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PHILADELPHIA DEPT OF MOLECULAR BIOLOGY.. G N LING
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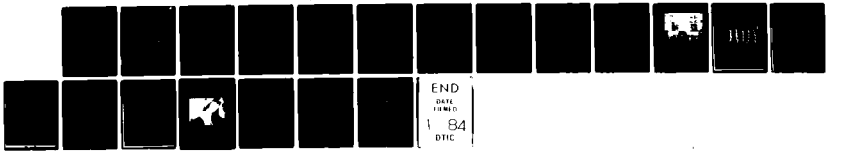

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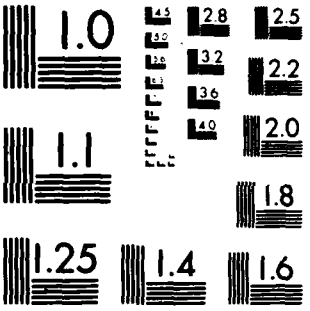

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Status Report

For Part of the Work Accomplished Between
November, 1980 to June, 1981
under ONR Contract N00014-79-C-0126

Principal Investigator:

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Status Report
November 1980 - June 1981

"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

The present status report roughly covers work conducted or initiated within the period of time from November, 1980 to June, 1981. For a clear presentation, two kinds of papers or manuscripts are enclosed: (i) publications (Publ. # 1, 2, etc.) are new publications or manuscripts and represents part of our achievements in this report period, and (ii) Exhibits (Exh. # 1, 2, etc.) are either earlier publications of our own or of other laboratories cited as background information and are not a part of our progress itself. The report is divided into two parts: laboratory and non-laboratory work.

Part I. Laboratory Work

A.) Investigations into the physical state of water in living systems and its role in the physiological as well as pathological behavior of cell systems.

In recent years we have demonstrated in model systems the long-range polarization of water in a matrix of more or less parallel chains of polymers (including proteins) containing oxygen atoms at regular intervals separated from its neighbors by distances roughly equal to the diameter of two water molecules (Exb. #1). As an invited speaker, I discussed the broad significance of this state of water on some basic functions of living cells at the International Congress on Cell Biology held in West Berlin last year (See enclosed Publ. #1).

The simplicity of the model system, polyethylene oxide $-(CH_2O-CH_2)_{\overline{n}}$ (Polyox) permits inquiries into the physical and chemical properties of water existing in this state not possible before. In the past half a year we have continued to carry out investigations in this general direction. Some highlights are summarized:

1. NMR of water existing in the state of polarized multilayers.

Without a clear and easily available example of water existing in the state of polarized multilayers, attempts to prove its existence or absence was greatly hampered and by and large, inconclusive. In work near completion, Randy Murphy and I have demonstrated that water in the state of polarized multilayers exhibits NMR relaxation times quite consistent with what theoretically had been expected. A brief communication by Ling and Murphy (Publ. #2) shows that water in the state of polarized multilayers does indeed suffer motional restriction but only to a moderate degree (not at all like that in solid ice, for example) in agreement with theory (Exb. #2).

Extensive data on all four polymers (polyox, poly-vinyl methyl ether, polyvinyl pyrrolidone and gelatin) showed that with increasing polymer content, water suffers an increasing degree of motional restriction as reflected in similar change of both T_1 and T_2. Such an identical or nearly identical values of T_1 and T_2 is explained by an increasing degree of motional restriction, and hence decreasing τ_c, rotational correlation time.

2. "Osmotic activity" of water in the state of polarized multilayers.

Other studies of the polymer oriented water includes the continued investigation of the measurement of the "osmotic activity" of polarized water by vapor pressure measurements. These studies too showed the expected behavior according to the association-induction hypothesis (AI Hypothesis). Before their introduction, some background materials may be helpful in bringing forth the significance of the new findings.

In recent work from this laboratory, from West Germany, and from Hungary, it has become established that the bulk of the major intracellular cation, K^+ in muscle cells is not free but is adsorbed in localized areas of the cell as shown in Figure 1 from Ling based on work of L. Edelmann.

According to the AI Hypothesis β and γ carboxyl groups, belonging respectively to aspartic and glutamic acid residues, provide the sites for selective K^+ adsorption. These sites are primarily located at the two edges of the A band and the Z-line of muscle cells and bind electron-dense uranium in the conventionally fixed and stained E.M. preparation of muscle cells. The bound uranium makes these areas dark in the E.M. plate (Fig. 1A). It was the confirmation of the expected localization of K^+ or its surrogate electron-dense Cs^+ or Tl^+ that has provided a crucial decisive evidence for the AI Hypothesis. Thus as illustrated in Figure 1 the top figure is a muscle cell stained with the conventional method with uranium after chemical fixation. The next two pictures, B and C, are similar muscle sections that had not been chemically fixed, but instead were loaded with either cesium (B) or thallium (C) while the muscles were still alive and then frozen-dried and dry cut. While in Figure A the darkness reflects the distribution of electron dense uranium, the darkened areas in Figures B and C reflect the distribution of electron-dense cesium and thallium respectively. A comparison of B and C with A leaves little doubt that the theoretical expectation based on the AI Hypothesis has been fully confirmed. That is, indeed, potassium or its surrogates cesium and thallium, which can displace K^+ stoichiometrically under normal physiological conditions, are not evenly distributed in the cell but are localized at areas predictable from the theory. This conclusion has been unanimously confirmed by three other techniques conducted in three different laboratories including our own: (a) radioautograms of either air-dried muscle cells or of frozen muscle cells gave results in complete agreement with the E.M. data presented in Figure 1. This work was in part done in our laboratory and in part at the University of Saarland by Dr. Ludwig Edelmann. (b) by dispersive x-ray microprobe analysis, which also showed the concentration of potassium (itself) (or cesium or thallium) in the A band and not in the I band, and (c) by the in vitro demonstration of selective uptake of potassium over sodium in frozen dried sections of muscle with the aid of both dispersive microanalysis and another new technique (Laser mass spectrometer microanalysis technique - LAMA). The demonstration of the localized distribution of the major intracellular cation potassium is at once a confirmation of the AI Hypothesis, and a disproof of the conventional, classic membrane theory according to which intracellular K^+ (as well as water) are primarily in the free state. More details are given in a recent review of Edelmann (Exb. #3). The evidence that the potassium or cesium is not localized but is adsorbed on one ion to one site has also been extensively discussed in Exb. #4).

