COLD INJURY-INDUCED SWELLING OF BRAIN AND OTHER TISSUES: ITS MOLECULAR MECHANISM

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• *Isolated mouse brain, kidney, and other tissues were incubated for from one to several days at 4° C in isotonic solutions containing varying mixtures of sucrose and NaCl (or Na_2SO_4, LiCl, Li_2SO_4, $MgSO_4$). The ischemic, cold-injured tissues swelled in proportion to the concentration of NaCl or LiCl. They swelled less in Na_2SO_4 and Li_2SO_4 and they swelled even less and/or shrank in $MgSO_4$ or sucrose. It was shown that in the presence of about 100 mM NaCl, the degree of swelling follows inversely the level of ATP present in the cells. The data were interpreted using the theory of cell swelling based on the association-induction hypothesis: NaCl- and LiCl-induced swelling in injured tissues and KCl-induced swelling in normal tissues, were both explained as the consequence of the dissociation of the volume-restraining effects of salt linkages among cell proteins. The depletion of ATP and the consequent increase of electron density or c-value of the fixed anionic groups turns NaCl (ineffective in causing swelling of normal tissues) into a fully effective agent for causing swelling of the injured tissues.*

Cell swelling and shrinkage were among the first physiological manifestations of the living cell that lent themselves to quantitative measurements. Studies of these cell volume changes led to the founding of the first major theory in cell physiology — the membrane theory. Early laboratory manipulations that produced these volume changes were usually achieved by altering the osmotic activity of the surrounding fluids. It also became known that cells can undergo extensive swelling within an environment with an osmotic activity equal to that of the cell interior, as they do in an isotonic KC1 solution (von Korosy, 1914) or in a natural isotonic environment rich in NaCl at 0° C (Stern, *et al,* 1949; Opie, 1949). Indeed, that injury can lead to tissue swelling is well known to physicians and lay people. Injury-induced swelling of brain is especially dangerous and sometimes fatal due to the confinement of the brain in a rigid box — the cranium.

Early theory of cell swelling and shrinkage was based on the assumption that the cell membranes were impermeable to solutes that can cause and maintain the shrunken state of the cells. A prime example of the impermeant solute thus defined was the Na^+ ion. This theory was disproven when it was shown that Na^+ is in fact permeant to the cell membrane (Cohn and Cohn, 1939; Heppel, 1940). The Na^+ pump was then postulated to maintain a low level of Na^+ in the cell and hence a constant cell volume (Lilly, 1923; Dean, 1941). Swelling due to injury or metabolic interference has been attributed to an interference with the functioning of metabolic pumps, that either continually drive out water (Opie, 1954; Robinson, 1952) or Na^+ (Tosteson, 1964). However, these theories became questionable when it was shown that the Na^+ pump alone would consume energy 15 to 30 times that available, and that the swelling of cells in hypotonic Ringer solution or in isotonic KCl remained unchanged after the intactness of the cell membrane was destroyed by cutting the cell into small segments (Ling and Walton, 1976). Under this condition, it was clearly established that there was no regeneration of a new membrane at the sites of amputation (Ling, 1973).

The basic concept that swelling of living cells may reflect behavior of the whole protoplasm rather than primarily the cell membrane can be traced back to Martin Fischer (1909). In more recent times, the association-induction hypothesis presented a unified conceptual framework to explain different types of swelling phenomena (Ling, 1962, 1969). The basic assumption, now supported by considerable experimental evidence (Ling, 1984), is that the osmotic activity of living cells (which is a measure of the reduction of the activity of cell water) is only to a minor extent provided by free intracellular ions and other solutes, notably K^+. This is so because virtually all of the cell K^+, the major cation found in the cell, (as well as most of the intracellular anions) exists in an adsorbed state (Ling, 1977a, Edelmann, 1977, 1981; Trombitas and Tigyi-Sebes, 1979). In this theory the reduction of cell water activity, measured as osmotic activity, is due primarily to certain cellular proteins, called matrix proteins. These matrix proteins exist in an extended conformation with their polypeptide NH and CO groups directly exposed to, and polarizing the bulk of, cell water (Ling, 1965, 1972, 1979). Water so polarized has reduced activity and exhibits osmotic activity far beyond its molar concentration predicted on the basis of the Boyle-van't Hoff Law (Ling, 1980, 1983). Experimental confirmation that water so polarized does indeed have high osmotic activity has just been published (Ling, 1983).

To put it differently, the behavior of water in living cells reflects its existence in the state of polarized multilayers. In support of this, Ling and Negendank (1970) demonstrated that 95% of the water in isolated frog muscles follows Bradley's multilayer-adsorption isotherm. Simple osmotic swelling in hypotonic solution is due to the higher water activity in a hypotonic solution and the natural tendency for water to seek the phase of lower water activity. Shrinkage in hypertonic solution is just the opposite. To explain swelling induced by an *isotonic* KCl solution, however, a more complex interpretation is involved: As a rule normal cells have a tendency to swell but this is held back by the presence of salt linkages which are formed between negatively charged side chain functional groups (e.g., β- and γ-carboxyl groups) and positively charged side chain functional groups (e.g., e-amino and guanidyl groups) of neighboring protein chains in the cells. Thus under normal conditions, salt-linkages between adjacent intracellular protein molecules prevent the full amount of water to be taken into the cell and adsorbed. However, KCl can split these salt linkages, as follows (Ling and Peterson, 1977):

$$f^+f^- + K^+ + Cl^- \rightleftarrows f^+Cl^- + f^-K^+. \qquad (1)$$

It was shown that this dissociation is autocooperative, i.e., all-or-none. That is, the salt linkages (f^+f^-) formed between fixed cations (f^+) and fixed anions (f^-) will resist the dissociation action of KCl until KCl reaches a certain critical concentration. At this concentration, the salt linkages all split up and the cell undergoes a step-wise swelling. As a rule, as one increases the KCl concentration in the external medium, gradually the cell undergoes several steps of swelling, interspersed with shrinkage due to the increase of total osmotic activity in the external medium with increase of total KC1 concentration. As mentioned above, after the intact muscle cells were cut into small segments 2 or 4 mm long with no membranes at their ends, 100 mM KCl in the external medium will continue to induce swelling while 100 mM NaCl will not (Ling and Walton, 1976).

