AD-A136 225 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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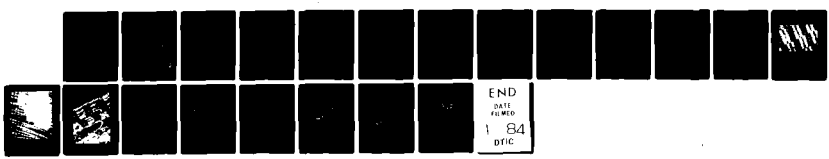

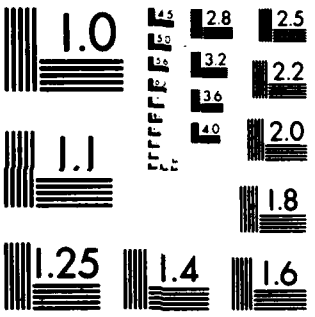

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Annual Progress Report

Work Accomplished Between
November, 1980 to October 1, 1981
under ONR Contract N00014-79-C-0126

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Annual Progress Report
November 1980 - October 1, 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

Since a fairly detailed report was submitted in June in the form of a "Status Report" this part of work accomplished will not be repeated (A copy is attached). Instead this report will concentrate on work that was not reported previously, including work accomplished within the period June 1 to October 1, 1981. Again the work will be described under two headings: laboratory work and non-laboratory work preceded by a brief sketch of the historical background.

Background Information

The most abundant components of the living cells are water, proteins and K^+. According to the membrane-pump theory intracellular water and K^+ exist largely in the free state. This theory has provided the theoretical framework for a great deal of brilliant achievements, including the theory of cellular potentials of Bernstein, Hodgkin, Huxley and many others.

According to the alternative, the "protoplasm theories" as championed by Moore, Fisher, and Gortner, a substantial part or all of cells K^+ and water may exist in a bound state. This view all but vanished in the early forties. The membrane-pump concept became almost universally accepted. But not everyone was so convinced.

Having discovered that iodoacetate plus anoxia failed to slow down the rate of Na^+ efflux in frog muscle (which in the membrane-pump theory largely represents Na^+ pumping rate) - a finding subsequently confirmed by Keynes, Conway and coworkers (1) - I suggested in 1951 a new theory including a molecular mechanism for the selective accumulation of K^+ over Na^+ in living cells as well as (sulfonate type) cation exchange resins. This theory comprises two postulates: (a) in living cells and some model systems there is a high degree of counterion (e.g., K^+) associated with fixed anionic sites and (b) electrostatic adsorption on fixed anionic sites (primarily β- and γ-carboxyl groups of cell protein) favors the smaller hydrated K^+ over Na^+ (2).

A variety of experimental studies were carried out to test if intracellular K^+ is free or "bound", including the measurement with a K^+ sensitive microelectrode of intracellular K^+ activity, the demonstration of an ability of pure natural cell membranes (e.g., red cell ghosts) or synthetic membrane vesicles (e.g., phospholipid vesicles containing K, Na activated-ATPase) selectively to accumulate K^+ or Na^+. These findings in addition to the apparent repudiation of early claims of demonstration of K^+ and Na^+ as well as H_2O binding by NMR led many scientists to the conclusion that the bulk of intracellular K^+ as well as water are in a free state.

More careful scrutiny of these findings over a period of many years, however, have gradually revealed that some of the evidence was incorrect; others equivocal (a full in-depth review will be given in my forth-coming book; for a briefer one, see ref (3)).

On the other hand neither the pure membrane "vesicle", par excellence, the squid axon membrane sac, nor white ghosts pump K^+ or Na^+ (3); whereas an effectively membraneless open-ended (EMOC) preparation of muscle cells as well as red ghosts containing large quantity of intracellular protein accumulates K^+ and extrudes Na^+ (3,4).

The most definitive experimental evidence that the bulk of intracellular K^+ exists in an adsorbed state came in the last four years. Using four new and different techniques, three different laboratories arrived at the conclusion that the bulk of intra-

cellular K^+ is localized primarily at the edges of the A band where myosin is located (and myosin is known to contain 60% of the β- and γ-carboxyl groups of the muscle cells).

(i) Edelmann (5, 6, 7): frozen dried- and dry-cut unfixed and unstained frog muscle cells with its K^+ replaced physiologically with electron dense surrogate, Ca^+ or Tl^+, reveal in transmission EM pictures localization of Cs^+, Tl^+ in A band and Z-line.

(ii) Ling (8): autoradiography of air dried single muscle fibers whose K^+ has been replaced by radioactive Cs^{134}-labelled Cs^+, or Tl^{208}-labelled Tl^+ shows localization of silver grains at A bands and Z-line. This finding was completely corroborated by Edelmann in autoradiographs of frozen single fibers (7).

(iii) Edelmann (9), using dispersive x-ray microprobe analysis showed concentration of surrogate Cs^+, Tl^+ as well as K^+ in normal K^+-loaded muscle in the A bands. This work was confirmed and extended by Trombitas and Tigyi-Sebes, who demonstrated concentration of K^+ in the A band of isolated air-dried honey been myofibrils (10).

(iv) Edelmann (11), using both x-ray microprobe analysis as well as laser microprobe mass spectrometer (LAMA) demonstrated selective uptake of K^+ and Ca^+ (over Na^+) in the A bands of freeze-dried muscle section when exposed to solution containing these ions.

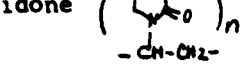
Other auxiliary experiments established that K^+, Cs^+, and Tl^+ compete for the same sites with widely different affinities in intact muscle cells, unaltered by the effective removal of cell membrane (8). Since K^+, Cs^+, Tl^+ and Na^+ are all univalent but differ only in short range attributes it was concluded that there must be close contact of the locally accumulated cation on anionic sites in the A band and Z-line (8).

