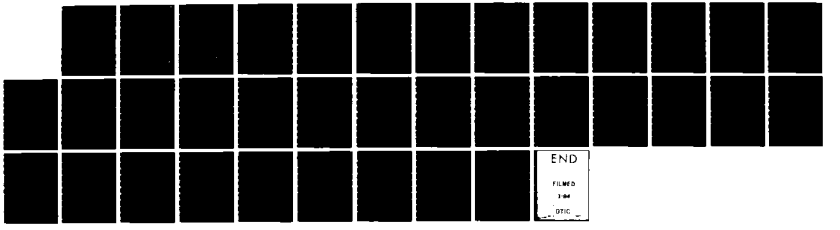
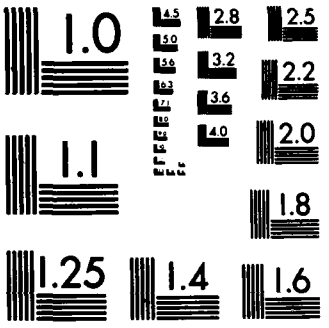
AD-A136 111 A UNITARY CAUSE FOR THE EXCLUSION OF NA(+) AND OTHER SOLUTES FROM LIVING..(U) EENNSYLVANIA HOSPITAL PHILADELPHIA DEPT OF MOLECULAR BIOLOGY.. 1/1

UNCLASSIFIED G N LING ET AL. 1983 N00014-79-C-0126 F/G 6/3 NL





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

by

Gilbert N. Ling

Cheryl L. Walton and Margaret M. Ochsenfeld

SHORTENED TITLE:

Na^+ and Sugar Effluxes from Dying Muscles

N00014-79-C-0126

Department of Molecular Biology

Pennsylvania Hospital

8th and Spruce Streets

Philadelphia, Pennsylvania 19107

SUMMARY

1. The effluxes of labelled $Na^{(+)}$, D-arabinose, and sucrose from normal muscle and muscle poisoned with low concentrations of iodoacetate were studied. The procedure involved repeated loading with isotope, followed by washing of the same muscle while still normal and at different stages of dying.

2. The rates of $Na^{(+)}$ efflux in both the fast and slow fraction remained either quite constant or showed some unpredictable, minor fluctuations. This was true for both $Na^{(+)}$ and the two sugars studied, confirming earlier conclusions that the steady levels of these solutes were not maintained by pumps.

3. In all cases studied, the efflux curves showed at least two fractions. It is the fast-exchanging fraction that steadily and consistently increased in magnitude as the muscles were dying, until finally the concentration of solute in this fraction reached and sometimes surpassed the labelled solute concentrations in the original labelled solutions in which the muscles were equilibrated. The slow fractions showed only a transient increase or none at all. These observations show that it is the fast fraction that represents solute dissolved in cell water and rate-limited by passage through the cell surface and that the partial exclusion of $Na^{(+)}$ and the sugars have a unitary cause - a reduced solubility in the cell water which in the presence of ATP exists in the state of polarized multilayers.

The low level of Na^+ in resting cells has been considered by many to be due to a continuous pumping mechanism. In the past this membrane-pump concept appeared to be supported by the best evidence available. However, in recent years findings of an opposite kind have been accumulating. Thus under carefully controlled conditions in frog muscle, the Na^+ pump alone would consume at least 15 to 30 times as much energy as the cell commands (Ling, 1962), a finding which has been confirmed in other cells (Jones, 1965; Minkoff and Damadian, 1973). None of the three remedial postulations made to bail out the pump hypothesis (exchange diffusion, sarcoplasmic reticulum sequestration of Na^+, and the non-energy consuming Na^+ pump) has received experimental support (see Ling et al, 1979). In addition, failure to demonstrate Na^+ transport against a concentration gradient in an axoplasm-free squid axon membrane sac (Ling 1977a) contrasts with success in demonstrating selective Na^+ exclusion and K^+ accumulation in an effectively membrane-pumpless open-ended cell (EMOC) preparation of frog sartorius muscle (Ling, 1978). Three different laboratories across the world - but especially that of Edelmann in West Germany - using a total of 4 different techniques have shown that the bulk of K^+ in muscle cells is not evenly distributed in a free state as demanded by the membrane-pump theory but is adsorbed on specific sites in the A band and Z-line (Edelmann, 1977, 78; Ling 1977b; Trombitas and Tigyi-Sebes, in press). This and other evidence is not readily reconcilable with the membrane-pump theory, and is generally speaking more in keeping with a much different general concept of the living cell entitled the Association-induction Hypothesis (AI Hypothesis).

According to this view the bulk of cell K^+ is adsorbed on β- and γ-carboxyl groups of cell proteins, a postulate supported by the findings just described. Water in a resting cell is not free but exists in the state of polarized multilayers (Ling, 1977a). In this state, water has solubility for solutes roughly in proportion to their sizes and complexity (Ling and Sobel, 1975). Thus the

equilibrium distribution coefficient, or q-value, for the large hydrated Na^+ (the ratio of the equilibrium concentration of free Na^+ in the cell water over the concentration of free Na^+ in the external medium) is low; the q-value for methanol on the other hand is close to unity.

In the AI Hypothesis the maintenance of the polarized multilayer state of cell water depends on the continued existence of certain proteins in an extended conformation forming a matrix occupying the entire cell interior. Confirmation of this hypothesis has been reported recently in experimental studies of both isolated proteins and synthetic polymers (Ling, Walton, and Bersinger, 1980; Ling, Ochsenfeld, Walton and Bersinger, 1980). The maintenance of such an extended conformation of the matrix proteins in living cells requires in turn the adsorption of ATP (and possibly other cardinal adsorbents) at key controlling cardinal sites on the proteins (Ling, 1969; Ling and Ochsenfeld, 1973; Ling, 1977a, b). In normal resting cells ATP is also required for selective K^+ adsorption on anionic sites of certain intracellular proteins.

According to the AI Hypothesis, there are two different ways that the cell may gain Na^+. In one, the Na^+ taken up replaces adsorbed K^+; in the other Na^+ is taken up within the cell water.

Following K^+-depletion or exposure to ouabain, muscle cells gain Na^+ and lose K^+. In these cases there is primarily a one-to-one Na^+ for K^+ exchange on adsorption sites (Ling and Cope, 1969; Ling and Bohr, 1971; Ling and Ochsenfeld, 1973). Neither K^+ depletion nor ouabain treatment seems to have significant effect on the polarized multilayer state of the cell water.