According to the association-induction (AI) hypothesis, this selective sensitivity to KCl but not to NaCl reflects a high preference of the fixed anions (f^-), or more specifically,

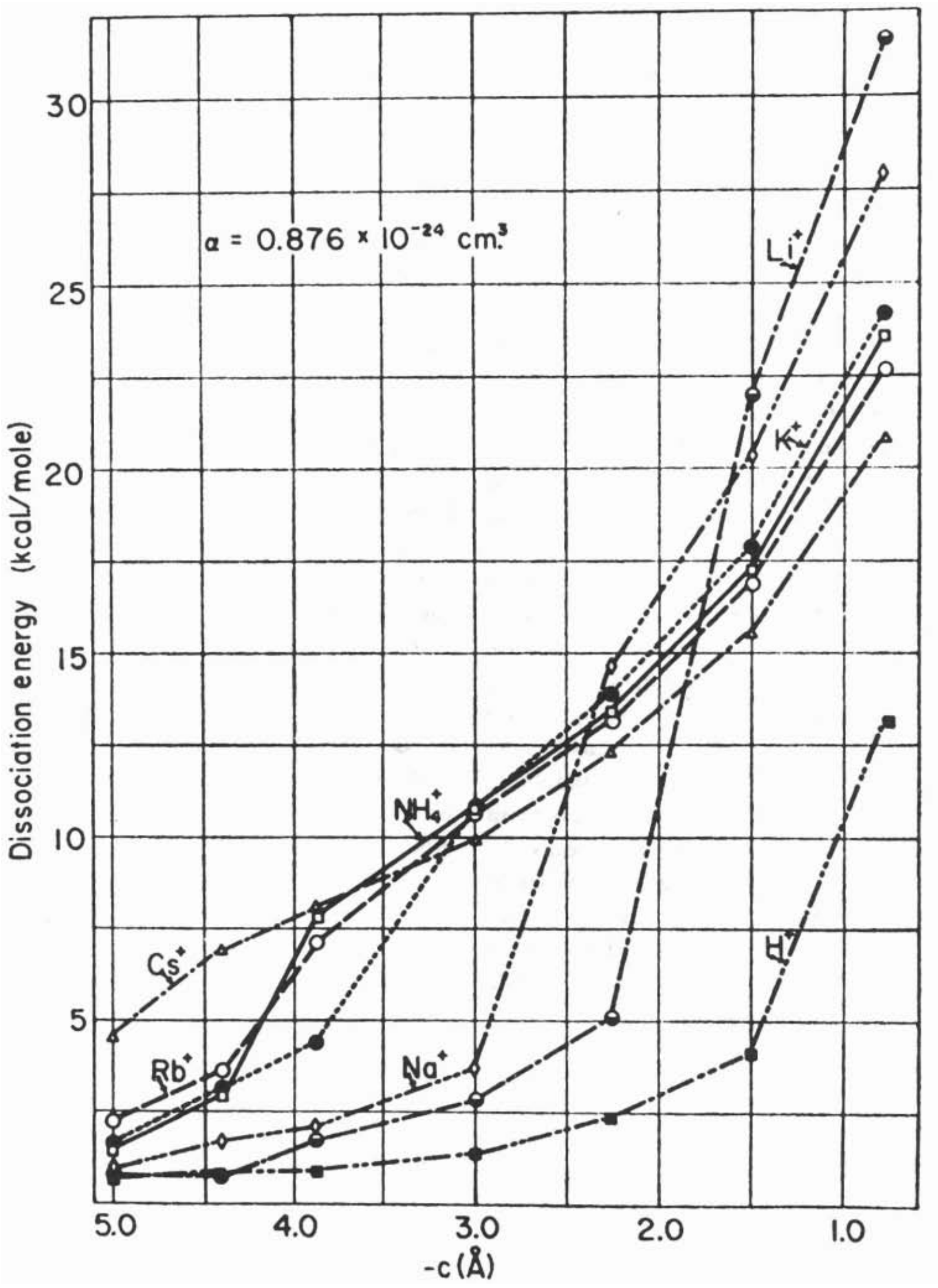

FIGURE I. Theoretical curves of the adsorption energies (ordinate) of various cations on an oxyacid when the electron density (expressed as c-value) of the oxyacid group changes. Lower c-value (toward the left side of the abscissa) represents the equivalent of lower pK_a value and vice versa (from Ling, 1962).

the β- and γ-carboxyl groups of the proteins, to adsorb K^+ over Na^+. In other words, the reaction represented by Equation 1 goes strongly to the right, while the reaction represented by Equation 2 below

$$f^+ f^- + Na^+ + Cl^- \rightleftarrows f^+ Cl^- + f^- Na^+ \quad (2)$$

goes only weakly to the right. In the AI hypothesis this cationic selectivity reflects a specific electronic density at the β- and γ-carboxyl groups, represented by the c-value (Ling, 1960, 1962). Figure 1 reproduces the theoretical curves showing that when the c-value is, say, around –3.5 Å, K^+ is greatly preferred over Na^+ in the selective adsorption onto the β- and γ-carboxyl groups.