The establishment of the adsorbed state of the major cation, K^+, demands a " new" source of osmotic activity inside the cell to balance that of free Na^+ in the external medium (12). Remembering that osmotic activity in fact is a measure of the decrease of water activity, this " new" source, in the AI-Hypothesis, is the third major component of the cells, the proteins. More specifically, it is the extended polypeptide chain of a matrix protein postulated to exist throughout the whole cell in all living cells. These protein matrix polarize in multilayers and hence reduce the osmotic activity of the bulk of cell water amounting to, on an average of about 5 layers of water molecules per chain. It is the reduced solubility of water for large hydrated ions (e.g., Na^+) and molecules (e.g., sugar, amino acid) that functions to maintain the low level of free Na^+ (and K^+) in muscle and nerve cells. Since there is no other osmotically active molecules or ions in the cell which match in concentration, that of K^+, the establishment of the adsorbed state of K^+ adds a compelling reason for the polarized multilayer theory of cell water. (for major new experimental evidence supporting this view, see 13, 14).

Laboratory Work is repeated on)

(1) V. Further investigation into the osmotic activity of water in the presence of several oxygen-containing polymers, → p.

In earlier work we demonstrated that a matrix of linear chains containing oxygen atoms (i) that are separated from each other by a distance of two water molecules diameter and (ii) that these oxygen atoms are not locked in intra- or intermacromolecular H-bonds but are directly exposed to bulk-phase water, has the ability of altering the water profoundly reducing its solubility for Na^+, sugars, and free amino acids to such an extent that more than one layer of water molecules are affected. Native proteins like

hemoglobin do not produce this effect because its backbone C=O groups (and NH group) are locked in H-bonds of helical and other secondary structures. It becomes effective when denatured with urea or guanidine HCl. Gelatine (denatured collagen) on the other hand, polarizes water because its proline, hydroxyproline, and glycine residues prevent the assumption of helical structure, reduces water solvency, so is a variety of synthetic and natural polymers satisfying the above-mentioned criteria. The most interesting among them are poly-vinyl pyrrolidone  and polyethylene oxide $[-CH_2-O-CH_2-]_n$.

In the "Status Report" part of this document we have already shown that while a 30% or = 5.3 mM (native) hemoglobin solution produces an osmotic effect equal to that of 13 mM NaCl solution a 0.5 mM or 30% polyethylene oxide solution can produce an osmotic effect equal to that of a 500 mM NaCl solution. Figure A and B further confirm these results. They show that polyvinyl pyrrolidone and exhaustively dialyzed gelatin exhibit similar high osmotic activity.

2) H. Further investigation into the molecular mechanism involved in the swelling of frog muscle induced by isotonic KCl, and → p. 4

Frog muscle swells to twice its normal size when soaked in a Ringer solution containing 100 mM KCl rather than 100 mM NaCl. This volume change could be quantitatively described by a theoretical model presented by Ling and Peterson, 1977 (15), involving cooperative salt linkage dissociation (see Fig. C). This theory is in harmony with the finding of Ling and Walton, 1976 (16) that frog muscle cells which represent parallel array of 3 cm long cells when cut into 2 and 4 mm long segments with open ends and no regeneration of new membrane (see Table 1) nevertheless can swell in isotonic KCl to an extent quantitatively indistinguishable from a similarly treated intact muscle. Clearly, these findings profoundly contradict the conventional membrane theory according to which cells without an intact membrane cannot be inflated. According to the AI Hypothesis, the degree of multilayer polarization can be measured by the equilibrium distribution coefficient or q-value of a probe molecule like non-metabolizable sugar, D-arabinose. Healthy muscle cytoplasm has a q-value for most pentoses studied equal to about 0.25, i.e., the equilibrium concentration of D-arabinose in the cell water is 25% of that in the external medium. A less exact parameter called the apparent equilibrium distribution coefficient (or ρ-value) which is a rough estimate of the maximal limit of the q-value since it may include adsorbed component that has not been sorted out and subtracted. The ρ-value of D-arabinose in frog muscles treated with 100 mM KCl is about 0.4 (Fig. D) higher than normal but still indicative of a generally polarized state. Yet here in these swollen muscles, there is twice as much water as a normal muscle. Thus all the additional water taken up must still be in an essentially similar state as water originally present in the cells.

It is long known that muscle swells largely in the lateral dimensions, with little change in length. An ordinary EM picture shows fully packed cells as shown in Fig. E. Where are the salt linkages that KCl dissociates to cause lateral expansion? Fig. F is an EM prepared by the standard method including fixation in glutaraldehyde and osmium oxide, in medium carefully set at unchanging osmolality. The fixed muscle was then imbedded in low viscosity Spurr medium and stained en bloc in uranium and lead. Note that large gaps now develop that look like normal myofibrils. Fig. G was an identically treated muscle which was, however, rapidly frozen and dried before infiltration with Spurr medium, by Edelmann at the University of Saarland, West Germany. Note that here the "myofibrils" are represented by much narrower bunches of myofilaments when compared to Fig.E . In between the filaments is a trabecular type of network. Our tentative conclusion is that isotonic KCl actually dissociates salt linkages which link the myofilaments

laterally under normal conditions. In the highly hydrated state, the standard fixation method causes the lateral " clumping" of much more dispersed filament bundles much better preserved in the frozen and dried section. In support, one may note that in the lower left hand corner of Fig. F one bunch of filaments start from the Z-line of one of the myofibrils but ends on that of another. We also suspect that the trabecular network between the filaments is also the end result of lateral clumping of what in the living state, may be a much more uniformly distributed " matrix protein" network where the chain-to-chain distances are closer than those visualized in even the best frozen-dried sections now available.

3) Further investigation into the relationship between swelling, salt ions, and ATP in cold-injured tissues,

In the " Status Report" part of the document, we have illustrated the work we performed on isolated mouse brain which swells enormously when exposed to cold (4° C). This swelling was shown to parralel changes in the level of ATP in the cells (Fig. 6) and is intimately related to the concentration of Na^+ and Cl^- in the surrounding medium (Fig. 5).

In Fig. H & I we have shown how different alkali-metal chlorides cause different degree of swelling of normal frog muscle. KCl and RbCl at 100 mM cause marked swelling; 100 mM NaCl, LiCl, on the other hand, had no effect. Fig. J shows while the swelling produced by 100 mM KCl is as marked in fresh, normal mouse kidney as in frog muscles; the swelling of fresh normal spleen is much less.