Since K^+ constitutes half of intracellular solutes the fact that the bulk of potassium is in the adsorbed state demands new solution of the osmotic balance problem, since the cell water would be highly hypotonic. As a result the cell is expected to shrink in a normal Ringer solution containing Na^+ of a concentration equal to that of cell K^+ but in a free solution. However, the cell does not shrink.

An answer to this dilemma has been provided by the AI Hypothesis, according to which the reduction of the activity of intracellular water is only to a small extent produced by the presence of free ions or other free solutes but is produced primarily by the cellular macromolecules - primarily the extended matrix proteins which may include actin and are as yet largely unidentified (see below).

It is in this context that the demonstration of large "osmotic activity" of polymer oriented water owes its primary significance. In work near completion, Ling and Bowes showed that a 30% aqueous solution of polyox exhibits an "osmotic pressure" corresponding to that of 1 molar sodium chloride, yet with a molecular weight of 600,000 the molar concentration of polyox is only 300/600,000 = 0.5×10^{-4} M and should have virtually no osmotic effect. Due to the interaction of long-range polarization on the oxygen sites , the long-range polarization by the polymer creates large lowering of water activity. According to the AI Hypothesis similar polarization of water by the matrix proteins in all living cells accounts for a major share of the "osmotic activity" of the cell cytoplasm. (For a preliminary note, see Publ. #3)

3. Swelling and shrinkage.

Another difficulty confronting the conventional membrane-pump theory of the living cell concerns the swelling and shrinkage behavior. The great difficulty lies in the fact that cells with portions of its cytoplasm not covered by a cell membrane when directly exposed to the external solution can swell and shrink much as an intact cell does (Exb. #5). In the enclosed preliminary note (MS #3) we have reported that water under the polarizing influence of Polyox can shrink and swell in hyper- and hypotonic solutions when the polymer water system is enclosed in a regular dialysis tubing which is fully permeable to the salt ions in question. Much of this piece of work is also near completion. (Figure 2)

(B.) What component of the cell surface serves as the permeability barrier of living cells? f, —> g, 4

According to the conventional view first clearly enunciated by E. Overton it is a continuous layer of phospholipid that endows the cells with selective permeability. This hypothesis has received important contributions from studies of permeability of the plant Nitella by R. Collander and many other investigators. Yet with increasing knowledge in general and more careful scrutiny of the cell membrane in particular there is serious doubt that the original Overton hypothesis is correct (for ref., etc., see Publ. #1). The cell membrane morphology revealed in an E.M. investigation, remained unchanged after the extraction of 95% of the lipid components of the membrane. A membrane of a similar trilaminar morphology was constructed from purely proteinoid material devoid of lipids. In addition the demonstration by Stillman and coworkers (1970) and by Maloff and coworkers (1978) have strongly indicated that the permeability barrier of squid axon and of the inner membrane of mitochondria cannot be that of phospholipids. These conclusions were drawn from the investigation and demonstration that K^+ specific ionophores monactin and valinomycin in the presence of varying external K^+ concentration had no noticeable effect at all on either the K conductance of the squid axon membrane or electrical conductance of mouse liver mitochondria inner membranes. These findings & their basic importance has not yet been recognized as they deserve to be. However we had continued investigation on this general subject and have by now nearly completed our study of three additional tissues - the frog oocyte as a prototype of all cells of mammalian origin, frog sartorius muscles, and the isolated human red blood cells. We investigated three major ionophores: valinomycin, monactin, and nonactin.

Valinomycin has no effect on K permeability in frog oocytes and frog muscles; it has a significant effect only on the K permeability of human red cells. However, monactin and nonactin both better K ionophores than valinomycin, had no effect on all three tissues (Fig. 3) Further investigation showed that the effect of valinomycin on human red blood cells is accompanied by cell injury (as indicated by the loss of total cell K^+), a not altogether surprising finding since valinomycin is a powerful poison. From these we reached the conclusion that we have completely confirmed the earlier work on squid axon and mouse and mouse liver mitochondria mentioned above. We further reached the conclusion that phospholipids do not form a continuous barrier in the cell membrane. The next question is, "What then does form the permeability barrier?" and "Where then is the phospholipid?"

To answer the second question first, we suspect that instead of the conventional picture of the cell membrane seen as an ocean of lipids in which are dispersed islets of proteins, in fact we have an ocean of protein-polarized water in which is dispersed pockets of phospholipids. Indeed Sjöstrand coworkers (Sjostrand & Barrnard, 1976) had come to exactly the same conclusion from their careful E.M. studies. The answer to the first question is: polarized multilayered water serves as the semipermeable cell surface barrier (see Publ. #1).

C. The molecular mechanism involved in brain swelling due to injury and of swelling of other similarly affected tissues, and [illegible] 5

Using a technique of an effectively membrane-pumpless open ended cell preparation or the EMOC technique (Exb. #6) and three independent methods we established that in the cut edge of a frog muscle fiber there is no membrane regeneration, either immediately or in the course of the next 48 hours at 25°C under sterile optimal conditions. A sartorius muscle contains fibers that run all the way from one end to the other (for evidence see article cited above). Using a razor blade we cut all the fibers into 2 mm and 4 mm sections. Muscle segments thus with both ends open were exposed to either hypotonic Ringer solutions or isotonic KCl. In either case they swelled to a degree not significantly different from similarly exposed intact cells. These observations indicate that the volume maintenance cannot be primarily a cell membrane function. (Exb. #5).