Since the fixed cations like α-amino groups, ϵ-amino groups and guanidyl groups are all modified NH_4^+ groups, the preference of some fixed β- and γ-carboxyl groups to form salt linkages may parallel the preference of other β- and γ-carboxyl groups for K^+ over Na^+. The former are engaged in maintaining cell volume, and the latter are engaged in selective accumulation of K^+ in the cells. As Figure 1 shows, the dissociation energies of NH_4^+ and K^+ have a very similar dependence on c-value. At the c-value of –3.5 Å, both NH_4^+ and K^+ have a much more favorable adsorption energy than Na^+.

Another major postulate of the AI hypothesis is that the c-values of the β- and γ-carboxyl groups function together in unison due to autocooperative interaction among the sites, and that the normal c-values of those sites described above are under the control of cardinal adsorbents, including ATP (for evidence, see Ling, 1977b). Evidence has also been presented showing that the function of ATP is to lower the c-value (Ling, 1981a, p. 86). Thus, if the concentration of ATP is reduced as a result of metabolic interference by cold or injury, the c-value may go to a higher value, say –2.4 Å (Figure 1). At this higher c-value, the theoretical curves show that K^+ and Na^+ are equally preferred at the f^- sites. Indeed there is evidence that interference with metabolism and the consequent ATP depletion does bring about a depolarization of cell water and an increase of free Na^+ as well as a transient increase of adsorbed Na^+ (Ling and Ochsenfeld, 1973; Ling *et al,* 1981).

As mentioned above, under normal conditions all cell ATP is adsorbed on cardinal sites on cellular proteins. This is a theoretical postulate by the AI hypothesis but it is made inevitable by the enormous binding constants of ATP on the cell protein, myosin (3.25 × $10^{11} M^{-1}$, Goody *et al,* 1977; Cardon and Boyer, 1978). The c-values of the f^- engaged in the salt linkages are low so that only KCl can cause the dissociation of those salt linkages and cell swelling; NaCl cannot. However, when ATP is depleted, the c-value of f^- rises. Now the normally ineffective 100 mM NaCl present in the Ringer solution or plasma, suddenly becomes as powerful as 100 mM KCl in causing $f^- f^+$ dissociation and cell swelling follows. This then is the theory of injury-induced swelling according to the AI hypothesis. It suggests a theoretical connection between swelling of normal cells in isotonic KC1 solution and swelling of cells injured by cold or poisons in normal plasma or Ringer solution. The present communication presents experiments designed to test this theory.

MATERIALS AND METHODS

All experiments were performed on isolated organs of Swiss (ICR) mice. Isolated brain and kidney are usually cut sagittally into halves, weighed on a torsion balance before being introduced into 5 ml of the experimental solution in a closed vial (to be described). These vials were kept at 4°C in a cold room without shaking. The tissues were taken out 24 hours later, blotted lightly and weighed then and on every day following for as many as six days. Low temperature combined with the antibiotics added kept bacteria from growing.

Table I gives the basic formula for the solutions used. Two major stock solutions (I and II) were each prepared by mixing three solutions (IA, IB, IE, etc.) which were kept apart to prevent precipitation and deterioration of the organic components in the GIB medium. The GIB medium was obtained in sterile dry powder form from Grand Island Biologicals (now GIBCO, Wilmington, DE). It is a chemically defined medium (for composition see Ling and Bohr, 1969).

Determination of ATP was by a firefly method essentially that described by Kahbhen and Koch (1967). The only significant modification was that the tissue extract was made with 5% TCA rather than boiling water. The light emitted by the luciferin-luciferase system in the presence of ATP was measured in a β-scintillation counter.

RESULTS

When isolated mouse kidney tissue was incubated at 4° C in a normal mammalian Ringer solution containing about 120 mM Na^+ it swelled rapidly in the first day. Intact kidneys increased their weights for another day before a gradual decline (Figure 2). On the other hand, if kidney halves were measured, the peak height of weight change was reached at one day and the decline was earlier. Since as a rule half organs were used, we chose one day equilibrium time as a rule. Half mouse brains, however, were an exception. It took two days to reach maximum

TABLE I. Composition of the two Stock Solutions used in preparing the solution of varying salt and sucrose concentrations. Each stock solution was prepared in three separate components which were mixed just before use. GIB medium was obtained from General Biological in the form of sterile powder free of NaCl or KCl and was dissolved in distilled water before mixing with the antibiotics. Experimental solutions were prepared by mixing Stock Solutions I and II (or its modification in which the NaCl had been replaced by LiCl, Li_2SO_4, $MgSO_4$ etc.) in different proportions.

		Conc. (M)	Stock Solution I Volume (ml)	Stock Solution II Volume (ml)
A	NaCl	5 × 0.154	26.76	—
	Sucrose	5 × 0.308	—	27.96
	KCl	0.154	5.50	4.30
	$NaHCO_3$	0.154	22.50	—
	Choline bicarbonate	0.154	—	22.50
	NaH_2PO_4	0.154	1.20	1.20
	H20		83.8	83.8
B	$MgCl_2$	0.11	1.2	1.2
	$CaCl_2$	0.11	2.75	2.75
	Glucose	0.308	5.6	5.6
E	GIB medium (KCl- and NaCl-free)		15.4	15.4
	H20		10.0	10.0
	Penicillin (Na)		13.0 mg	13.0 mg
	Streptomycin		13.0 mg	13.0 mg

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weight and then they remained at these peak values for two more days. For this reason the brain weight was obtained at two to four days.