Thus cold injury induces changes which led to a drastic decline of the ATP content of the tissue and along with this change, the cells normally not swollen by the exposure to 100 mM NaCl (see Fig. 6 of " Status Report ") - which is a normal component of the cell environment, i.e., plasma - now they become so. Again there is no way to explain the phenomenon in conventional membrane-pump theory terms because these injured cells are freely permeable to and are leaking considerable amount of intracellular proteins - indicating profound increase of membrane permeability with cell deterioration.

These phenomenon can be explained by the AI Hypothesis without ad hoc postulations: ATP acts as a cardinal adsorbent and allosterically controls the electronic (and steric) conformation of the key proteins so that the electron density of β- and γ-carboxyl groups carried by aspartic and glutamic residues (measured in terms of the parameter, called the c-value) is maintained at a value (indicated by a in Fig. K) at which K^+ and Rb^+ tolerance exceeds that of Na^+ and Li^+. At this c-value NH_4^+ is also preferred over Na^+ and Li^+. This is highly significant because NH_4^+ which is the prototype of the fixed cations of ε-amino group and guanidyl groups carried respectively by lysine and arginine residues, and because it offers a possible explanation why K^+, Rb^+ can dissociate salt linkages formed between β- and γ-carboxyl groups and ε-amino groups while Na^+ and Li^+ cannot.

Another basic concept introduced by the AI Hypothesis is that when ATP is lost, as it does during cold exposure of mammalian tissues, the c-value of the fixed anions shifts to a higher value (say at point indicated by b of Fig. K). At this c-value the β- and γ-carboxyl groups have similar preference for Na^+ and Li^+ as for NH_4^+. As a result 100 mM NaCl which is ineffective in causing swelling under normal, muscles becomes as effective as KCl in causing swelling. Fig. L shows that the sensitivity of cold-injured kidney and liver have similar swelling response to the presence of NaCl, while spleen, shows also a minimal response just as under normal physiological condition it shows low sensitivity to 100 mM KCl. In further support, one finds that LiCl is as effective as NaCl in causing swelling of cold-injured mouse brain and kidney (Fig. M).

We now turn to the significance of anions in the cold-induced swelling. In all cases studied, swelling of cold-injured tissues depends not only on the right cation (Na^+, Li^+, but not Mg^{++} for example (Fig. M) but also the right anion. Thus NaCl and LiCl caused swelling while Li_2SO_4 and Na_2SO_4 at the same osmotic activity caused much less swelling. This also agrees with the general concept that ϵ-amino group and guanidyl groups tend to bind Cl^- more tightly than SO_4^{2-} (see Fig. N).

II. Non-laboratory Work

A. I have now finished the first draft of the new book, now given the following title: " In Search of the Physical Basis of Life".

B. Additional Work published:

Ling, G. N., Water and the Living Cell as Seen from the Viewpoint of a New Paradigm, in International Cell Biology 1980-1981, ed. H. G. Schweiger, Springer-Verlag, Berlin, pps. 904-914 (1971)

Ling, G. N., Oxidative Phosphorylation and Mitochondrial Physiology: A Critical Review of Chemiosmotic Theory, and Reinterpretation by the Association-Induction Hypothesis, Physiol. Chem. Phys., 13:29-96 (1981)

References

1. Ling, G. N. and M. M. Ochaenfeld, Physiol. Chem. Phys. 8:389 (1976)
2. Ling, G. N., Amer. J. Physiol. 167:806 (1951)
   Ling, G. N., in Phosphorous Metabolism (Vol. II), W. D. Mc Elroy and B. Glass, Eds., The John Hopkins University Press, Baltimore, MD, 21218, pgs. 748-795 (1952)
3. Ling, G. N. and W. Negendank, Persps. in Biol. & Medicine 23:215 (1980)
4. Ling, G. N., J. Physiol. 280:105 (1978)
5. Edelmann, L. Physiol. Chem. Phys. 9:313 (1977)
6. Edelmann, L. J. Microscop. 112:243 (1978)
7. Edelmann, L. Intern. Cell. Biol. 1980-1981, Ed. H. G. Schwieger, Springer, Berlin, 1981
8. Ling, G. N., Physiol. Chem. Phys. 9:319 (1977)
9. Edelmann, L. Microscop. Acta Suppl. 2:166 (1978)
10. Trombitas, K. and A. Tigyi-Sebes, Acta Bioch. Biophys., Acad. Hung. 14:271 (1979)
11. Edelmann, L., Physiol. Chem. Phys. 12:509 (1980)
12. Ling, G. N., Physiol. Chem. Phys. 12:383 (1981)
13. Ling, G. N., M. M. Ochsenfeld, C. Walton, and T. J. Bersinger, Physiol. Chem. Phys. 12:3 (1980)
14. Ling, G. N., C. Walton, and T. J. Bersinger, Physiol. Chem. Phys. 12:111 (1980)
15. Ling, G. N. and K. Peterson, Bull of Math. Biol. 39:721 (1977)
16. Ling, G. N. and C. L. Walton, Science 191:293 (1976)

	Uptake of labelled Sucrose through cut end (2 hrs; 25°C) (μmoles/g)	Uptake of labelled Na through cut ends (28 hrs; 25°C) (μmoles/g)	Uptake of labelled D-arabinose through cut ends (27 hrs; 25°C) (μmoles/g)
Immediately after cut	0.174 ± 0.031 (4)	22.3 ± 5.1 (4)	3.53 ± 0.20 (5)
After Incubation	0.169 ± 0.016 (4) (24 hrs.;25°C)**	22.7 ± 2.5 (4) (51 hrs.; 25°C)**	3.28* ± 0.20 (5) (43 hrs.; 25°C)**

* After incubation, muscles were cut 1 cm from cut end and the freshly cut surface exposed.

** Time referred to duration of incubation after ends of muscles were amputated and before exposure to labelled solutions.

Table 1

Data showing that the exposed surface of a frog sartorius muscle after razor blade cuts do not regenerate a new cell membrane as indicated by similar rate of permeation of probe molecules, sucrose, Na^+ and D-arabinose immediately after cut and after varying length of (sterile) incubation in a Ringer-tissue culture medium capable of preserving frog muscle in physiological condition for at least 8 days (25° C).