ATP-depletion, brought on by any one of a large variety of metabolic poisons also causes cells to lose K^+ and gain Na^+ (Ling, 1962; Gulati et al, 1971). Changes in the cellular Na^+ concentration in this case parallels a similar gain in sucrose in the cell, and there is not a one-for-one exchange of Na^+ for K^+,

suggesting that Na^+ gain brought about by ATP depletion involves a different mechanism: an increase of the q-value for free Na^+ as a result of the depolarization of cell water (Ling and Ochsenfeld, 1973). However, these conclusions were based on the study of <u>total</u> concentrations of Na^+ and of sucrose in the cell while according to the AI Hypothesis only a part of them, the <u>free</u> Na^+ and <u>free</u> sucrose are involved. Fortunately, there is now a technique which permits the separation of the free cell Na^+ from the adsorbed or compartmentalized fraction by the efflux analysis method to be described next.

In 1948 Levi and Ussing described the efflux of labelled Na^+ from frog sartorius muscle and recognized the existence of more than one fraction of Na^+ (Levi and Ussing, 1948). Their assumptions - that the fast fraction is from the extracellular space and that the slower fraction, with a half-time of exchange of 30 minutes at room temperature, represents Na^+ within the cell - have been accepted by many later investigators of this subject (Harris, 1950; Keynes, 1954; Keynes and Maisel, 1954; Johnson, 1955; Hodgkin and Horowicz, 1959; Mullins and Frumento, 1963; Keynes and Steinhardt, 1968).

In the interim a variety of experimental data has been presented showing that the original assumption of Levi and Ussing might have been only partially correct. Thus by comparing the volume occupied by the fast exchanging Na^+ with the volume of extracellular space determined with three other more rigorous methods (Ling et al, 1973), by studying Na^+ efflux of a single muscle cell, which has no "extracellular space"* (Ling et al, 1973), and by studying the Na^+ efflux alone or simultaneously with K^+ efflux after removing extracellular fluid by the centrifuga-

* A recent assertion of Neville and Mathias (1979) that this fast fraction we reported came from the attached tendons of the isolated single fibers ignored the fact that in these studies free floating muscle fibers were used and that tendons were removed beforehand.

tion method (Ling and Walton, 1975b), we reached the conclusion that the fast fraction is not extracellular, and is rate-limited by passage through the cell surface barrier.

This evidence, as well as that derived from studies of Na^+ and sugar effluxes from single frog ovarian eggs (Ling and Ochsenfeld, 1977), support the notion that the fast fraction represents free Na^+ dissolved in the cell water and the slow fraction (or fractions) represents Na^+ either adsorbed on proteins or other macromolecules or sequestered in subcellular compartments (Ling, 1962). A recent comprehensive analysis of the Na^+ efflux from human lymphocytes led Negendank and Shaller to a similar conclusion (Negendank and Shaller, in press). If this interpretation of the significance of the fast fraction of Na^+ efflux is correct, it provides a tool to distinguish the free from the adsorbed (or sequestered) fraction of cell Na^+ and to estimate each quantitatively.

We tested the following predictions of the Association-induction Hypothesis:

(1) As frog muscles are dying slowly in a low concentration of iodoacetate, the ATP concentration falls slowly; this decline of ATP concentration should be accompanied by a progressive increase of the q-value for Na^+, revealed as a progressive rise of the intercepts of the fast fraction of the Na^+ efflux.

(2) The progressive rise of the q-value for Na^+ in the dying muscle should be paralleled by a progressive rise of the q-value for any other normally excluded solutes, such as D-arabinose and sucrose, also revealed as a progressive rise of the intercepts of the fast fraction of the effluxes of these solutes (the "Universality Rule", Ling, 1979).

MATERIALS AND METHODS

All experiments were performed on isolated sartorius muscles of North American leopard frogs (Rana pipiens pipiens, Schreber).

Incubation and isotope loading of muscles were carried out in a Ringer-GIB medium equilibrated with 5% CO_2 and 95% O_2; this medium contained Na^+, 100 mM; K^+, 2.5 mM; Ca^{++}, 1.0 mM; Mg^{++}, 1.2 mM; PO_4^{3-}, 2.7 mM; HCO_3^-, 15.7 mM; Cl^-, 88.7 mM; SO_4^{2-}, 0.8 mM; NO_3^-, 0.1 mM: glucose, 23.3 mM besides 21 amino acids, 14 vitamins and oxalacetic acid. A more complete description was given by Ling and Bohr (1971). The Ringer phosphate solution used for washing the isotope-loaded muscles contained the following: Na^+, 113.3 mM; K^+, 2.5 or 10 mM; Ca^{++}, 1.0 mM; Mg^{++}, 1.2 mM; Cl^-, 97.2 mM; HCO_3^-, 6.6 mM; SO_4^{2-}, 1.2 mM; PO_4^{3-}, 3.2 mM.

Two techniques of efflux studies were used (Ling, 1962, p. 198; and Ling and Ochsenfeld, 1965, p. 785): The U-tube method was used for studies of effluxes of γ-emitting isotopes only (e.g., ^{22}Na). The second technique was used to study the efflux of β-emitting ^{14}C or ^{3}H labelled sugars and sometimes also that of ^{22}Na: the loaded tissue was washed in successive portions of non-radioactive Ringer solution while being agitated by a stream of air bubbles. The details of these techniques as well as the procedure for correcting for the contributions of the connective tissue, blood vessels, etc. were as described in an earlier publication (Ling and Walton, 1975b).

The muscle efflux data were presented in the conventional manner where the concentrations of radioactively labelled Na^+, or sugar in the muscle in μmoles per gram of fresh tissue was plotted semilogarithmically against the duration of washing.

The overall accuracy of the method in assessing exponential-peeling fraction size was investigated and found to be about 3% for the data to be dealt with in this paper where the time constants of the faster and slower fraction differ by more than a factor of 10 (Ling, 1962, p. 327; 1980; Huxley, 1960).