An interpretation was offered on the basis of the AI Hypothesis. Osmotic behavior reflects the ability of cell water to exclude solutes. This concept was substantiated by the observation that in a polymer (polyethylene oxide)-water system, the volume of the system varies with the concentration of electrolytes in the surrounding medium even though the electrolyte (sodium citrate) is quite permeable to the cellophane casing enclosing the polymer water. Equilibrium is reached when the osmotic activity of the polymer itself and that of (the low level of) sodium citrate in the bag yields a total osmotic activity equal to that of the external solution. Other osmotic activity measurements of the PEO water system indicates apparent osmolarity of 1 OsM or higher when the total actual molar concentration of PEO is only one millimolar or lower. Thus the polymer has powerful effect in reducing the activity of water several orders of magnitude greater than its classical osmotic activity based on total molar concentration of the polymer. (Fig. 4)

There is reason to believe that PEO water systems we studied can sustain all levels of volume without the assistance of restraining pressure. In frog muscle, on the other hand, a restraining force seems to be operative under the resting condition. This restraining force is according to the AI Hypothesis primarily provided by salt linkages formed between β- and γ-carboxyl groups on one hand and the ϵ-amino groups and guanidyl groups on the other. Under normal

physiological conditions when the cells are equipped with normal ATP the c-values of the β- and γ-groups is such that potassium and ammonium are preferred over sodium. There is also reason to believe that ammonium is a prototype of the ε-amino groups and guanidyl groups. Therefore in normal muscle the β- and γ-carboxyl groups are divided into two classes some adsorbing K^+, others adsorbing ε-amino and guanidyl groups thereby forming volume restraining salt linkages. Increase of KCl concentration to 0.1 M displaces the equilibrium, causing dissociation of salt linkages; as a result the cell swells. Isotonic sodium chloride does not have this affect nor does isotonic potassium sulphate. This is explained by the higher preference for potassium over sodium and of chloride over sulphate.

According to the AI Hypothesis, deterioration of tissue leads to a fall of the level of ATP which serves as the critically important cardinal adsorbent maintaining the normal c-value ensemble of the cell proteins. As a result of the ATP fall in concentration the c-value shifts to a higher level, at which the greater preference of the β- and γ-carboxyl groups for potassium and ammonium gives way to one in which the sodium preference is substantially increased. This shift to higher sodium preference now endows the sodium chloride with the ability of causing swelling in much the same way that KCl causes swelling in normal cells. This theoretical interpretation has been confirmed by studying the relative effects of different anion-cation combinations in conjunction with nonswelling inducing sucrose and by measurements of ATP levels and in its relation to the degree of swelling in dying and dead tissues. (Figs. 5 and 6)

(D.) Selective accumulation of K^+ and extrusion of Na^+ in erythrocyte ghosts.

K and Na are chemically very much alike yet they are extensively segregated in all living cells, cells being rich in K and poor in Na while its surrounding medium is rich in Na and poor in K. This assymetry in fact is the best example of natural "probes" of the physical-chemical makeup of the living cell. It is in many ways a most sensitive monitoring system for the living system; this attribute immediately disappears the moment the cell dies.

The membrane-pump theory argues that the assymetry is due to the continual operation of membrane pumps; the AI Hypothesis argues that it reflects the reduced solubility of Na^+ (and K^+) in cell water and selective adsorption on β- and γ-carboxyl groups of cell proteins of K^+. We have repeatedly pointed out that to sustain the operation of the many pumps needed to keep the living cells in a steady state would command much more energy than the cell commands. Indeed one pump alone, the Na pump, would consume energy 15 to 20 times more than the cell has available. (Publ. #1) This work first published in 1962 has been confirmed in general principle by two different laboratories and has not been disputed in print. However three remedial postulations have been put forth in order to keep the membrane theory afloat. These include Ussing's exchange diffusion mechanism, the Na sequestration in the sarcoplasmic reticulum, and finally the nonenergy consuming pump of Glynn. All three have been experimentally disproven. (For ref. see Publ. #1)

In further support of the AI Hypothesis and against the membrane pump theory is the failure, in spite of extensive effort of some of the most skilled workers in the field, of demonstrating in the squid axon membrane sacs, whose axoplasm has been removed, accumulation of K and exclusion of Na. In contrast, a muscle cell preparation (the EMOC), which has intact cytoplasm but whose membranes and postulated pumps have been made nonfunctional, continues to accumulate K and exclude Na much as normal cells do (see Exb. #6).

However, in support of the membrane pump theory, it has often been quoted that human red blood cells and various other cytoplasm-free membrane vesicles can demonstrate selective K accumulation and Na exclusion. To clarify the situation

I have (1) in cooperation with Dr. W. Negendank, extensively reviewed the subject and published the review under the title "Do Isolated Membranes and Purified Vesicles Pump Sodium? A Critical Review and Reinterpretation", (Exb. #1). In this review we came to the conclusion that much of the claims mentioned above are based either on data that are selfcontradictory or are based on experimental work on vesicles that does not satisfy the criterion of being cytoplasm-free membranes. For example, almost all the natural vesicles studied contained total protein materials at the level equal to or higher than the original cells from which the socalled vesicles or pure membranes, as often quoted, are derived. Data from these preparations are no more enlightening than from intact cells and offer no clarification on the issue, whether or not it is the membrane or the cytoplasm that is the seat of K accumulation and Na exclusion.