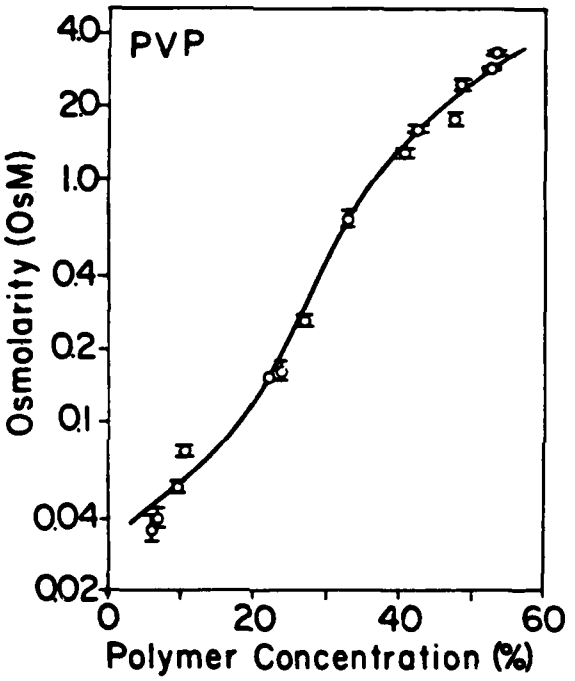


Figure A. Osmotic activity of aqueous solutions of polyvinylpyrrolidone. Osmotic activity measured with a Wescor osmometer at room temperature.

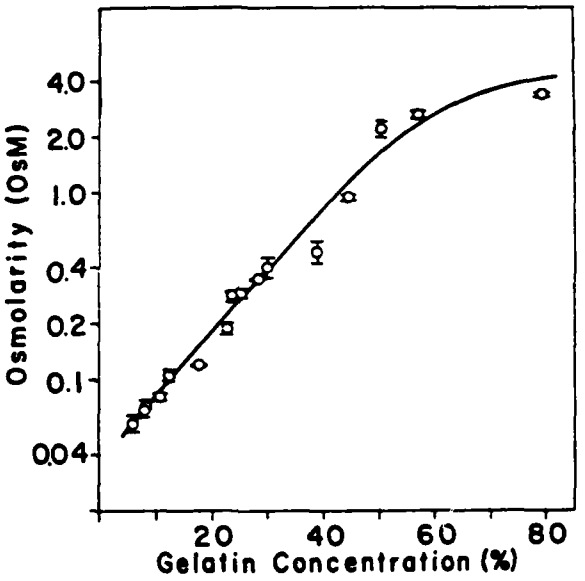


Figure B. Osmotic activity of aqueous solutions of exhaustively dialyzed gelatin.

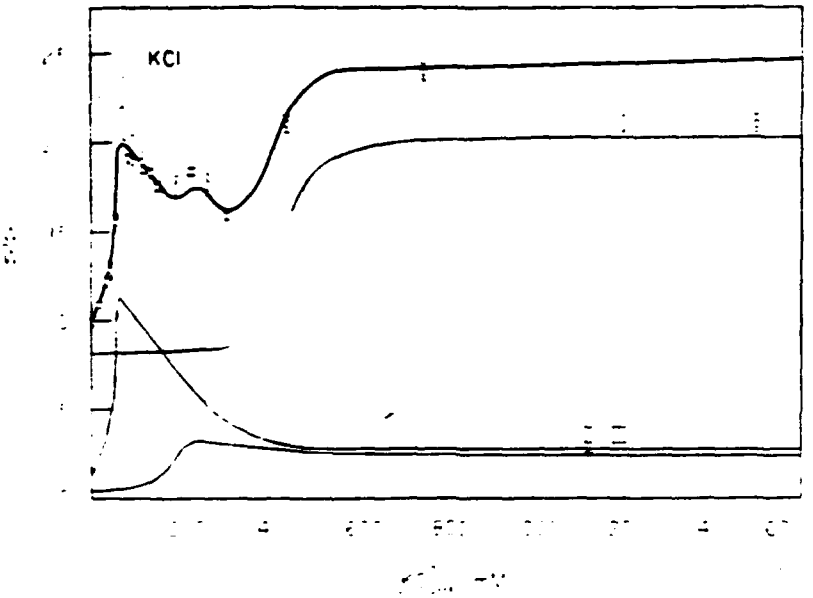


Figure C. Demonstration of agreement between theoretical curves and experimental data on the swelling of muscles in KCl. The numerical values used in the construction of the theoretical curves I, II, and III are as follows: Curve I: $K = 420$, $\theta = 0.01$, $\phi = 0.6$, $a_1 = 1.35$, $b_1 = 3$, $b_2 = 0.84$; Curve II: $K = 55$, $\theta = 0.035$, $\phi = 0.52$, $a_1 = 1.35$, $b_1 = 3$, $b_2 = 0.36$; Curve III: $K = 18$, $\theta = 0.01$, $\phi = 0.135$, $a_1 = 1.35$, $b_1 = 3$, $b_2 = 0.25$