To avoid confusion, the corrected efflux curves and their resolved fractions were presented in units of μmoles per gram of total fresh tissue weight which included the connective tissue weight, and correction was not made for the

extracellular space fluid in the reproduced efflux curves (Figures 1, 3, 6, 7). However, in subsequent presentation of the magnitudes of the slow and fast fraction in Figure 4A and B, an 8% correction for the extracellular space fluid was taken into account (Ling and Walton, 1975a). The fast fraction is then obtained from the initial count of the efflux curve (after connective tissue correction) minus counts belonging to the slow fraction and counts equal to that in an extracellular space containing the same concentration of labelled Na^+ as in the external solution. To capture the successive stages in the gradual depolarization of water, we slowed down the dying process by using a very low concentration of Na iodoacetate (0.05 to 0.2 mM) and sometimes by reducing the temperature. The basic procedures used were of two types. In Procedure I, the muscles, loaded with isotopes under sterile conditions for 42 hours (25° C), were exposed to IAA only during the washing, which took place also at 25° C. At the conclusion of the first washing period, the muscles were again exposed to ^{22}Na in a Ringer-GIB solution containing no IAA for 22 hours to 69 hours and washed in IAA-containing Ringer solution again at 25° C. This loading and washing was then repeated for a total of from 3 to 6 times. About one-half the experiments were performed using Procedure I. In one variation of this procedure the second, third, and subsequent isotope loadings were all carried out at 0° C (Procedure IA). In another, only the third and subsequent loadings were at 0° C while the second loading was for 2.5 hours (Procedure IB). In Procedure II the loaded muscles were not exposed to IAA during washing. Instead, they were exposed to IAA at the second and subsequent isotope loading steps only, all at 25° C. The duration of each IAA exposure was from 3 to 21 hours.

^{22}Na was obtained from ICN, Isotope and Nuclear Division (Lot Nos. 34, 35, and 36H), D-arabinose[5-^{3}H] om ICN (Lot No. 619265), and sucrose [U-^{14}C] from ICN (Lot No. 640171).

The loading solution as a rule contains 1 μCi/ml of ^{22}Na. For studies involv-

ing sugars, the incubating solution contained 5 mM of nonlabelled sugar and 2 μCi/ml of labelled sugar.

For the measurement of resting potentials of single muscle cells, the method of Ling and Gerard (1945) was used.

RESULTS

Successive Na^+ efflux in a sartorius muscle dying from repeated exposure to IAA

Figure 1 shows an example of the successive Na^+ efflux curves of a sartorius muscle as it was slowly dying from exposure to low concentrations of IAA. Figure 2 shows a set of efflux curves of loose connective tissues loaded with labelled Na^+ and treated exactly as the muscles of Figure 1. In both Figures 1 and 2, the curve labelled A was the first efflux curve, B the next, and so on. The heavy lines go through most of the experimental points and give the general contours of the efflux curves after correction had been made for contribution of Na^+ from connective tissue elements given in the data shown in Figure 2. The corrected curves were then resolved into two or more fractions; the slowest fraction is referred to as Fraction I, the next slowest as Fraction II, and so on. Note that as the cells were dying, the total Na^+ in the cell steadily increased until it reached the concentration in the external medium (ca. 100 mM). Contrary to expectations based on the original assignment of Levi and Ussing, this steady increase was in the fast fraction (s) rather than the Fraction I.

Figure 1 and 2 near here

Figure 3 shows an example of a different type of Na^+ efflux curve, though the muscles were treated in essentially the same way as in Figure 1. There was also the same steady gain of Na^+ in the fast fraction II. The difference lies in the slow Fraction I. In Figure 1, the magnitude of ^{22}Na in Fraction I as indicated by the intercept at 0 time remained more or less constant and then

fell. In Figure 3, Fraction I first rose and then fell.

Figure 3 near here

Figures 4A and 4B show plots of the magnitude of Fraction I (also to be referred to as the "slow fraction") represented as empty circles and the "fast fraction"* represented as solid circles in all the experiments performed. In Figure 4 A the slow fraction remained more or less constant before falling (12 out of 27 cases) while in Figure 4B the slow fraction first rose and then fell (the remaining 15 cases). Variation of K^+ concentration in the washing solution between 2.5 mM and 10 mM does not seem to have a statistically significant effect on the rise of the slow fraction. In all cases, the fast fraction steadily rose till it reached a concentration approaching or equalling that of Na^+ in the incubation medium.

Figure 4 near here

Figure 5 presented the rates of both fractions of Na^+ efflux represented as the half-time of exchange ($t_{\frac{1}{2}}$). As the muscle cells progressed from being perfectly normal to dead neither Fraction I nor Fraction II shows consistent or marked slowing-down to be expected on the basis of the pump model. This observation was in full accord with previous findings demonstrating that metabolic poisons which brought about minimal change of total cell K^+ and Na^+ concentrations in frog muscle cells (0° C) or large changes in them (25° C) produced no alteration in the Na^+ efflux rate (Ling and Ochsenfeld, 1976). Findings of this sort were the earliest observation that led one of us to suspect the validity of the Na-pump hypothesis.

---

* This fast fraction is in essence Fraction II but is obtained by subtracting from the total Na^+ (as indicated by the initial point of each curve) Fraction I and labelled Na^+ in the extracellular space (8 percent) (Ling and Walton, 1975a).

Figure 5 near here

Efflux of D-arabinose and sucrose from slowly dying muscles

In Figures 6 and 7, the efflux rates of D-arabinose and sucrose, respectively, from dying muscles are presented. In general the efflux curves were quite similar to those shown for Na^+ effluxes. There was a slow fraction (Fraction I) and fast fraction (Fraction II). There was also no significant slowdown of either Fraction I or Fraction II in the efflux curves of D-arabinose or of sucrose. Figure 8 presents magnitudes of the Fraction I and the fast fraction from all four sets of data on sugar effluxes. Again the most consistent observation from these studies is that as the muscle cells were dying, there was a steady increase of the fast fraction of both D-arabinose and sucrose until their concentrations in cell water approached that in the external medium, which was 5 mM. A comparison of Figures 6, 7 and 8 with Figures 1, 3, and 4 shows that parallel changes in the fast fraction were indeed seen for all these solutes in general agreement with the expectations from the Association-induction Hypothesis.

Figures 6, 7, & 8 near here

Before drawing a final conclusion from these data, one may raise the question: "Could the gradual increase of the fast fraction be explained as reflecting an increasing number of dead muscle cells?"