Figure 3 shows the weights of three mouse tissues after one day incubation at 4° C in various solutions containing different proportions of NaCl and sucrose. The ordinate represents the one-day weight represented as the ratio of the final weight, W_{fin} divided by the initial weight (W_{init}). In spite of the fact that all solutions used were isotonic, marked differences in W_{fin}/W_{init} occurred. In all cases the highest ratios were in those organs in the solution containing the most NaCl and the least sucrose. However the degree of swelling in the presence of the high NaCl concentration varied considerably among the three tissues studied here. It follows the order: kidney > liver > spleen.

Also clearly notable is the considerable shrinkage of all three organs after exposure to the Na-free sucrose Ringer solution, represented by the left-hand point along the abscissa. However, this shrinkage in Na-free sucrose Ringer solution is not universal. Other tissues like the brain did not undergo shrinkage in any solution studied (see below).

Figure 4 compares the shrinkage-swelling curves of mouse kidney in various solutions in which the sucrose components (Stock Solution II) were similar but the NaCl con-

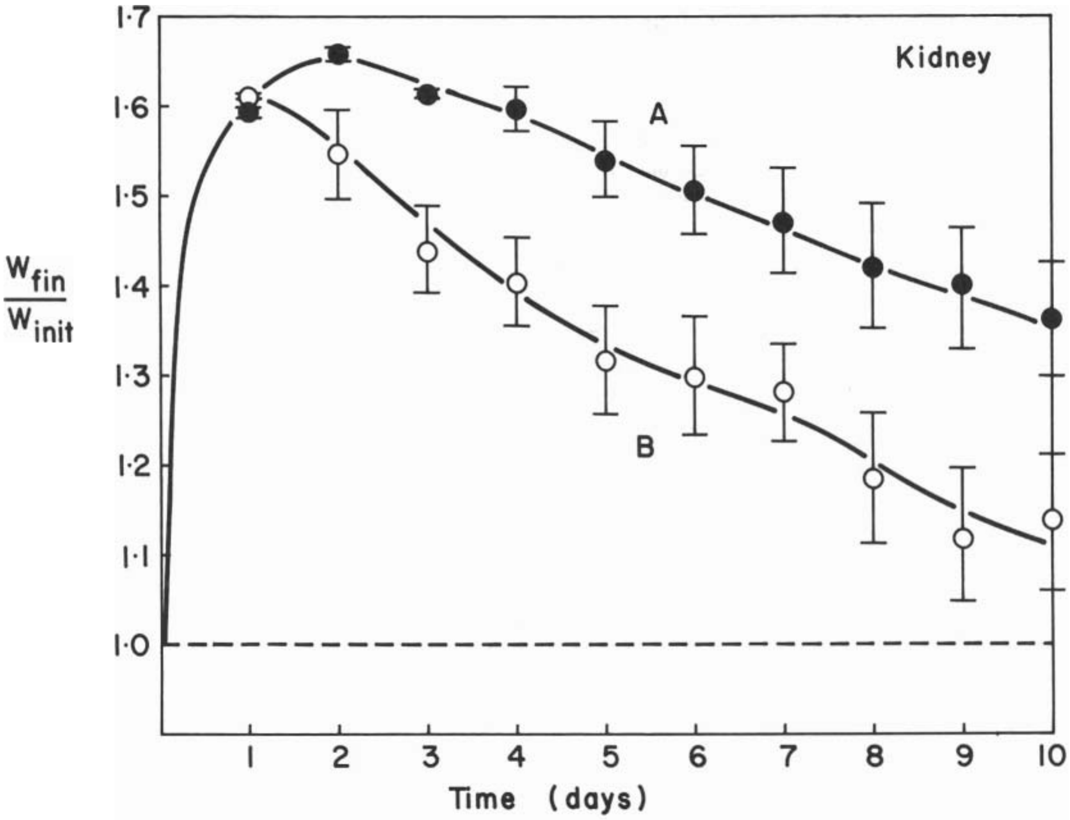


FIGURE 2. Time course of weight change of isolated intact mouse kidney (A) and of mouse kidney cut into halves (sagittally) (B). Four sets of tissues were weighed repeatedly after light blotting on wetted filter paper. The bathing solution is a normal mammalian Ringer solution containing 118 M NaCl; its composition is described in Table I. Ordinate represents the relative volume, obtained by dividing the weight after a certain length of time (W_{fin}) by the initial fresh weight (W_{init}).

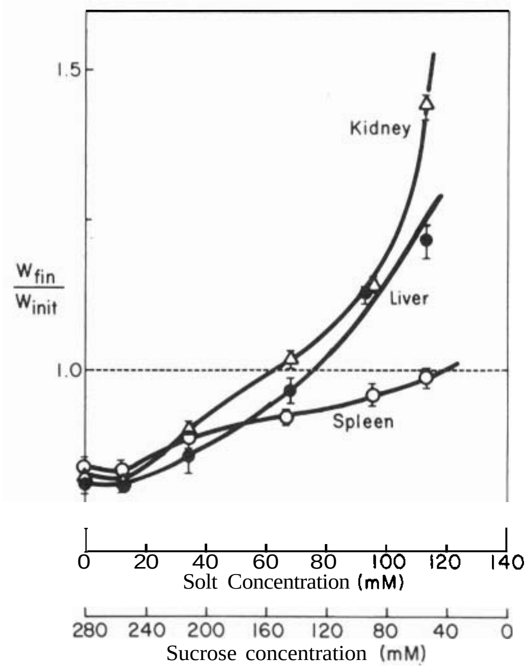


FIGURE 3. The relative weight of isolated mouse kidney, liver, and spleen following incubation for I day in mixtures of the two stock solutions (I and II) described in Table I. All solutions are isotonic, but those to the left of the abscissa have more sucrose while those to the right have more NaCl (referred to in the abscissa as salt). The far right experimental point of each of the 3 curves corresponds to that of a normal mammalian Ringer solution containing 118 M NaCl.