For further clarification we have carried out a rather extensive study of K^+ and Na^+ distribution in the human red cell ghosts. Interestingly enough, the most concrete evidence for the few such ghosts, widely regarded as cytoplasm-free membranes, which can accumulate K^+ and extrude Na^+ actually came out of my own laboratory in the hands of my former graduate student, Jeffrey Freedman. Soon after his publication we found out that the red blood cell ghosts prepared by Freedman, following a conventional procedure designed by Bodeman and Passow, did not produce a pure membrane vesicle. Instead much proteins remain inside the membrane, and ghosts are red, and in an E.M. plate, the ghosts look solid. (Fig. 7) We then found that the is only the ghosts that accumulate K^+ and extrude Na^+ significantly. On the other hand, perfectly intact pure membrane vesicles without much cytoplasm such as those produced by the method of Dodge et al. were completely devoid of these activities. (Figs. 8 & 9, taken from an as yet unpublished paper). In more recent times the early E.M. demonstration of Ling and Balter of the solidness of red cell ghosts used by Freedman in contrast to the hollowness of those prepared by the Dodge et al. method, have been confirmed by Hazlewood et al. (1979)

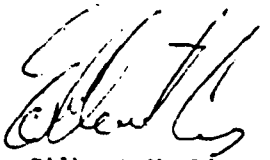
In still more recent work (as yet not published) Ling and Zodda have produced further evidence that the seat of the K accumulation and Na exclusion is not the cell membrane but the remaining cytoplasmic proteins. Thus using the same procedure but different erythrocyte donors, we were able to analyze the data collected as in Figure 10, in which both the K reuptake of the ghosts that had once lost its accumulated K during hypotonic lysis and the Na extrusion are related to the amount of proteins (primarily hemoglobin) which have remained in these ghosts, which perhaps for genetic and other physiological reasons varied a great deal.

Part II. Non-laboratory work.

As a preamble to the new book I am writing (see below), I have in the past year or so written altogether four major review papers. The first one was written in conjunction with Dr. Negendank and has already been mentioned (Exb. #7). The other three bear the titles as follows: "Oxidative Phosphorylation and Mitochondrial Physiology: A Critical Review of the Chemiosmotic Theory, and Reinterpretation by the Association-Induction Hypothesis"; "Active Solute Transport Across Frog Skin and Epithelial Cell Systems According to the Association-Induction Hypothesis"; "The Cellular Resting and Action Potentials: Interpretation Based on the Association-Induction Hypothesis". (A copy of each is enclosed as Publ. # 4, 5, and 6). The need for these efforts arises from the fact that if the AI Hypothesis has validity, then it should not only be able to explain experimental data derived from the subject that has been of central interest to this laboratory but it must also be able to explain new as well as old findings from fields we have not tackled before.

I am now also near the end of another (seventeen chapter) monograph. I have not decided on a final title yet but it is not a more up-to-date version of my first book. Rather it represents results of a much larger effort, in which I have

attempted to bring into one volume, the essence of the living phenomenon as perceived not only by myself but by many others both working now and those who have long departed. It is an attempt at a full synthesis starting from the very beginning of mans' recognition of the living cell as the physical basis of all life. If everything goes well I hope the book may be in a more or less complete form by the end of this year.



Gilbert N. Ling, Ph.D.
Principal Investigator

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Hazlewood, C. F.; D. B. Singer, and P. Beale, Physiol. Chem. Phys. 11:181-184 (1979)

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Maloff, B. L.; S. P. Scordilis, and M. Tedeschi, Science 199:568 (1978)

Additional Published Papers

Ling, G. N., "The Role of Multilayer Polarization of Cell Water in the Swelling and Shrinkage of Living Cells", Physiol. Chem. Phys. 12:383-384 (1981)

Ling, G. N., C. L. Walton, and M. M. Ochsenfeld, "A Unitary Cause for the Exclusion of Na^+ and Other Solutes from Living Cells, Suggested by Effluxes of Na^+, D-Arabinose, and Sucrose from Normal, Dying, and Dead Muscles", J. of Cell. Physiol. 106:385-398 (1981)