A statistical interpretation of the steadily rising fast fraction demands that in the low concentrations of IAA, each muscle cell either remains normal or is completely dead. This requirement arises from the data in Figure 5, which shows that half-times of the fast fraction and those of the slow fraction (Fraction I) are clearly separated at all times. Indeed the statistical interpretation demands that there are - at all times, two populations of cells - one with normal slow

solute permeability ($t_{\frac{1}{2}}$) of 20 to 40 minutes for Na^+, the other with "dead" solute permeability ($t_{\frac{1}{2}}$) of 2 to 10 minutes for Na^+. Similar segregation would be expected from the sugar efflux data. In order to prevent confusion in the minds of the reader, we must emphasize that we do not believe that there is any validity in this idea for reasons some of which were cited in the introduction. To cite one, the segregation into fast and slow fractions exists in perfectly normal muscle (even single fibers). Nevertheless, the test described in the next section represents extra precautions.

Does the slowly increasing fast fraction represent progressive events that occur in every single muscle cell, or does it represent a statistical increase in the number of dead cells?

To further help answer this question, we employed microelectrodes to measure the resting potential of individual muscle cells randomly chosen in sartorius muscles whose efflux curve had just been measured and in muscles whose identically-treated pairs were used in efflux studies. In all, we studied two perfectly normal muscles, two completely dead muscles, and a total of 16 muscles in intermediate stages of IAA poisoning. Representative detailed data are shown in Table 1.

Table 1 near here

It is immediately clear that the randomly chosen cells do not show a separation into two populations. Indeed within the limitations of the small population, each set of data represents essentially normal populations as indicated by the rough correspondence between the observed and the theoretically expected population density within the range of mean $\pm 1\sigma$, mean $\pm 2\sigma$, and mean $\pm 3\sigma$ (Table 2). These findings rule out the statistical interpretation of the rising fast fraction.

DISCUSSION

This investigation confirmed the two predictions of the Association-induction Hypothesis: as the muscle cells deteriorated, the fast exchanging fraction of Na^+ increased until it reached a concentration equal to that in the external solution; and this progressive increase of the fast fraction occurred with essentially the same time course for two other normally-excluded solutes, D-arabinose and sucrose.

The steady level of Na^+ in cells and the influx and efflux rates.

The present findings confirm the earlier conclusion that the fast fraction represents Na^+ movement, rate-limited by its passage through the cell surface. We have previously pointed out that the Na^+-efflux rate was not slowed down by metabolic poisons - even though the steady level of Na^+ of these muscles was steadily rising (Ling, 1962; Ling and Ochsenfeld, 1976). This constancy of the rate of Na^+ efflux of dying muscles was mostly seen in the slow fraction of Na^+ efflux (Figure 5, open circles). However, the present data clearly show that there is no slow down of the fast fraction either (Figure 5, solid circles).

The question may be raised, If the efflux rate does not change, how can the cell Na^+ level be gaining? The answer lies in a difference in time scale. The increase of total Na^+ in the dying muscles occurred over a period of hours and days, while the rate of Na^+ exchange is much faster, in terms of minutes. A very minor increase in the influx rate will cause an easily observable increase in the level of Na^+ in the cell over the much longer period of time.

The Universality Rule

According to this rule, the relative exclusion of solutes by living cells has a unitary cause - the multilayer polarization of the cell water. Therefore if for one reason or another, the q-value of one of the excluded solutes should rise or fall, parallel changes in the q-value of all other normally excluded solutes should occur (Ling, 1979); Ling and Ochsenfeld, 1977). This rule, which is confirmed

that the increase of Na^+ in response to ATP decline is primarily due to a change of free Na^+ in consequence of increasing the q-value of the bulk-phase cell water rather than by steady increase of adsorbed Na^+ (see below). However, this is not the whole story. In half of the muscles studied, the slowly exchanging Na^+ also increased, sometimes to a level as high as 40 moles/kg of fresh muscle cells, before falling to low level again. This could be explained as a transient gain of adsorbed Na^+, displacing adsorbed K^+. Why this gain of slowly exchanging Na^+ occurs only in half of the muscles studied is not clear. Attempts to reduce the slowly exchanging fraction by raising external K from 2.5 to 10 mM did not produce a significant difference.

Are the cell proteins normally adsorbing K^+ the same proteins which polarize the bulk of cell water?

As mentioned earlier, almost all the intracellular K^+ in normal resting muscle is adsorbed, primarily on the A bands and the Z-line (Edelmann, 1977, 1978). Yet we have shown clearly that the loss of adsorbed K^+ in dying muscle cells was replaced primarily by free Na^+ in the cell water. Both the level of adsorbed K^+ and the level of free Na^+ appear to depend quantitatively on the level of ATP. How can these two different phenomena be so closely associated?

The simplest model, one that had been discussed earlier, is that the protein which provides the β- and γ-carboxyl groups for the selective adsorption of K^+ (over Na^+) also provide backbone NHCO sites to polarize water (Ling, 1969). However, there is reason to doubt that this is the whole answer. In voluntary muscle cells, myosin provides the major part of K^+ adsorbing sites. Except for the Z-line, which also adsorbs K^+, there are few or no K^+ adsorption sites in the I bands. If myosin alone provides sites for K^+ adsorption and water polarization and the proteins making up the I band provide no site for water polarization, then the relative volume occupied by the I bands would set a lower limit to the q-value of any excluded permeant solute. This limit may be as high as 0.5 for a stretched

here once more, is useful because it sets apart the simplicity of the mechanism of solute exclusion in the association-induction hypothesis from the ad hoc mechanisms in the conventional membrane-pump theory: e.g., a pump for Na^+ (Glynn and Karlish, 1975), mitochondrial membrane impermeability for D-arabinose (Miller, 1974) and/or a D-arabinose permease (Cohen and Monod, 1957), and absolute plasma membrane impermeability to sucrose.

The dependence of K^+ accumulation and Na^+ exclusion on ATP

According to the association-induction hypothesis, the maintenance of the polarized multilayer state of water in living cells depends on ATP functioning as a cardinal adsorbent (Ling, 1977a).

When metabolic pathways are blocked, the level of ATP declines. With the fall of ATP concentration, the key proteins undergo cooperative changes to a lower-energy state with major shift in the electron distribution. The effect of ATP depletion on K^+ and Na^+ distribution are twofold: (i) More water-polarizing protein molecules assume an α-helical or other conformation in which the polarizing sites, NH and CO, become internally neutralized by other CO and NH groups; as a result more water reverts to a state close to that assumed by normal liquid water and the q-values of all excluded solutes rise. (ii) The electron density of anionic sites shifts to a value which favors K^+ adsorption less than in the normal resting cells.