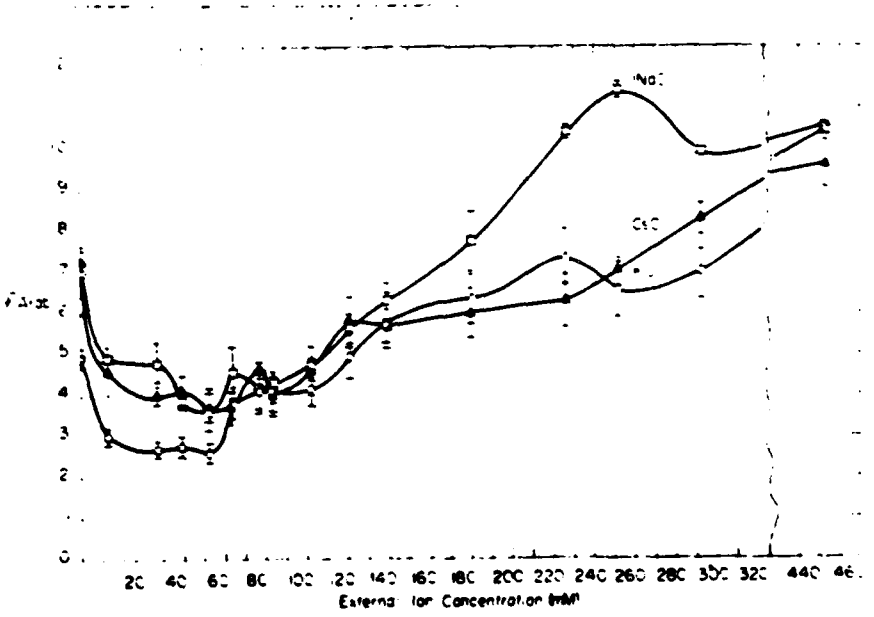


Figure D. The apparent equilibrium distribution coefficient or p-value of D-arabinose in tissue water of muscles exposed to the external environment. Muscles were exposed for six days at 4 C to NaCl, KCl, and CsCl solutions containing 5 mM D-arabinose and then for two more days at 0 C in the same solution to which radioactive D-arabinose had been added

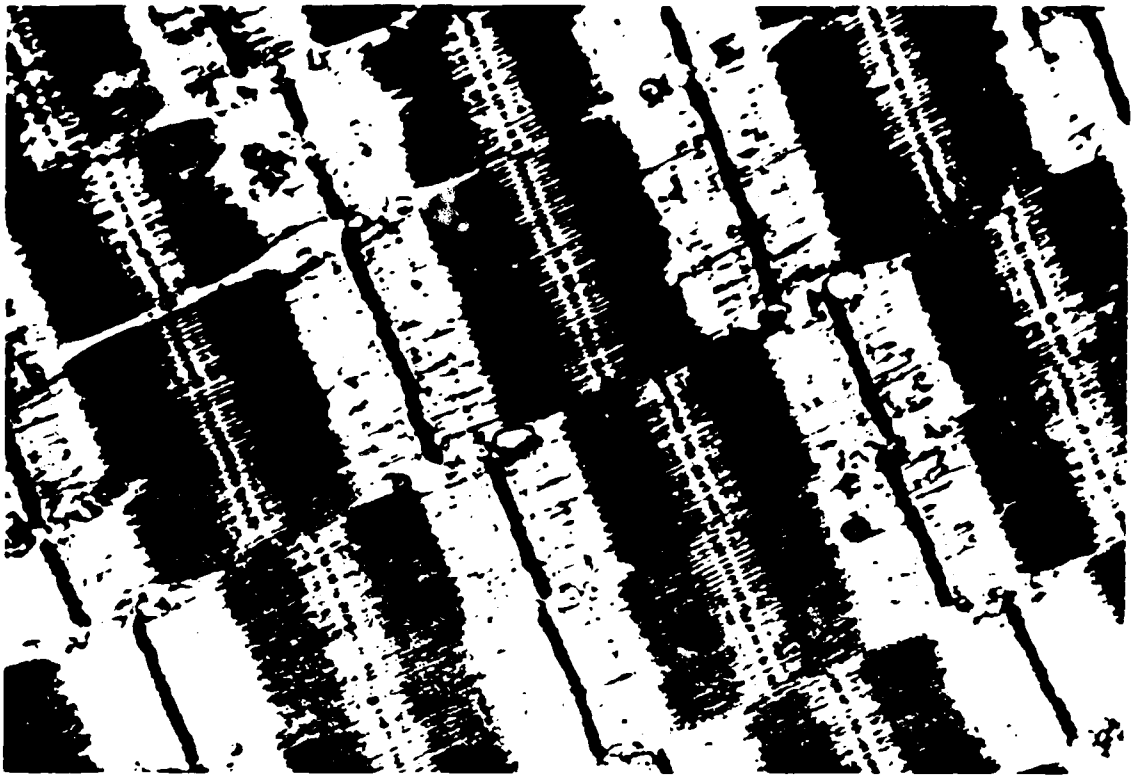


Figure E. EM of a section of a normal frog sartorius muscle showing darkened A bands and light I-band bisected by the dark, thin Z-line.