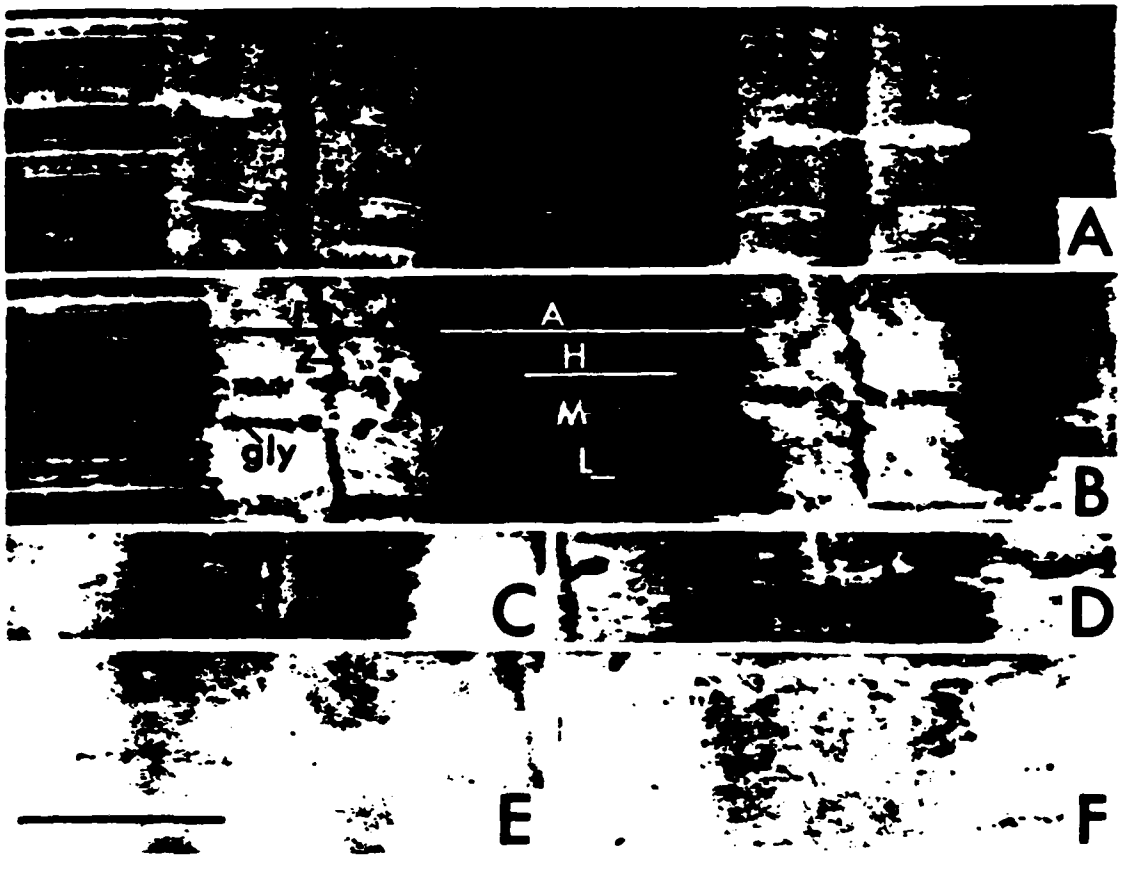


FIGURE 1

Electron micrographs of frog sartorius muscle. (A) Muscle fixed in glutaraldehyde (only) by stained with uranium by conventional procedure. (B) EM of section of freeze-dried Cs^+-loaded muscle without chemical fixation or staining. (C) $T1^+$ loaded muscle without chemical fixation or staining. (D) SAME as C after exposure of section to moist air, which causes the hitherto even distribution of thallium to form granular deposits in the A band. (E) Section of central portion of B after in distilled water. (F) Normal "K^+-loaded" muscle. A: from Edelmann (unpublished) B to F: from Edelmann by permission of Physiol. Chem. Phys. (9:217,1977)

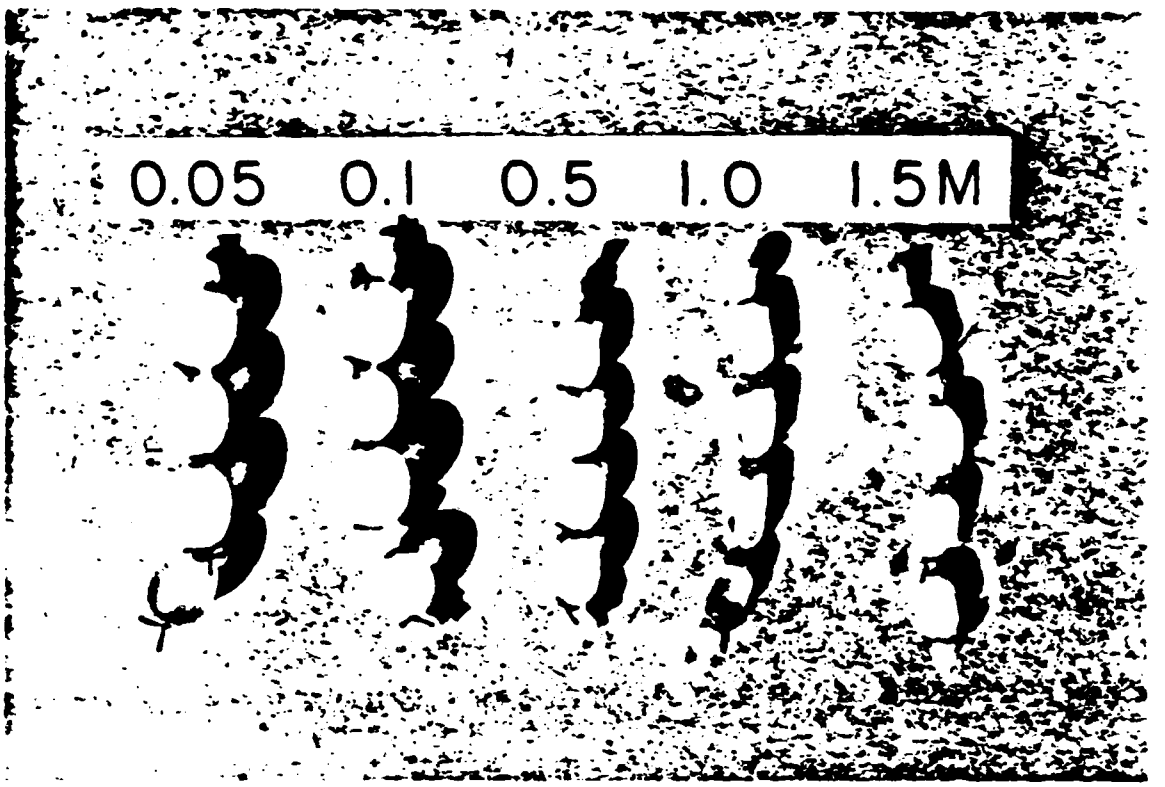


FIGURE 2

Swelling and shrinkage of a non charged polymer water system in the presence of varying concentrations of sodium citrate. Note that at equilibrium the bags containing initially similar concentration of the polymer, swelled in dilute salt solution but shrank in the more concentrated salt solution. Dialysis tubing used is readily permeable to sodium citrate and are used only to hold the polymers. The data indicate that a polymer water system can shrink and swell in the same manner as the living cells when they are placed in hyper- or hypotonic solutions. No semi-permeable membrane is needed. All that is required is water polarized and thus having a low q value.