The K^+ concentration in the poisoned frog muscle has been shown to be quantitatively dependent on the level of ATP in the cells if enough time is allowed for ionic distribution equilibrium to keep pace with ATP decline (Ling, 1962, 1973, 1974; Gulati et al, 1971) Furthermore the sum of the concentration of K^+ and Na^+ remain roughly constant in the deteriorating cells (Gulati et al, 1971).

The variation of cell Na^+ level could reflect changes in either adsorbed or free Na^+ in the cell. The studies presented in this paper clearly establish

muscle. The real q-value for Na^+ can be estimated from the Na^+ efflux curve from perfectly normal muscle cells presented in this and earlier papers, i.e., .05 to 0.1. This difference suggests that either actin (Ling, 1979) which makes up the major component of the I band, or some other matrix protein present in the I band and elsewhere must also provide seats of multilayer water polarization.

The related and yet separate mechanism for K^+ accumulation and Na^+ exclusion reminds one of the K^+ and Na^+ level changes in the course of muscle tissue maturation. Thus Hazlewood and coworkers showed that as maturation proceeds from prenation to maturity the gain of rat muscle K^+ is strictly related to the gain of total fat free solids, mostly proteins. In contrast, there was a steady decrease of cell Na^+, suggesting a gradual lowering of the q-value accompanying an increasing extent of multilayer polarization of water (Hazlewood et al, 1969).

ACKNOWLEDGEMENTS

The present work was supported by NIH Grants 2-R01-Ca16301-03, 2R01-GM1142-13, and Office of Naval Research, Contract Number N00014-71-C-0178. The John H. Hartford Foundation provided many of our basic facilities.

LEGENDS

Figure 1: Successive Efflux Curves of Labelled Na^+ in a Dying Frog Sartorius Muscle Repeatedly Loaded with Labelled Na^+ and Washed in Ringer's Solution Containing 10 mM KCl and 0.05 mM Na Iodoacetate.

The schedule used was Procedure IB. In this and the following graphs, Curve A was the first washing curve, Curve B, the next, and so on. Heavy solid lines are the best-fitting curve to the experimental points after subtracting the contribution of the connective tissue (see Figure 2). These corrected curves were then resolved into a slow fraction (I) and fast fraction (II) or fractions (II, III).

Figure 2: Successive Efflux Curves of Labelled Na^+ in "Connective Tissue" Loaded with Labelled Na^+ and Washed in Ringer's Solution Containing 10 mM KCl and 0.05 mM Na Iodoacetate.

Isolated "connective tissue" was treated the same way as the sartorius muscle in Figure 1 and the data plotted in a similar way except the curves were not resolved.

Figure 3: Successive Efflux Curves of Labelled Na^+ in a Dying Frog Sartorius Muscle Repeatedly Loaded with Tracer Na^+ and Washed in Ringer's Solution Containing 10 mM KCl and 0.05 mM Na Iodoacetate.

The schedule used was Procedure IB. Other procedures leading to the separation of Fraction I and II are the same as described under Figure 1, with the exception that the corrected curves are shown as thin curved lines in A, B, C while in D and E the corrections were too small to be distinguished from the heavy lines going through the experimental points.

Figure 4: The Concentration of Labelled Na^+ in the Fast and Slow Fraction from the Successive Efflux Curves of Sartorius Muscles Repeatedly Exposed to IAA.

In A, the slow fraction did not show significant increase. In B, the slow fraction shows transient increase. Solid circles represent Na^+ concentration in the fast fraction; empty circles represent labelled Na^+ concentration in the slow fraction. The schedule of poisoning for the first 8 sets of experiments (3E18A to 3F5D) was Procedure IA; for the remaining sets of experiments, Procedure IB was used.

Figure 5: The Half Time Exchange ($t_{\frac{1}{2}}$) of the Slow and Fast Fraction of Labelled Na^+ from Successive Efflux Curves of Sartorius Muscles Repeatedly Exposed to IAA.

Solid circles represent $t_{\frac{1}{2}}$ of labelled Na^+ in the fast fractions. Empty circles represent $t_{\frac{1}{2}}$ of labelled Na^+ in the slow fraction. (For details of experimental procedures used, IAA, and K^+ concentrations, see Legend of Figure 3).

Figure 6: Successive Efflux Curves of Labelled D-Arabinose in a Dying Frog Muscle Repeatedly Equilibrated with Labelled D-Arabinose and Exposed to IAA.

The schedule of poison exposure and washing used was Procedure II. IAA concentration was 0.2 mM. Other procedures leading to the separation of Fraction I and II are the same as described under Figure 1, except the connective tissue curves were not presented.

Figure 7: Successive Efflux Curves of Labelled Sucrose in Dying Frog Sartorius Muscle Repeatedly Equilibrated with Labelled Sucrose and Exposed to IAA.

The schedule of poison exposure and washing used was Procedure II. IAA concentration was 0.2 mM. Other procedures leading to the separation of Fraction I and II are the same as described under Figure 1, except the connective tissue curves were not presented. (Expt. No. 3K15A).

Figure 8: The Concentration of Labelled D-Arabinose or Sucrose in the Fast and Slow Fractions from the Successive Efflux Curves of Sartorius Muscles Repeatedly Exposed to IAA.

Solid circles represent labelled Na^+ concentration in the fast fraction; empty circles represent labelled Na^+ concentration in the slow fraction. All schedules used were Procedure II. All IAA concentrations were 0.2 mM.

Table 1: Resting Potentials of Randomly Chosen Single Muscle Cells in Normal, Dying, and Dead Frog Sartorius Muscles.

Table 2: Analyses of the Population Distribution Pattern of Resting Potentials Measured Among Randomly Chosen Single Muscle Cells in Normal, Dying, and Dead Sartorius Muscles.

Fractions of items in a perfectly normal distribution between mean $\pm\sigma$, mean $\pm 2\sigma$, and mean $\pm 3\sigma$ are shown on the bottom line. Good accord of experimental data with this ideal distribution indicates that the resting potential randomly measured in normal and dead muscle cells as well as in cells of muscles at various stages of dying are normal; at no time was there noted a separation into groups.

REFERENCES

Cohen, G. N. and J. Mon d 1957 Bacterial Permeases. Bacteriol. Rev. 21:169

Edelmann, L. 1977 Potassium adsorption sites in frog muscle visualized by cesium and thallium under the transmission electron microscope. Physiol. Chem. Phys. 9:313-317.