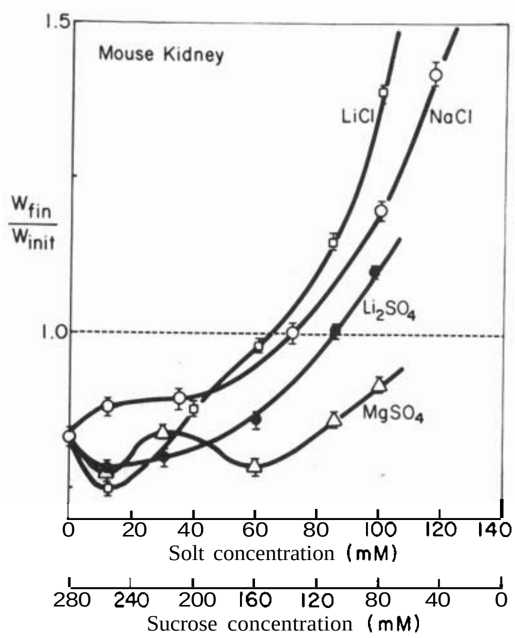


FIGURE 4. The relative weight of isolated mouse kidney following incubation for one day at 4° C in mixtures of sucrose-Ringer solution (Stock Solution I) and isotonic Ringer solution (Stock Solution II) or its variants containing NaCl, LiCl, Li_2SO_4 or $MgSO_4$.

centrations were either the same as in the experiments described in Figure 3 or were replaced by an isomostic equivalent of LiCl, Li_2SO_4, or $MgSO_4$. LiCl and $MgSO_4$ replaced NaCl mole for mole. On the other hand, each mole of NaCl was replaced by 0.67 moles of Li_2SO_4.

Note that mole-for-mole, LiCl was more effective than NaCl in causing swelling. Yet Li_2SO_4 was much less effective, suggesting that the swelling depends not only on the specific nature of the cation, but the anion as well. The virtually complete ineffectiveness of $MgSO_4$ further confirms this view. The kidney consistently shrank in the sucrose-Ringer.

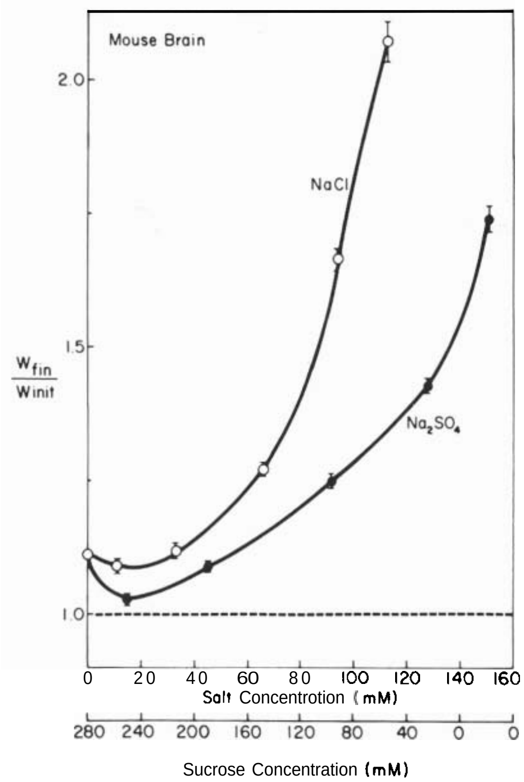
Figures 5 and 6 show the swelling of mouse brains in NaCl and Na_2SO_4 Ringers, and in LiCl and in Li_2SO_4, respectively. The greater swelling in the chloride salt than the sulfate salt is in full agreement with the conclusion from the kidney study (Figure 4). In contrast to kidney, liver and spleen, however, brain tissues did not undergo shrinkage in the sucrose Ringer solution. Figure 6 shows that LiCl caused more swelling than isoosmatic concentrations of Li_2SO_4.

In Figure 7 we have plotted the water contents of half brains against the ATP concentrations measured in the tissues after they have been incubated at 4° for various lengths of time, as indicated by the number of hours in brackets beside each experimental point. The incubation solution used contained the full amount of NaCl of a normal mammalian

Ringer (118 mM). The data shows that the swelling of the brain in the presence of a high concentration of external NaCl follows closely the decline of the ATP contents of the tissue.