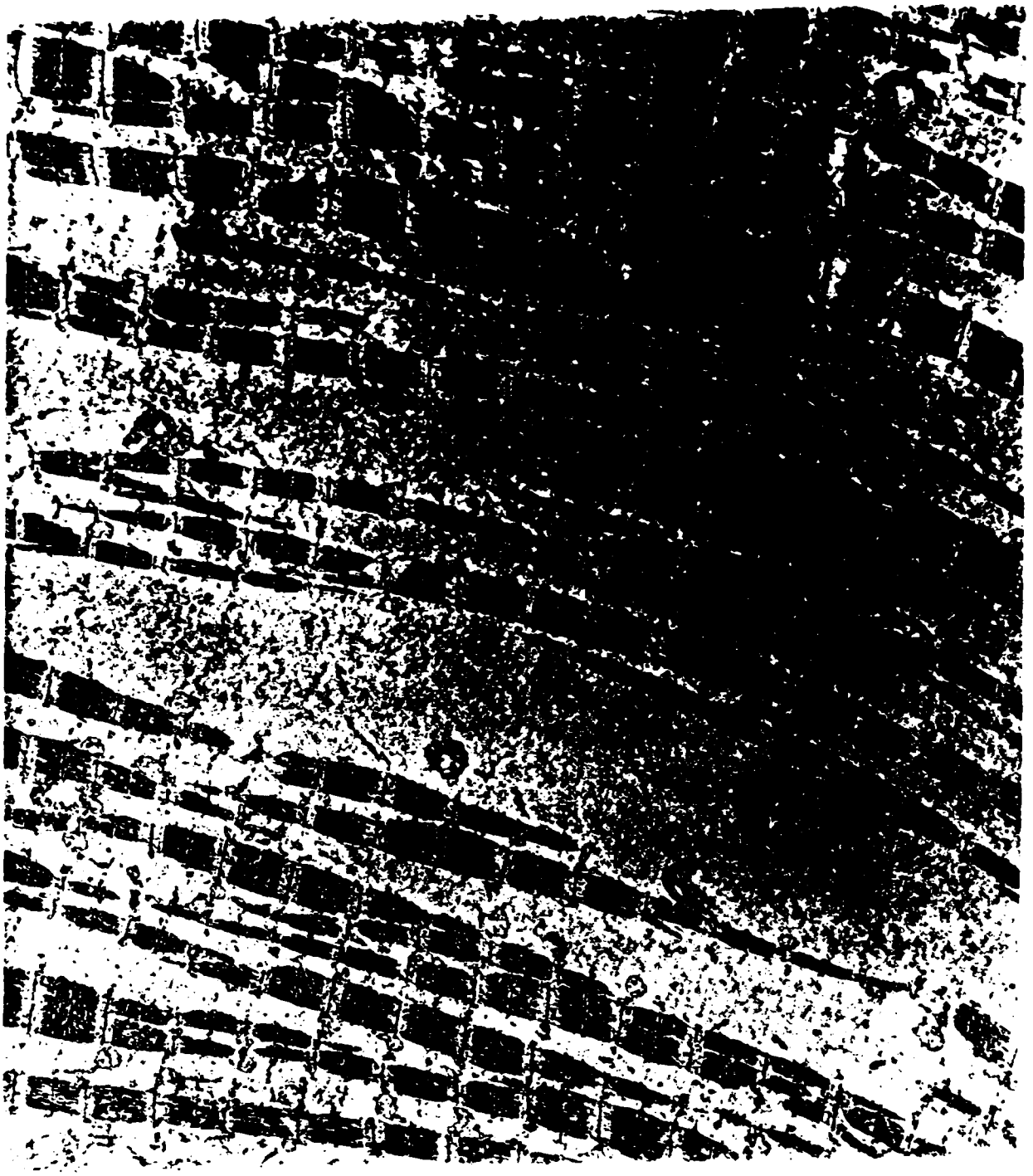


Figure F. Frog muscle pretreated with a Ringer solution containing 93 mM KCl (4^{0} C) before fixation, imbedding, etc. by the conventional method

Figure G. Frog muscle pretreated with a Ringer solution containing 93 mM KCl (4° C) but frozen dried (Picture provided by Dr. L. Edelmann, who cooperated with us in this work.)

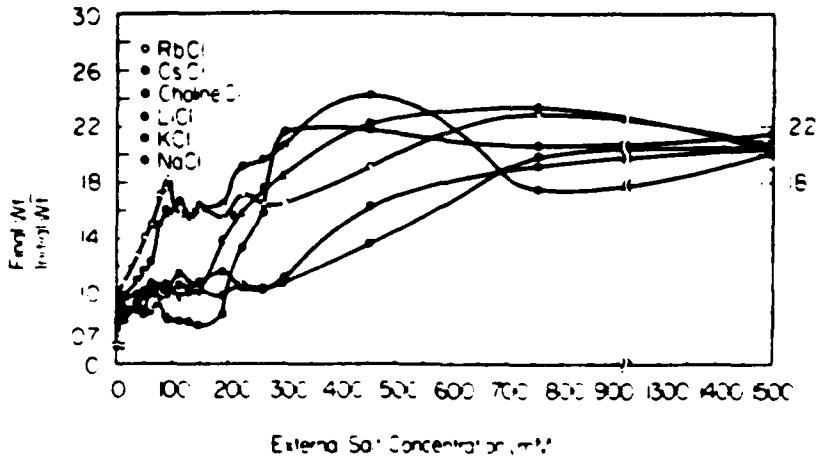


Figure H. Swelling of frog voluntary muscles in varying concentrations of LiCl, NaCl, KCl, RbCl, CsCl, and choline chloride, demonstrating the segregation into roughly three types of curves. Each point represents the average of four determinations from one of each of the four different types of muscles. To avoid confusion the standard errors of the means are not shown here but are shown in the more detailed Figures (6 and 7)

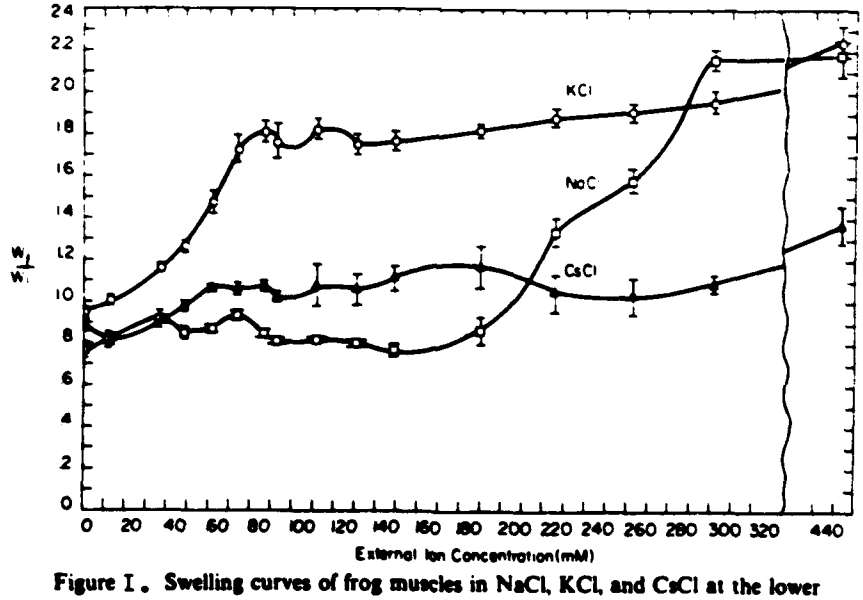


Figure I. Swelling curves of frog muscles in NaCl, KCl, and CsCl at the lower concentration range