____ 1978 Visualization and x-ray microanalysis of potassium tracers in freeze-dried and plastic embedded frog muscle. In: Microprobe Analysis in Biology and Medecine, Microscop. Acta. Suppl. 2, eds. P. Echlin and R. Kaufman, S. Hirzel Verlag, Stuttgard.

Glynn, I. M. and S. J. D. Karlish 1975 The sodium pump. Ann. Rev. Physiol. 37:13-55.

Gulati, J., M. M. Ochsenfeld, and G. N. Ling 1971 Metabolic cooperative control of electrolyte levels in adenosine triphosphate in the frog muscle. Biophys. J. 11:973-980.

Harris, E. J. 1950 The transfer of sodium and potassium between muscle and surrounding medium. Part II. The sodium flux. Trans. Farad Soc. 46:782-882.

Hazlewood, C. F. and B. L. Nichols 1969 Changes in muscle sodium potassium, chloride, water. and voltage during maturation in the rat: An experimental and theoretical study. Hopkins Med. J. 125:119-133.

Hodgkin, A. L. and P. Horowicz 1959 Movement of Na and K in single muscle fibers. J. Physiol. 145:405-432.

Huxley, H. E. 1960 Mineral Metabolism, vol. 1, eds. C. L. Comar and F. Bromner, Academic Press, pp. 163-166.

Johnson, J. 1955 Kinetics of release of radioactive sodium, sulfate and sucrose from a frog sartorius muscle. Am. J. Physiol. 181:263-268.

Jones, A. W. 1965 The water and electrolyte metabolism of the arterial wall. Ph.D. Thesis. University of Pennsylvania

Keynes, R. D. 1954 The ionic fluxes in frog muscle. Proc. Roy. Soc. B. 142:359-382.

Keynes, R. D. and G. W. Maisel 1954 The energy requirement for sodium extrusion

from a frog muscle. Proc. Roy. Soc. B. 142:383-392.

Keynes, R.D. and R.A. Steinhardt 1968 The components of the sodium efflux in frog muscle. J. Physiol. (London) 198:581-599.

Levi, H. and H. H. Ussing 1948 The exchange of sodium and chloride ions across the fibre membrane of the isolated frog sartorius muscle. Acta. Physiol. Scand. 16:232-249.

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

_____ 1969 A new model for the living cell: A summary of the theory and recent experimental evidence in its support. Int'l. Rev. Cytology. 26:1-61.

_____ 1973 What component of the living cell is responsible for its semi-permeable properties: Polarized water or lipids? Biophys. J. 13:807-816.

_____ 1974 An answer to a reported apparent contradiction in the predicted relation between the concentration of ATP and K in living cells. Physiol. Chem. Physics. 6:285-286.

_____ 1977a The physical state of water and ions in living cells and a new theory of the energization of biological work performance by ATP. J. Molec. and Cell. Biochem. 15:159-172.

_____ 1977b K^+ localization in muscle cells by autoradiography, and identification of K^+ adsorbing sites in living muscle cells with uranium binding sites in electron micrographs of fixed cell preparations. Physiol. Chem. Physics. 9:319-328.

_____ 1978 Maintenance of low sodium and high potassium levels in resting muscle cells. J. Physiol. 280:105-123.

_____ 1979 The polarized multilayer theory of cell water and other facets of the association-induction hypothesis concerning the distribution of ions and other solutes in living cells. In: The Aqueous Cytoplasm, ed. A. D. Keith, Marcel Dekker, Inc., New York.

_____ 1980 Underestimation of Na permeability in muscle cells: Its implications regarding the theory of cell potential and energy requirement of the

Na pump. Physiol. Chem. Physics. 12 (in press)

Ling, G. N. and G. Bohr 1971 Studies on ion distribution in living cells: III. Cooperative control of electrolyte accumulation by ouabain in the frog muscle. Physiol. Chem. Physics. 3:431-447.

Ling, G. N. and F. W. Cope 1969 Potassium ion: Is the bulk of intracellular K^+ adsorbed. Science. 163:1335-1336.

Ling, G. N. and R. W. Gerard 1949 The normal membrane potential of frog sartorius fibers. J. Cell. Comp. Physiol. 34:383-396.

Ling, G. N., C. Miller and M. M. Ochsenfeld 1973 The physical state of solutes and water in living cells according to the association-induction hypothesis. Ann. N. Y. Acad. Sci. 204:6-50.

Ling, G. N. and M. M. Ochsenfeld 1965 Studies on the ionic permeability of muscle cells and their models. Biophys. J. 5:777-807.

_____ 1973 Control of cooperative adsorption of solutes and water in living cells by hormones, drugs, and metabolic products. Ann. N.Y. Acad. Sci. 204: 325-336.

_____ 1976 Na^+ and K^+ levels in living cells: Do they depend on the rate of outward transport of Na^+? Physiol. Chem. Physics. 8:389-395.

_____ 1977 Confirmation of "universality rule" in solute distributions: Studies of simultaneous efflux of Na^+ and D-arabinose from single frog eggs, living, dying, and dead. Physiol. Chem. Physics 9:405-426.

Ling, G. N., M. M. Ochsenfeld, C. Walton and T. J. Bersinger 1980 Mechanism of solute exclusion from cells: The role of protein-water interaction. Physiol. Chem. Physics. 12:3-10.

Ling, G. N. and A. M. Sobel 1975 The mechanism for the exclusion of sugars from the water in a model of the living cell: The ion-exchange resin: Pore size or water structure? Physiol. Chem. Physics. 7:415-421.

Ling. G. N. and C. Walton 1975a A simple rapid method for the quantitative separation of the extracellular fluid in frog muscles. Physiol. Chem. Physics. 7:215-218.

_____ 1975b Simultaneous efflux of K^+ and Na^+ from frog sartorius muscle freed of extracellular fluids: Evidence for rapidly exchanging Na^+ from the cells. Physiol. Chem. Physics. 7:501-515.

Ling, G. N., C. Walton and T. J. Bersinger 1980 Reduced solubility of polymer-oriented water for sodium salts, sugars, amino acids and other solutes normally maintained at low levels in living cells. Physiol. Chem. Physics. 12:111-138.

Ling, G. N., C. Walton and M. R. Ling 1979 Mg^{++} and K^+ distribution in frog muscle and egg: A disproof of the Donnan theory of membrane equilibrium applied to the living cells. J. Cell. Physiol. 101:261-278.

Miller, C. 1974 Nonelectrolyte distribution and reversal of the insulin effect. Biochem. Biophys. Acta. 339:71-84.