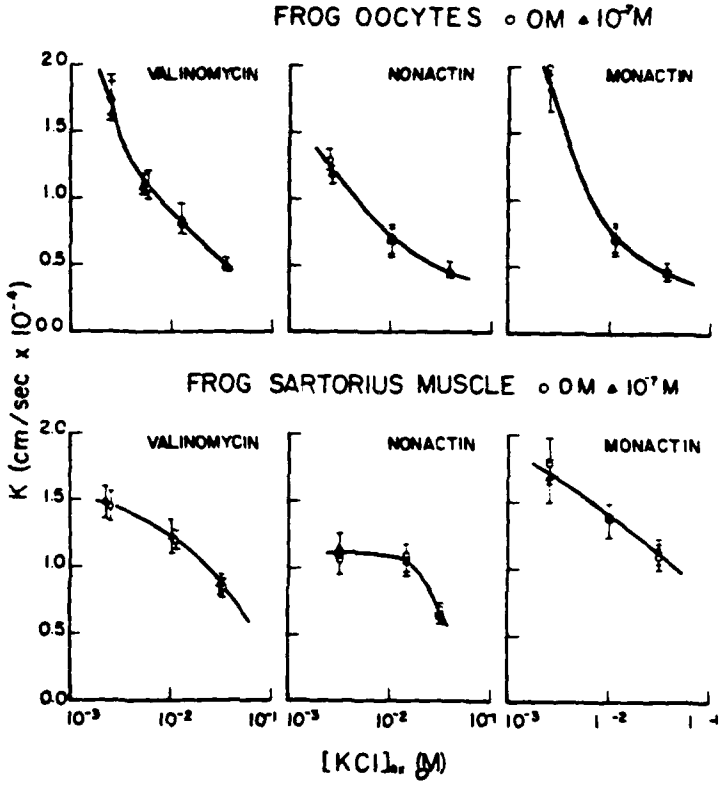

FROG OOCYTES ○ 0M ▲ 10⁻⁷M
VALINOMYCIN
NONACTIN
MONACTIN
FROG SARTORIUS MUSCLE ○ 0M ▲ 10⁻⁷M
VALINOMYCIN
NONACTIN
MONACTIN
K (cm/sec x 10⁻⁴)
2.0
1.5
1.0
0.5
0.0
10⁻³
10⁻²
10⁻¹
[KCl]ₒ (M)

FIGURE 3

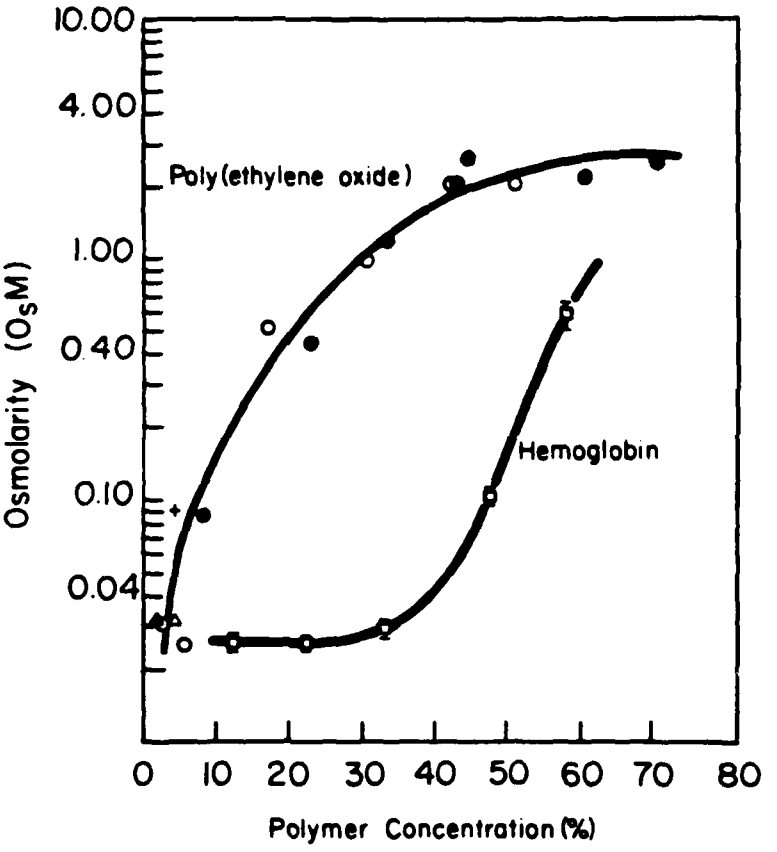

10.00
4.00
1.00
0.40
0.10
0.04
Osmolarity (O_sM)
Poly(ethylene oxide)
Hemoglobin
0
10
20
30
40
50
60
70
80
Polymer Concentration (%)