DISCUSSION

According to the AI hypothesis, water in resting, normal living cells is not free water in which free K^+ salts are dissolved. Rather, water exists in the cells in the state of polarized multilayers. Most of the intracellular ions, of which K' is predominant, is adsorbed singly on anionic protein sites (Ling, 1981b). The maintenance of the normal amount of cell water reflects the balance of two opposing forces: an expansive force originates from the tendency of the cells to acquire additional layers of water, and a contracting force due to restrictive bonds between neighboring protein chains, largely in the form of salk-linkages (Ling and Peterson, 1977). When the cardinal adsorbent ATP is present at a normal level and all the cardinal sites for ATP, Ca^{++}, etc. are fully occupied, KCl (and RbCl) at about 100 mM concentration causes extensive swelling because from a free-energy standpoint it is more favorable for the reaction described in Equation 1 to proceed to the right. In con-



FLGURE 5. The relative weights of isolated half mouse brain following incubation for two days at 4° C in mixtures of sucrose-Ringer (Stock Solution I) and either Stock Solution II containing 118 M NaCl or a modified Stock Solution II in which Li_2SO_4 has replaced the NaCl at isosmotic strength (i.e., 2/3 M of Li_2SO_4 replaced 1 M NaCl).

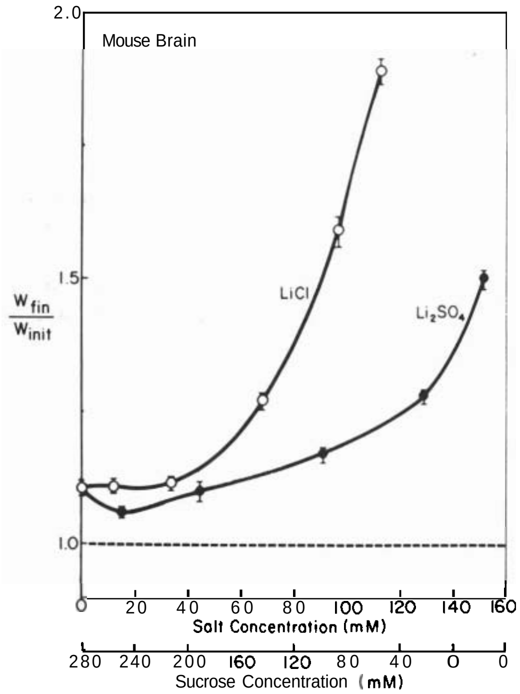


FIGURE 6. The relative weights of isolated half mouse brain following incubation for four days at 4° C in mixture of sucrose-Ringer (Stock Solution I) and modified Stock Solution II in which the NaCl was replaced either by LiCl (1 mole for 1 mole) or Li_2SO_4 (2/3 M Li_2SO_4 for 1 M NaCl).

trast, an isosmotic concentration of sucrose is totally ineffective. K_2SO_4 or NaCl are also ineffective or less effective. The sulfate ion is known to be less strongly adsorbed than Cl^- on the ϵ-amino group and guanidyl group (see Ling, 1962, p. 172). Na^+ and Li^+ are less preferred by the fixed β- and γ-carboxyl groups when these groups are held at a relatively low c-value at which K^+, NH_4^+, and the variants of NH_4^+ — the a-amino, ϵ-amino, and guanidyl groups — are preferred. Most of these theoretical expectations have been verified elsewhere experimentally (Ling and Peterson, 1977).

According to the AI hypothesis, any kind of metabolic interference of or injury to the living cell, whether brought on by chilling, mechanical damage or metabolic poisoning, usually leads to a diminished ability of the cell to regenerate ATP and thus to keep its cardinal sites occupied by this critically important cardinal adsorbent. Incubation of isolated mouse tissue at a low temperature without its normal efficient supply of oxygen and other essential exchange of nutrients and water leads to gradual ATP depletion, as Figure 7 shows. We would also like to suggest that the observed swelling of slices of liver, kidney, and brain by exposure to 0° C temperature, metabolic inhibitors like cyanide and 2,4-dinitrophenol, and various mercurials, produces NaCl-dependent swelling by a similar mechanism.

Let us next discuss the special requirement of NaCl, the major component of the blood plasma, for the injury-induced swelling.