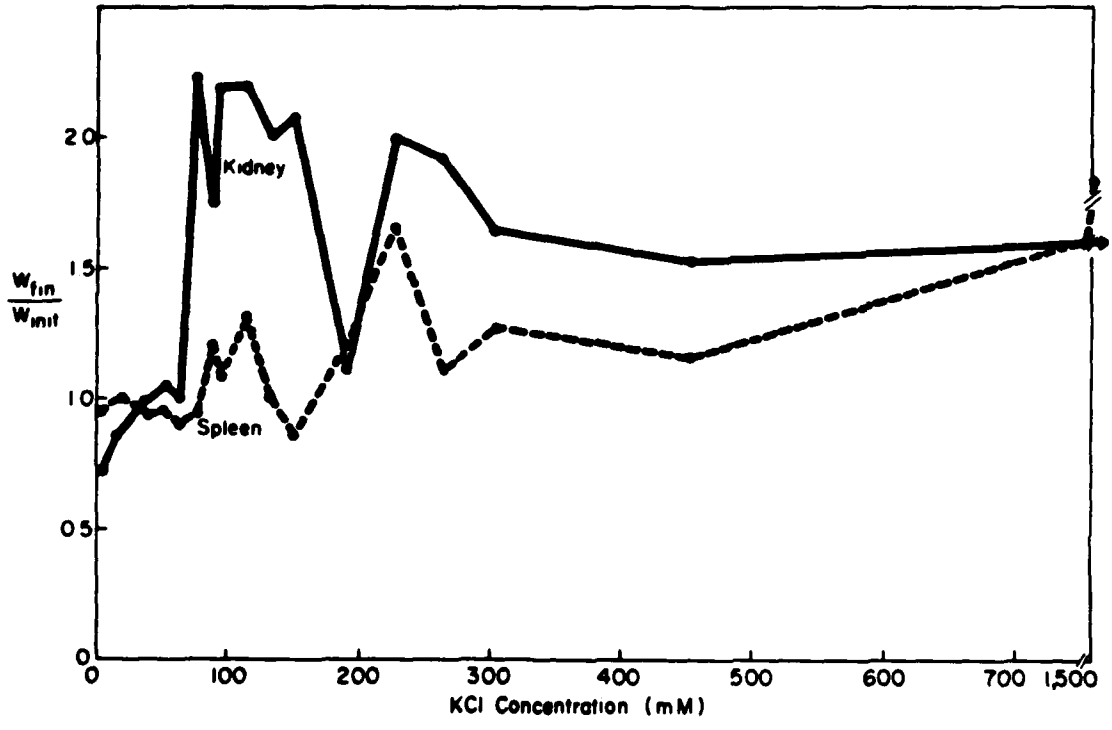


Figure J. Swelling of mouse kidney and spleen in Ringer solution containing increasing concentration of KCl.

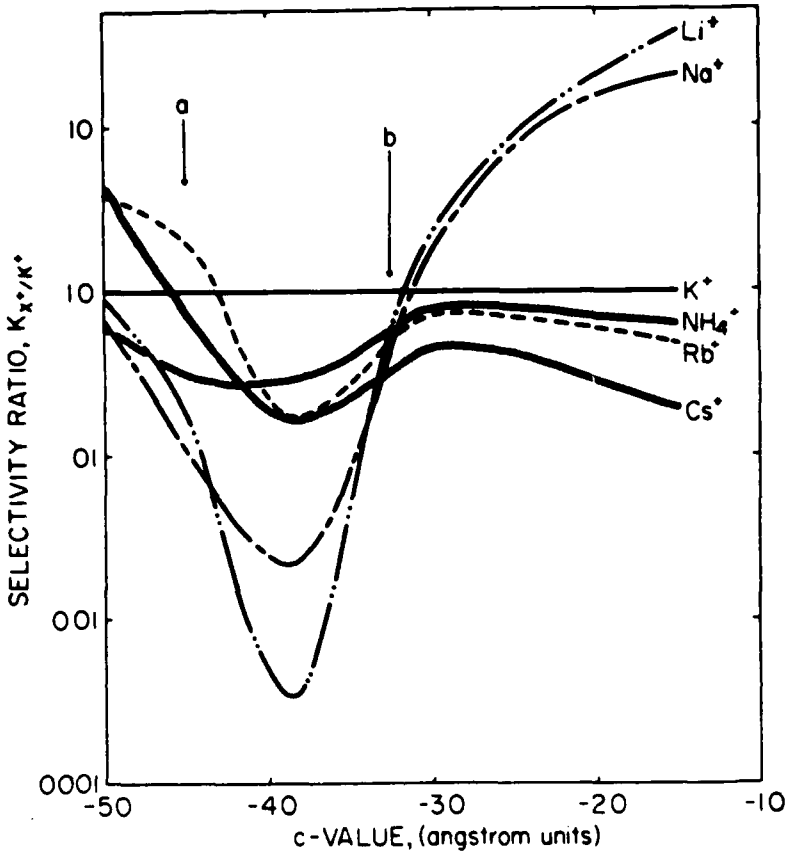


Figure K. Theoretical plot of relative affinity of fixed anionic groups with different charge density expressed as the c-value (High c-value is equivalent to high pK_a value) (Ling, 1962)

