depolarization of water produces T_1-lengthening. This is then gradually over-powered by the secondary T_1-shortening effect of the changing protein-paramagnetic ion complex of the cells. In support of this view I found that water extracts of frog muscle ashes added to a solution of bovine serum albumin reduce its T_1.

B. The freezing properties of water in the state of polarized multilayers.

Major progress has also been made in the study of freezing properties of water, in the state of polarized multilayers. These studies reveal other features of water in this state which run closely parallel to the solvency effect described. One recalls that water in a 20% solution of native globular proteins (see Table 1) has an entirely normal solvent property: the apparent equilibrium distribution coefficient (ρ-value) of probe molecules like Na^+ citrate is unity. When these pro-

teins have been denatured by exposure to 10 M urea, the ρ-value for the same probe molecules fall below unity. Similarly water in solutions of gelatin, PVP, PEO, PVME, etc. have reduced ρ-value. We also pointed out how these findings came from and are in agreement with the theoretical expectations of the theory of polarized cell water as part of the AI hypothesis.

(1) Parallel phenomena of reduced solvency for probe molecules and the phenomenon of warming exothermic reaction (WEX)

Normal liquid water freezes during cooling; it is an exothermic reaction. Normal ice thaws during warming; it is an endothermic reaction. If a dilute solution of PVP is frozen and then thawed it will first give off heat during freezing and absorbs heat during thawing (see Fig. A). However, as one increases the PVP concentration, a remarkable phenomenon occurs. That is, as the frozen PVP solution is thawed, an exothermic reaction (WEX) takes place, which appears as a sharp downward (exothermic) peak shown in Fig. A. An extensive study was made, from which we concluded that water associated with PVP, PEO, PVME and with 9 M urea denatured globular proteins all demonstrate WEX when the concentration of polymer was high enough. However, equally concentrated solutions of normal globular proteins show no WEX. Our tentative interpretation is that during freezing, water in the presence of PVP, urea-denatured proteins, etc. is caught in the state of polarized multilayers. It will not be able to overcome the energy barrier to assume an ice I or an undefined but energetically more favorable state until the temperature is high enough. That is why " freezing" occurs with warming in this situation.

(2) Does non-freezing water exist as a constant component of the polymer-water system? Is the non-freezing water the same water we suggested as existing in the state of polarized multilayers?

It is possible with the Perkin-Elmer DSC-2 differential scanning calorimeter to measure the enthalpy (ΔH) of thawing of polymer-water systems. By extrapolating plots of ΔH per gram of ploymer against the number of grams of water per gram of polymer, one can obtain the so-called non-freezing water of the polymer-water systems (Fig. B). This nonfreezable water in the presence of PVP, PEO, etc. is quite large., often at values of 1 gm H_2O/gm of polymers. For PVME (poly-vinyl methyl ether), this signifies that water in a 50% solution of PVME is completely non-freezing at any temperature studied. The question is, " How is the non-freezing water related to the water in the state of polarized multilayers?"

Fig. C shows plots of the ρ-value of Na citrate in aqueous PVME solutions of different concentrations. Note that at 50% concentration the ρ-value has become less than 10%. In other words a 50% solution of PVME has lost virtually all its solvency property for Na citrate.

Comparing the non-freezing water with the non-solvency one is led to conclude that they are the same. Thus non-freezing and non-solvency are different expressions of the same basic phenomena created by the presence of the polymers which bear oxygen atoms at distances 2-water-diameters apart and thus satisfying the theoretical requirement for multilayers polarization.

Now if this non-freezing, non-solvent water exists in quantity at fixed ratio to the weight of the polymer one can deduce the ρ-value for polymer-water system containing more water. This is shown as the dashed line in Fig. C.

Actually the q-value measured is much lower than that predicted by the dashed line in intermediate concentration ranges. Thus at 35%, the ρ-value predicted by the dashed line is 0.33 while the observed value is 0.11. This discrepancy signifies that the water affected by the polymer is not constant and that in a large range of polymer concentration much more water is affected than that one can predict from the concept of a fixed amount of non-freezing, non-solvent water. In contrast the data agree well with the polarized multilayer concept: When polymers are too far apart there is relatively little water polarized; when polymers are too close, the total number of water polarized again is also reduced. A high degree of cooperativity exists among the water molecules and through them the polarizing actions of the fixed polarizing sites.

III. Non-laboratory Work

A. I have now completely finished my new book, which is 200,000 words in length, divided into 5 major sections, 20 chapters containing about 400 text figures. Under the title, " In Search of the Physical Basis of Life", it is expected to be published in the summer of 1983 by Plenum Publishing Corporation, New York.