Minkoff, L. and R. Damadian 1973 Calorie catastrophe. Biophys. J. 13:167-178.

Mullins, L.J. and A. S. Frumento 1963 The concentration dependence of sodium efflux from muscle. J. Gen. Physiol. 46:629-654.

Negendank, W. G. and C. C. Shaller 1980 Multiple fractions of sodium exchange in human lymphocytes. J. Cell. Physiol. (in press)

Neville, M. C. and R. T. Mathias 1979 The extracellular compartments of frog skeletal muscle. J. Physiol. 288:45-70.

Trombitas, K. and A. Tigyi-Sebes 1980 X-ray microanalysis of the isolated myofibrils from the honey-bee flight muscle. Acta. Physiol. Acad. Sci. Hung. (in press)

	Experiment Number	IAA Concentration (mM)	Incubation Time (hrs)	Temp. (°C)	Ψ (mV)					
normal	7-D-29A	-	-	-	90	90	90	92	93	90
					90					
	7-D-29B	-	-	-	92	96	90	90	97	93
					95	93	93	93		
dying	7-D-29C	0.2	5	25°	56	57	60	59	64	56
					54	57	65	64	56	62
					52	53	55	60		
	7-D-29D	0.2	5	25°	46	46	54	48	50	54
					53	45	64	51	55	44
					50	65	57	58	60	66
					59	57	60			
	7-D-30C	0.2	4.7	25°	61	69	74	70	60	76
					78	83	58	58	78	58
					68	78	71	80	76	
	7-D-30D	0.2	4.7	25°	84	85	70	78	80	85
					76	71	68	78	71	62
					82	65	84	75	82	79
					86	85	60	70		
	*7-E-3A	0.2	4.5	25°	40	30	33	57	55	45
					49	55	57	37	50	43
					30	30	31	38	39	49
	*7-E-3B	0.2	4.5	25°	59	48	33	55	55	42
					32	41	62	34	35	35
					59	50	45	56	38	51
					48	44				
	*7-E-3C	0.2	4.5	25°	38	48	36	44	28	35
					48	53	45	55	60	60
					52	54	46	44	46	58
					48	49				
	*7-E-3D	0.2	4.5	25°	35	44	32	38	36	61
					45	58	65	57	42	58
					50	35	30	33	53	45
					58	29				
dead	7-D-30A	0.2	23	4°	4	8	5	6	5	6
					7	5				
	7-D-30B	0.2	23	4°	2	6	8	8	6	5
					9	5				

*reading after efflux

TABLE 1

	n	Ψ (mV.) $\bar{X} \pm \sigma$	Portion of Ψ Values within Limits $\bar{X} \pm \sigma$ %	$\bar{X} \pm 2\sigma$	$\bar{X} \pm 3\sigma$
6-E-5H	16	61.4 ± 11.0	62.5	100	100
6-E-5B	16	52.5 ± 10.0	56.2	100	100
6-E-5C	16	45.6 ± 7.1	87.5	93.8	100
6-E-5D	16	52.8 ± 6.1	75.0	93.8	100
6-E-10A	15	35.4 ± 6.3	80.0	100	100
6-E-10B	15	47.1 ± 8.6	67.0	100	100
6-E-10C	15	41.3 ± 8.4	86.7	93.3	100
6-E-10D	15	38.2 ± 6.4	80.0	93.3	100
7-D-29A	7	90.7 ± 1.2	71.4	100	100
7-D-29B	10	93.2 ± 2.2	60.0	100	100
7-D-29C	16	58.1 ± 4.0	68.8	100	100
7-D-29D	21	54.4 ± 6.6	66.7	100	100
7-D-30C	17	70.4 ± 8.5	76.0	100	100
7-D-30D	22	76.2 ± 8.0	64.0	95	100
7-E-3A	18	42.7 ± 9.8	56.0	100	100
7-E-3B	20	46.1 ± 9.7	70.0	100	100
7-E-3C	20	47.4 ± 8.5	65.0	95	100
7-E-3D	20	45.2 ± 11.5	60.0	100	100
7-D-30A	8	5.8 ± 1.3	75.0	100	100
7-D-30B	8	6.1 ± 2.2	75.0	100	100
Population within Limits		Observed (mean ± S.E.)	70.1 ± 2.1	98.2 ± 0.63	100 ± 0.0
		Expected for Normal Distribution in Large Population	68.3	95.5	99.7

TABLE 2

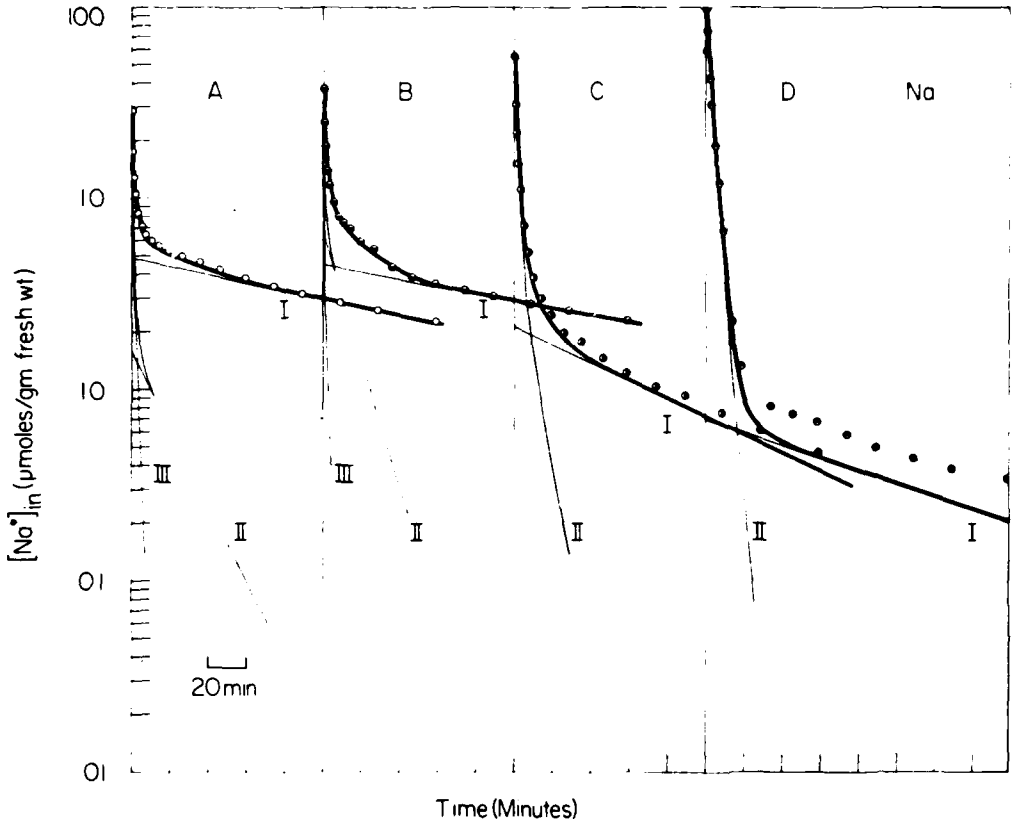

100
10
10
01
01
A
B
C
D
Na
I
I
I
I
III
III
II
II
II
II
I
20min
$[Na^+]_{in}$ (μmoles/gm fresh wt)
Time (Minutes)