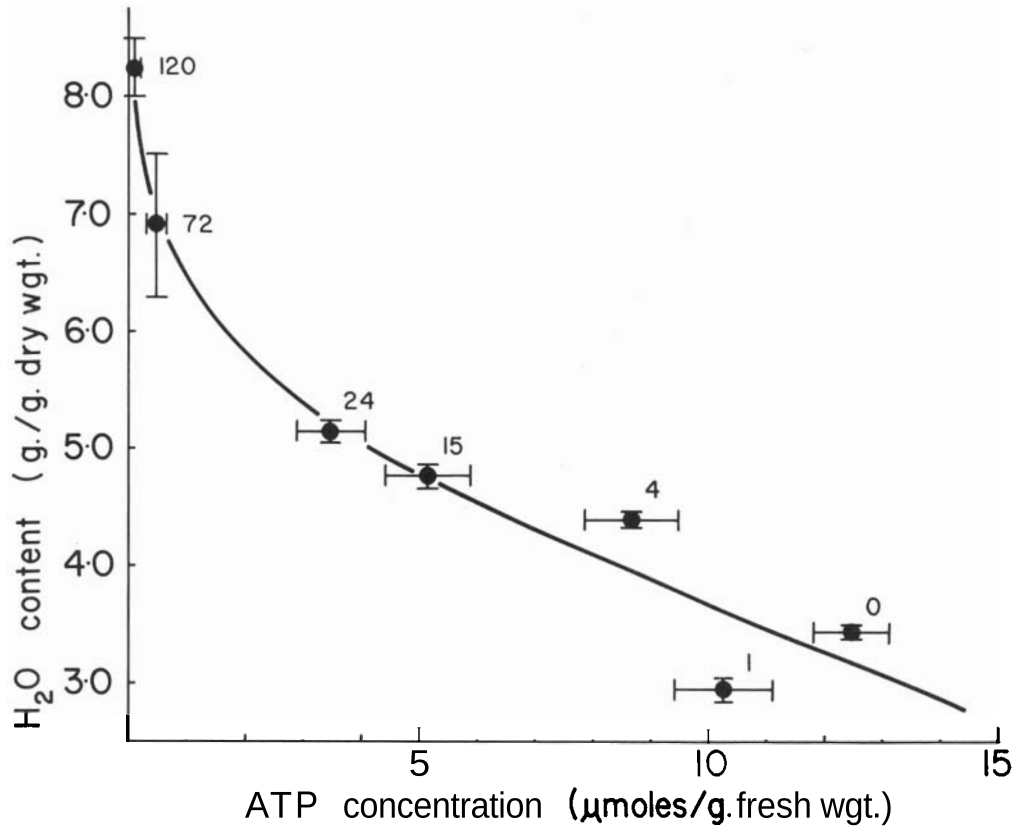


FIGURE 7. The relation between the water contents of mouse brains and their ATP contents after the isolated half brains were incubated for various lengths of time in hours (indicated by numbers in brackets near each point). Each point represents average of 3 or 4 determinations ± S.E.

Figures 3 to 6 show quite consistently that tissues exposed to low temperature in Ringer solution whose NaCl had been replaced by an isoosmotic concentration of sucrose, showed little swelling. Indeed, often there was shrinkage. This special requirement for Na^+ in injury-induced swelling was recognized by Saladino, Bentley, and Trump (1969). One asks, "Is this substitution of two moles of sucrose for one mole of NaCl truly accurate enough in terms of osmotic activity?" The answer is yes. Thus Negus (see Fraser, 1927) gave the partial vapor pressure of 0.15 M NaCl as 0.995. The partial vapor pressure of 0.30 M sucrose is also 0.995 (Bear, see Fraser, 1927). Clearly the widely different effect of sucrose Ringer solution and NaCl Ringer solution on swelling is not a simple matter of osmotic activities of the two solutions, which are in fact identical. Nor can this special requirement for NaCl be due to the net electric charges of the particles: Na^+ and Cl^-. Thus isoosmotic $MgSO_4$ bearing net charges is just as ineffective as isoosmotic sucrose bearing no net charges in causing injury-initiated swelling of mouse kidneys.

The failure of sucrose and $MgSO_4$ to produce swelling is a clear affirmation of a high degree of specfic (Na^+) preference in the reaction described by Equation 2, just as there is a high degree of specific K^+ preference in the reaction described by Equation 1, applicable to normal rather than injured tissues.

That Mg^{++} cannot replace Na^+ in causing swelling is first and foremost a question of relative adsorption energy. As pointed out earlier the Mg^{++} and other divalent cations are ineffective in the depolarizing of the electrical potential of living muscle cells and model systems (Ling, Walton, and Ling, 1979). The interpretation was that divalent ions are only very weakly adsorbed onto the isolated single β- and γ-carboxyl groups which adsorb monovalent cations. We believe that in all probability this is the case here. According to this theory, if the β- and γ-carboxyl groups occur in pairs or clusters, Mg^{++} salts would be as effective as or more effective than Na^+ salts in causing swelling. But clearly, this is not the case here.

The ineffectiveness of $MgSO_4$ in causing swelling is also partly due to the ineffectiveness of SO_4^{2-}. That SO_4^{2-} is as a rule very weakly adsorbed on amino types of cationic groups has been emphasized often in the presentation of the AI hypothesis and is fully borne out by the comparative study of the relative binding energies on weak amino types of ion exchange resins and on isolated proteins (Ling, 1962, p. 172).

Another strong support for the theoretical interpretation offered here for injury-induced swelling is the effectiveness of LiCl in promoting injury-induced tissue swelling. An examination of the theoretical plots of c-value vs. relative adsorption energy on fixed anionic groups in Figure 1 shows that the observation is theoretically predicted: at such a c-value that Na^+ is effective in causing swelling, Li^+ should also be effective.

We want to ask next why do injured tissues actually undergo shrinkage in sucrose Ringer solution. This is not the first time that isoosmotic sucrose-Ringer solution was found to induce cell shrinkage. Ling, Walton, and Ling (1979) noted the same phenomenon in normal, uninjured frog muscles when NaCl in a normal Ringer solution was replaced by an isoosmotic concentration of sucrose. The following explanation is offered. In normal cells, a small number of salt linkages are kept dissociated by the NaCl, as illustrated in Equation 2. It is possible that the anionic components of these salt linkages are different from the majority of their counterparts and have a higher c-value at which Na^+ is preferred, so that in the presence of 100 mM or so of NaCl in the normal environment the NaCl is able to sustain the dissociated state of these particular salt linkages and allow a corresponding number of multilayers of water to exist in the cells. When this NaCl is replaced by sucrose, the reaction represented by Equation 2 goes to the left and the cell then loses water, as observed.

Finally we would like to point out that in the combined ischemia- and cold-induced injury seen here, the initial swelling may well be reversible (see Stern et al, 1949; Robinson, 1950; Whittam and Davies, 1953). But as time progresses, the cell certainly became irreversibly damaged. Thus the molecular mechanism as represented by Equation 2 is more fully applicable to the initial state of swelling. During this stage, the polarized multilayered state of water is still maintained. Augmented by the liberated adsorbed ions, the high total osmotic pressure provides the basic force for swelling when the restraining force of salt linkages is simultaneously broken down by the NaCl present. However, this process would continue only for a certain length of time. As ATP begins to reach very low concentration, cell water depolarizes and the initial excessive osmotic activity of the injured cells, due to the combined contribution to the osmotic activity of polarized water and liberated K^+, declines. Although still apparently swollen, this state is only the remnant of the swollen state: extended, overblown "shells" filled with free water and free salt ions. In support of this view, Ling and Walton (1976) had shown that centrifugation for 4 minutes at 1000 g. of living frog tissues, whether intact or cut into segments, removed only water in the extracellular space. But when the final state of injury and deterioration occurred, the swollen dead tissues readily gave up their water during a similar centrifugation.