B. Additional Work Published

Ling, G. N., " Active Solute Transport Across Frog Skin and Epithelial Cell Systems According to the Association-Induction Hypothesis", Physiol. Chem. Phys. 13:356-384 (1981)

Ling, G. N., " The Cellular Resting and Action Potentials: Interpretation Based on the Association-Induction Hypothesis", Physiol. Chem. Phys. 14:47-96 (1982)

Ling, G. N., " Synchronous Control of Metabolic Activity by K^+ Transiently and Reversibly Liberated from Adsorption Sites During Muscle Contraction: An Extension of Association-Induction Theory," Physiol. Chem. Phys. 13:565-566 (1982)

Table 1. p-values of Na^+ in water containing native proteins (A), gelatin (B), PVP (C), and polyethylene oxide) (PEO) (D) (25 °C) (from Ling et al. 1980 by permission of *Physiological Chemistry and Physics*)

Group	Polymer	Concentration of medium (M)	Number of assays	Water content (%) (mean ± SE)	p-Value (mean ± SE)
(A)	Albumin (bovine serum)	1.5	4	81.9 ± 0.063	0.973 ± 0.005
	Albumin (egg)	1.5	4	82.1 ± 0.058	1.000 ± 0.016
	Chondroitin sulfate	1.5	4	84.2 ± 0.061	1.009 ± 0.003
	α-Chymotrypsinogen	1.5	4	82.7 ± 0.089	1.004 ± 0.009
	Fibrinogen	1.5	4	82.8 ± 0.12	1.004 ± 0.002
	γ-Globulin (bovine)	1.5	4	82.0 ± 0.16	1.004 ± 0.004
	γ-Globulin (human)	1.5	4	83.5 ± 0.16	1.016 ± 0.005
	Hemoglobin	1.5	4	73.7 ± 0.073	0.923 ± 0.006
	β-Lactoglobulin	1.5	4	82.6 ± 0.029	0.991 ± 0.005
	Lysozyme	1.5	4	82.0 ± 0.085	1.009 ± 0.002
	Pepsin	1.5	4	83.4 ± 0.11	1.031 ± 0.006
	Protamine	1.5	4	83.9 ± 0.10	0.990 ± 0.020
	Ribonuclease	1.5	4	79.9 ± 0.19	0.984 ± 0.006
(B)	Gelatin	1.5	37	57.0 ± 1.1	0.537 ± 0.013
(C)	PVP	1.5	8	61.0 ± 0.30	0.239 ± 0.005
(D)	Polyethylene oxide)	0.75	5	81.1 ± 0.34	0.675 ± 0.009
		0.5	5	89.2 ± 0.06	0.623 ± 0.011
		0.1	5	91.1 ± 0.162	0.754 ± 0.015
(E)	Methylcellulose	0.1	4	83.4 ± 0.43	0.689 ± 0.008

TABLE 1

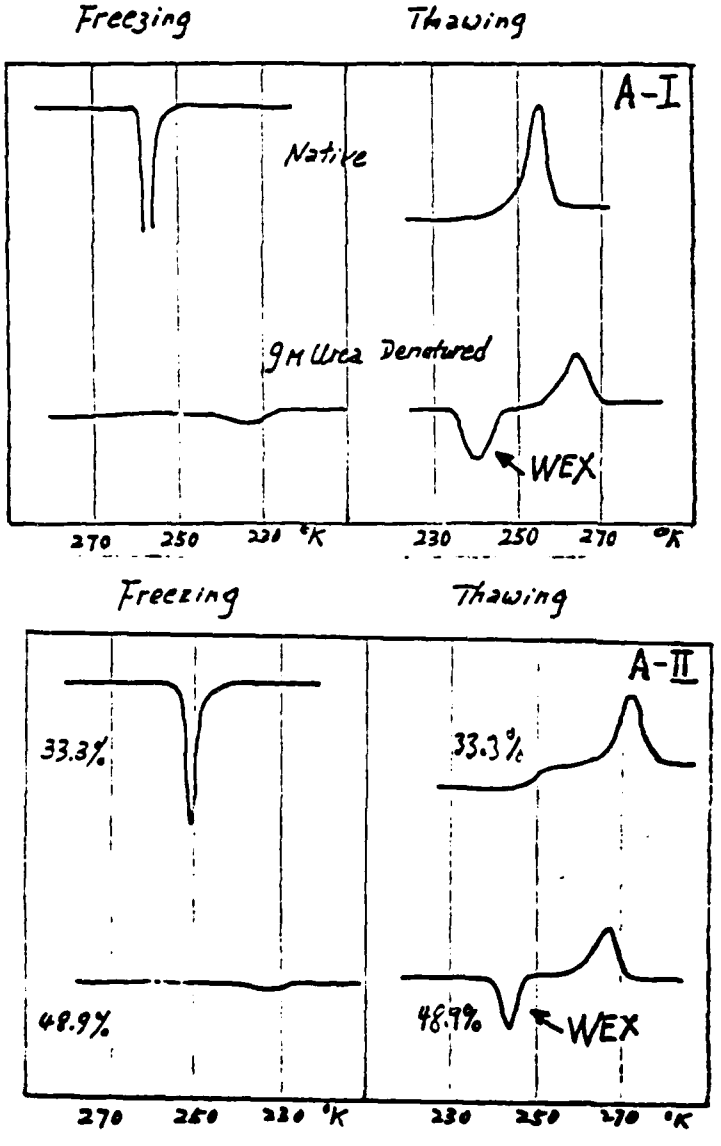

FIGURE A

AI Top: Thermograms of solutions of native hemoglobin showing standard endothermic (upward peak) thawing and exothermic freezing (downward) (WEX).