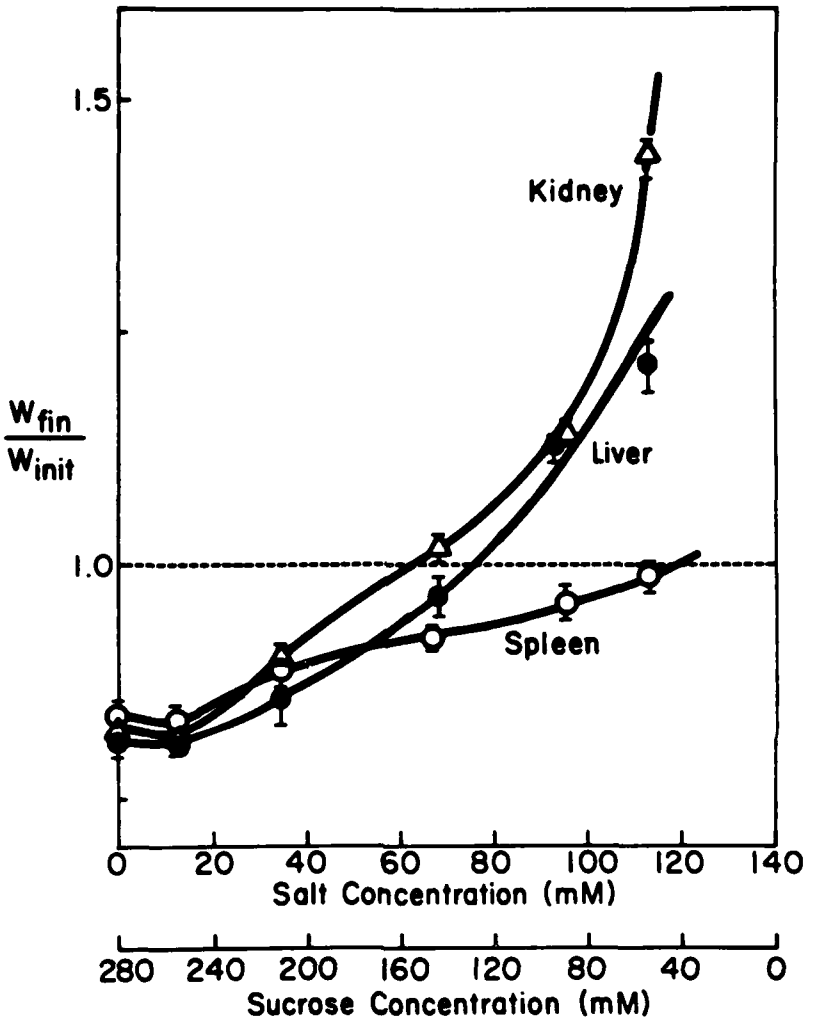


Figure L. Effect of external NaCl and sucrose concentration on the swelling of mouse tissues injured by cold (4° C, 24 hrs.)

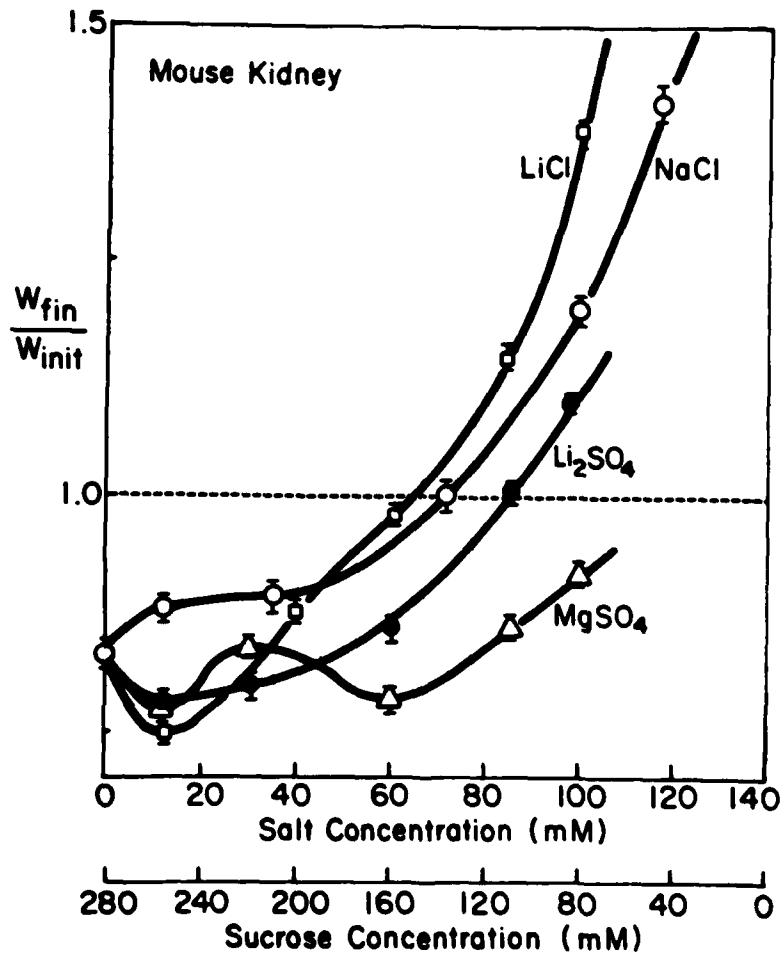


Figure M. Effect of Varying Cations and Anions on the Swelling of Mouse Kidney after Cold Injury (4° C, 24 hrs.)

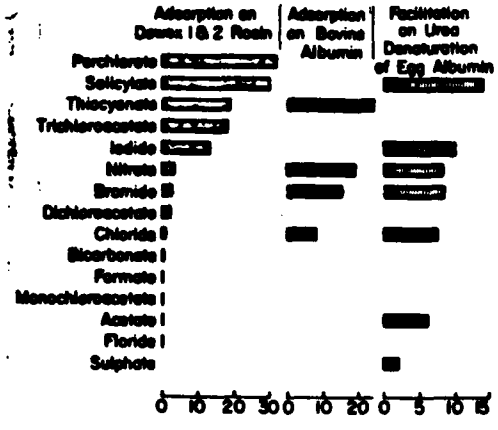
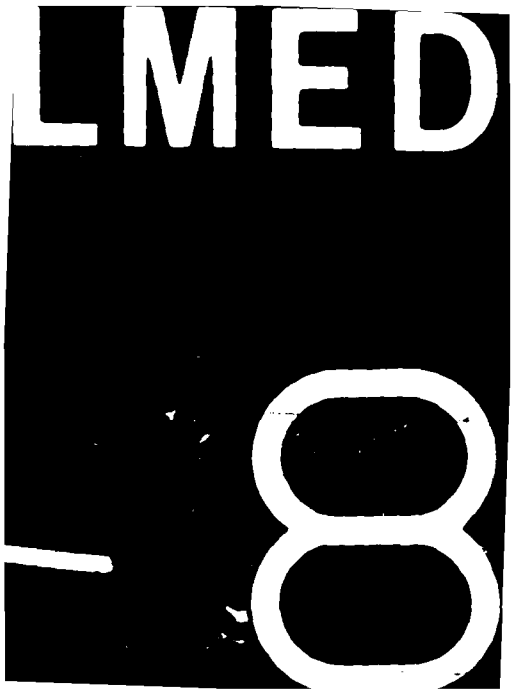


Figure N. The relative binding affinities of various anions (lyotropic sieves) on proteins and a model (amino type ion exchange resin, DOWEX 1, 2) (Ling, 1962)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