FIGURE 4

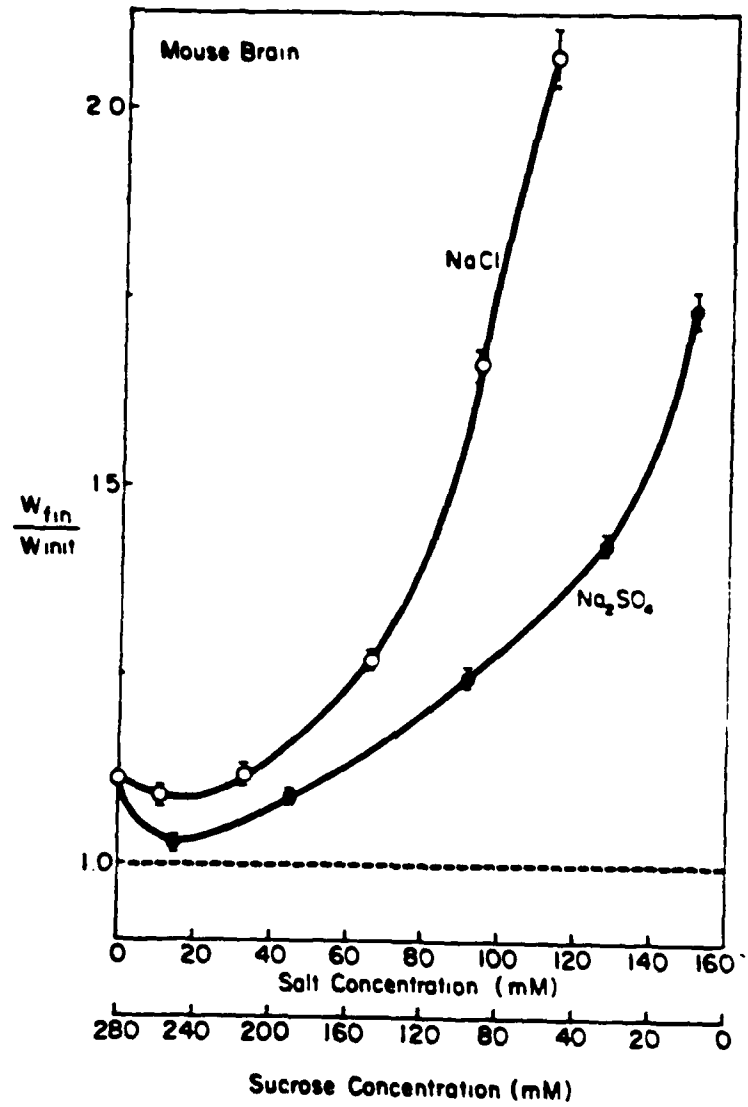


FIGURE 5

The effect of salt or sucrose concentrations on the swelling of dying mouse brain. As represented on ordinate solutions used for incubation ranged from one containing 0 mM salt and 280 mM sucrose to one containing 140 mM salt and 0 mM sucrose.

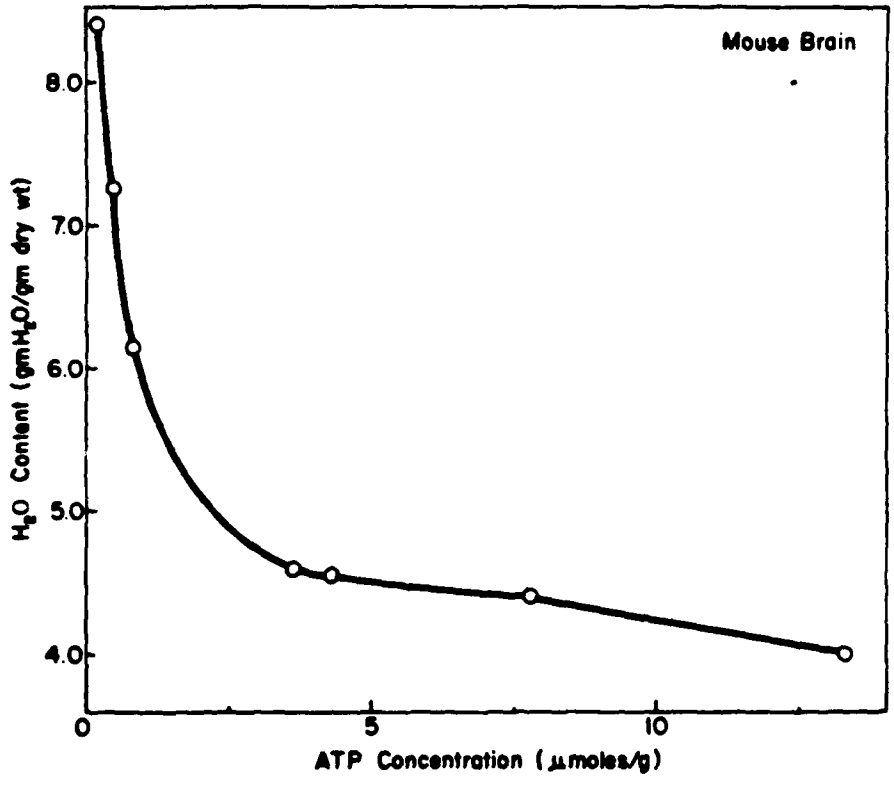
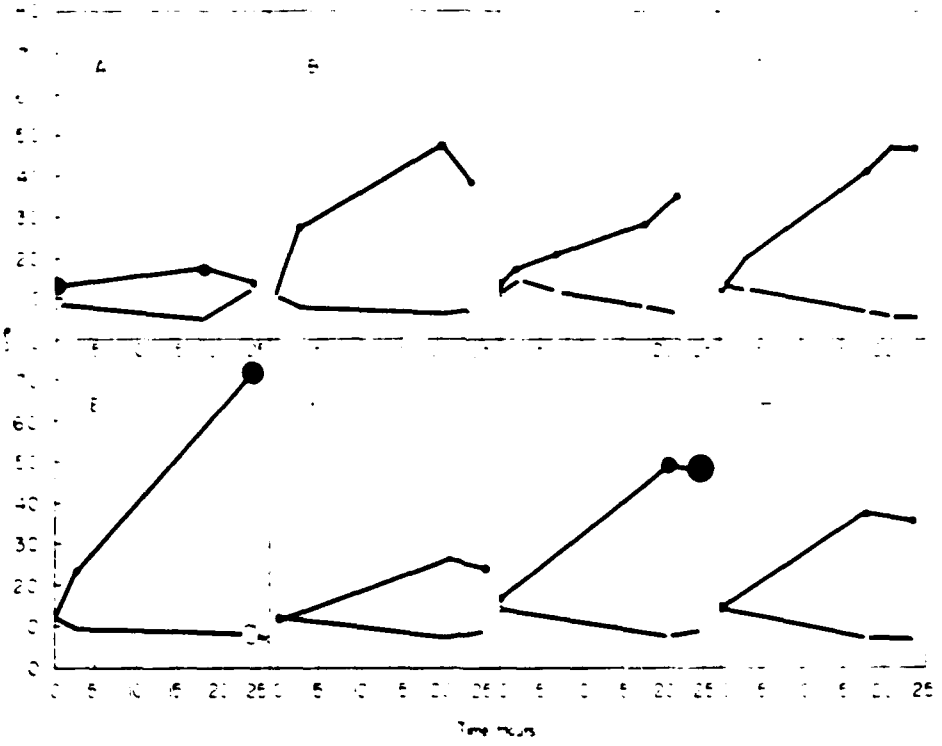