This investigation was primarily supported by the Office of Naval Research Contract N00014-79-0126; and to a lesser extent by NIH Grants 2-R01-CA16301-3 and 2-ROI-GM11422-13; and the Pennsylvania Hospital General Research Support.

REFERENCES

Cardon, J. W., and Boyer, P. D., 1978, ***Europ, J. Biochem.*** 92:443.

Cohn, W. E., and Cohn, E. T, 1939, ***Proc. Soc. Expt. Biol. Med.*** 41:445.

Dean, R. B., 1941, ***Biol. Symp.*** 3:331.

Edelmann, L., 1977, ***Physiol. Chem. Phys.*** 9:313.

Edelmann, L., 1981, in ***Intern. Cell Biol. 1980-1981,*** (Schweiger, H. G., ed.) p. 941, Springer Verlag, Berlin.

Fischer, M. H., 1909, ***Trans Coll. Phys. Phila.*** 31:457.

Frazer, J. C. W., 1927, ***The Direct Measurement of Osmotic Pressure,*** Columbia University Press, New York.

Goody, R. S., Hofmann, W., and Mannherz, H. G., 1977, ***Europ. J. Biochem.*** 78:317.

Heppel, L. A., 1940, ***Amer. J. Physiol.*** 128:449.

Kahbhen, D. A., and Koch, H. J., 1967, ***Z. klin. Chem. u klin, Biochem.*** 5:299.

Lillie, R. S., 1923, ***Protoplasmic Action and Nervous Action,*** University of Chicago Press, Chicago.

Ling, G. N., 1960, ***J. Gen. Physiol.*** 43:149.

Ling, G. N., 1962, ***A Physical Theory of the Living State: The Association-Induction Hypothesis,*** Blaisdell, Waltham.

Ling, G. N., 1965, ***Ann. NY Acad. Sci.*** 125:401.

Ling, G. N., 1969, ***Int'l. Rev. of Cytol.*** 26:1.

Ling, G. N., 1972, in ***Structure and Transport Processes in Water and Aqueous Solutions,*** (Horne, A., ed.) p. 201, Wiley-Interscience, New York.

Ling, G. N., 1973, ***Physiol. Chem. Phys.*** 5:295.

Ling, G. N., 1977a, ***Physiol. Chem. Phys.*** 9:310.

Ling, G. N., 1977b, ***J. Mol. Cell, Biochem.*** 15:159.

Ling, G. N., in ***The Aqueous Cytoplasm,*** (Keith, A. D., ed.) p. 23, Marcel Dekker, Inc., New York.

Ling, G. N., 1980, ***Physiol. Chem. Phys.*** 12:383.

Ling, G. N., 1981a, ***Physiol. Chem. Phys.*** 13:39.

Ling, G. N., 1981b, in ***Intern. Cell Biol. 1980-1981,*** (Schweiger, H. G., ed.) p. 904, Springer Verlag, Berlin.

Ling, G. N., 1983, ***Physiol. Chem. Phys.*** 15:155.

Ling, G. N., 1984, ***In Search of the Physical Basis of Life,*** Plenum Publishing Corporation, New York.

Ling, G. N., and Bohr, G., 1969, ***Physiol. Chem. Phys.*** 1:591.

Ling, G. N., and Negendank, W., 1970, ***Physiol. Chem. Phys.*** 2:15.

Ling, G. N., and Ochsenfeld, M. M., 1973, ***Ann. NY Acad. Sci.*** 204:325.

Ling, G. N., and Peterson, K., 1977, ***Bull of Math. Biol.*** 39:721.

Ling, G. N., and Walton, C. L., 1976, ***Science*** 191:293.

Ling, G. N., Walton, C. L., and Ling, M. R., 1979, ***J. Cell Physiol.*** 101:261.

Ling, G. N., Walton, C. L., and Ochsenfeld, M. M., 1981, *J. of Cell Physiol.* **106**:385.
Opie, E. L., 1949, *J. Exp. Med.* **89**:185.
Opie, E. L., 1954, *J. Exp. Med.* **99**:29.
Robinson, J. R., 1950, *Proc. Roy. Soc.* **137**:378.
Robinson, J. R., 1952, *Nature,* **169**:713.
Saladino, A. J., Bentley, P. J., and Trump, B. F., 1969, *Amer. J. Pathol.* **54**:421.
Saladino, A. J., and Trump, B. F., 1969, *Amer. J. Pathol.* **52**:737.
Stern, J. R., Eggleston, L. V., Hems, R., and Krebs, H., 1949, *Biochem. J.* **44**:410.
Tosteson, D. C., 1964, in *The Cellular Functions of Membrane Transport,* (Hoffman, J. F., ed.) p. I, Prentice-Hall, Englewood.
Trombitas, C., and Tigyi-Sebes, A., 1979, *Acta Physiol. Acad. Sci. Hung.* **14**:271.
von Korosy, K., 1914, *Z. Physiol. Chem.* **93**:154.
Whittam, R., and Davies, R. E., 1953, *Biochem. J.* **55**:880.

(Received in revised form September 22, 1983.)