AI Bottom: Thermograms of urea-denatured hemoglobin. Solution of the same Hb concentration showing downward exothermic peak during warming.

AII Top: Thermogram of low concentration of PVP (33.3%).

AII Bottom: Thermogram of high concentration of PVP (48.9%) showing ing downward exothermic peak (WEX) during warming.

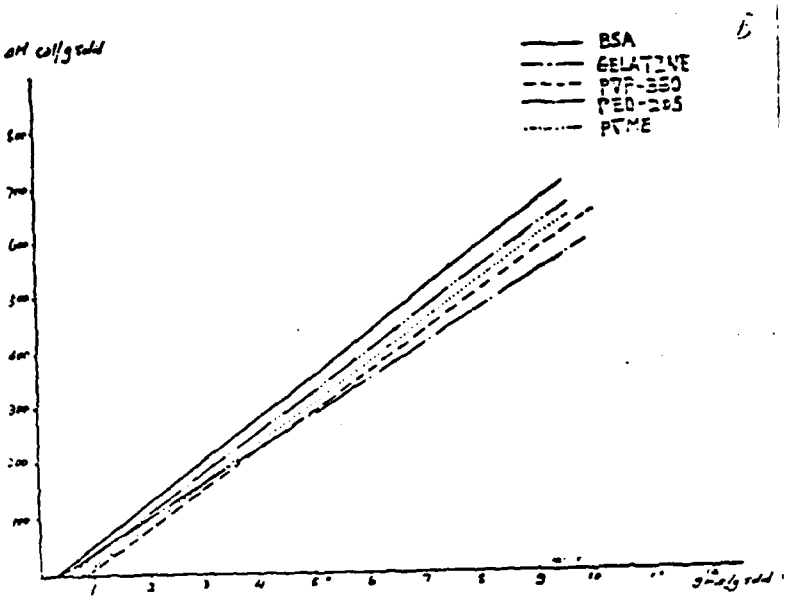

FIGURE B

Plots of enthalpy of melting per gram of polymer against water per gram of polymer. By extrapolation, the intercept on the abscissa yield the non-freezing water.

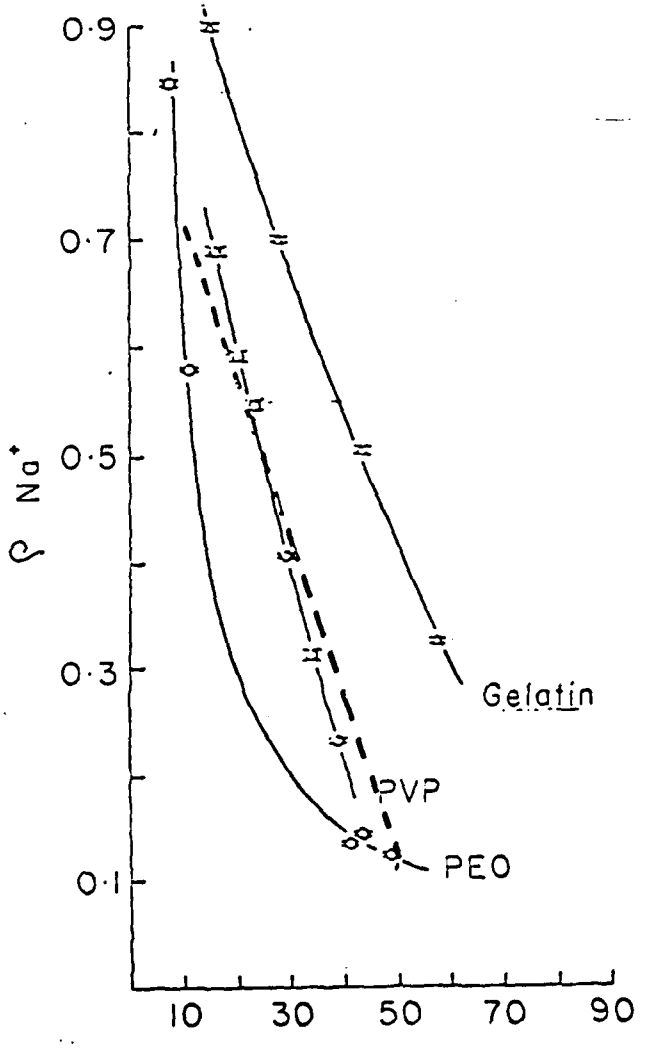

FIGURE C

Plots of the apparent equilibrium distribution constant (ρ-value) of sodium citrate against the concentration of poly-ethylene oxide (PEO). Dashed line is a theoretical ρ-value curve calculated on the assumption that there is a fixed ratio of non-freezing and non-solvent water per gram of PEO.

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