Mouse Brain
8.0
7.0
6.0
5.0
4.0
H_2O Content (gm H_2O/gm dry wt)
0
5
10
ATP Concentration (μmoles/g)

FIGURE 6

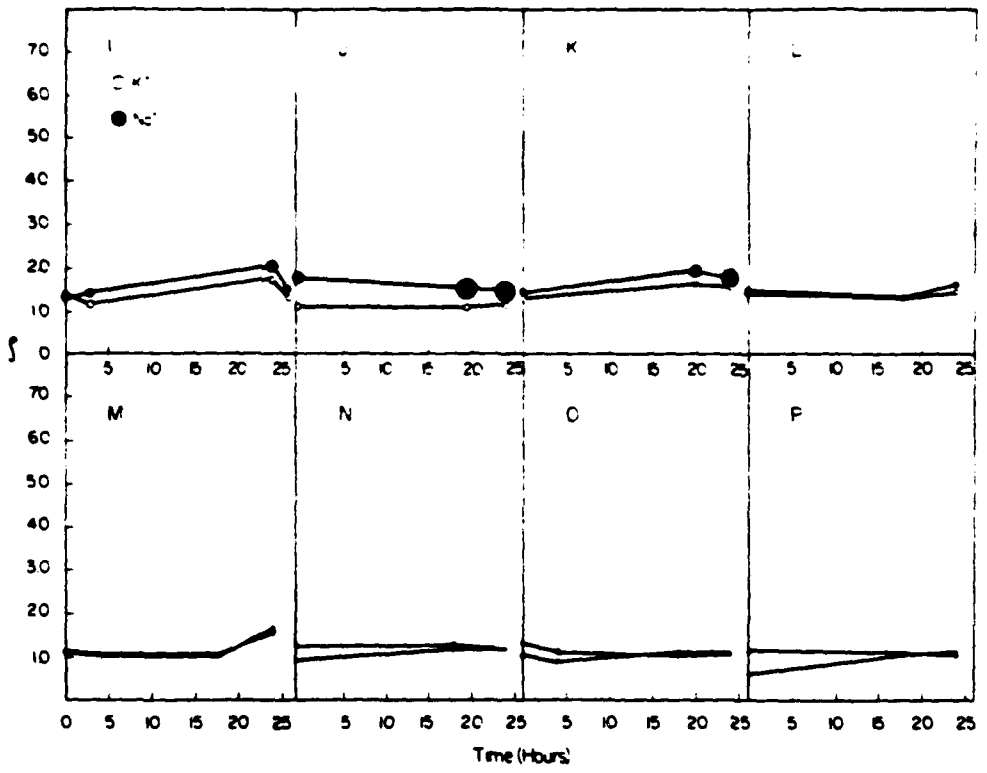
Electron micrograph of human red cell ghosts prepared by the method of Freedman ("Freedman ghosts"). (Magnification: 18,000x)

FIGURE 7



Demonstration of active transport of k^+ and Na^+ against concentration gradients in the Freedman ghosts. The ordinate represents the ratio of the concentration of K^+ and Na^+ ion in the ghost water over the concentration of the same ion in the incubation media. This ratio is called the ρ-value. Each point is the average of at least four determinations. The diameter of the solid circles (Na^+) and hollow circles (K^+) represents twice the standard errors.

FIGURE 8



Demonstration of a lack of active transport of K^+ and Na^+ against concentration gradients in the Marchesi-Palade ghosts. The ordinate represents the ratio of the concentration of K^+ or Na^+ ion in the ghost water over the concentration of the same ion in the incubation media. This ratio is called the p-value. Each point is the average of at least four determinations. The diameter of the solid circles (Na^+) and hollow circles (K^+) represent twice the standard error.

FIGURE 9

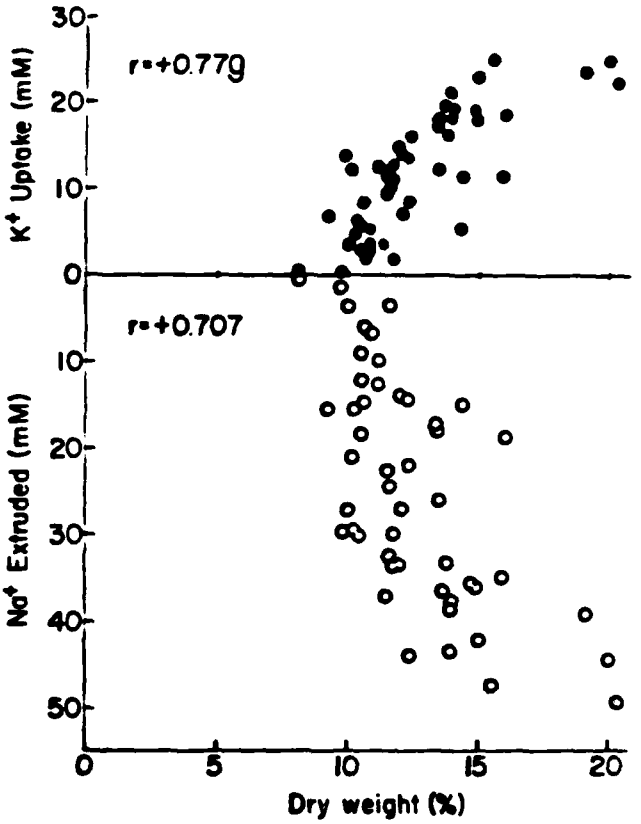

30
20
10
0
10
20
30
40
50
K+ Uptake (mM)
Na+ Extruded (mM)
r=+0.779
r=+0.707
0
5
10
15
20
Dry weight (%)

FIGURE 10