FIGURE 1

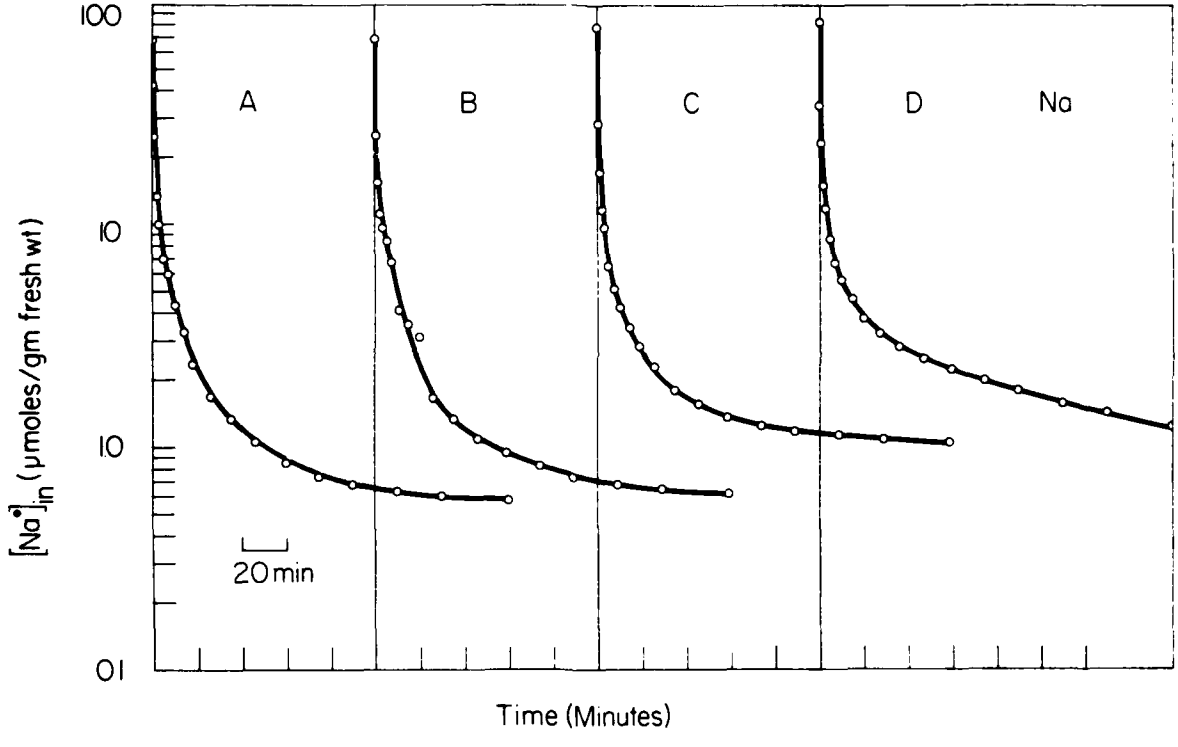

100
10
1.0
0.1
A
B
C
D
Na
20 min
$[Na^+]_{in}$ (μmoles/gm fresh wt)
Time (Minutes)

FIGURE 2

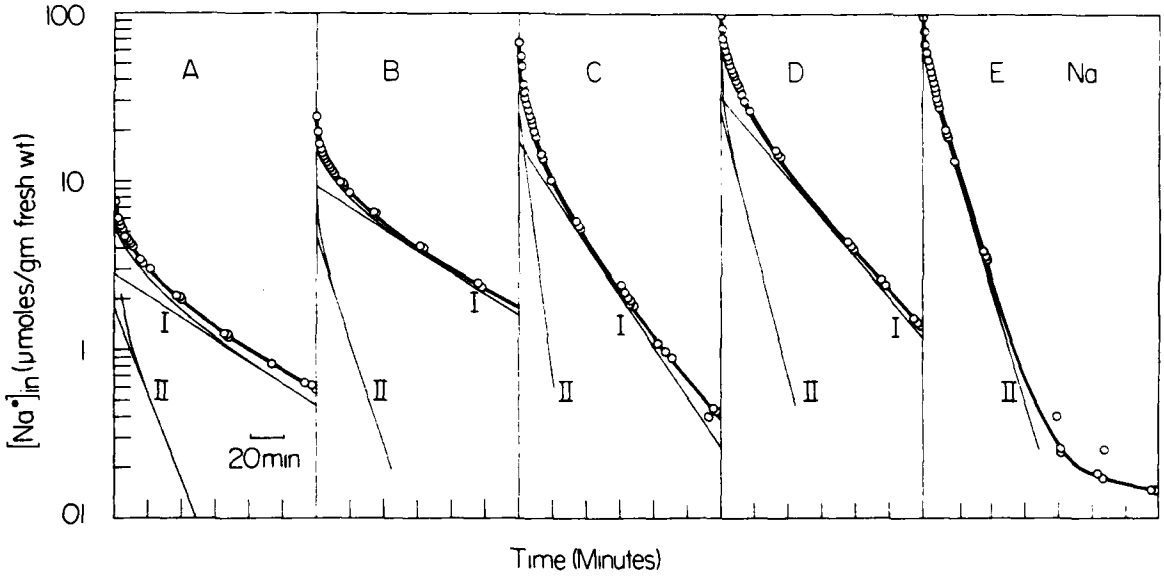

100
10
1
01
A
B
C
D
E
Na
I
II
20min
[Na+]in (μmoles/gm fresh wt)
Time (Minutes)

FIGURE 3

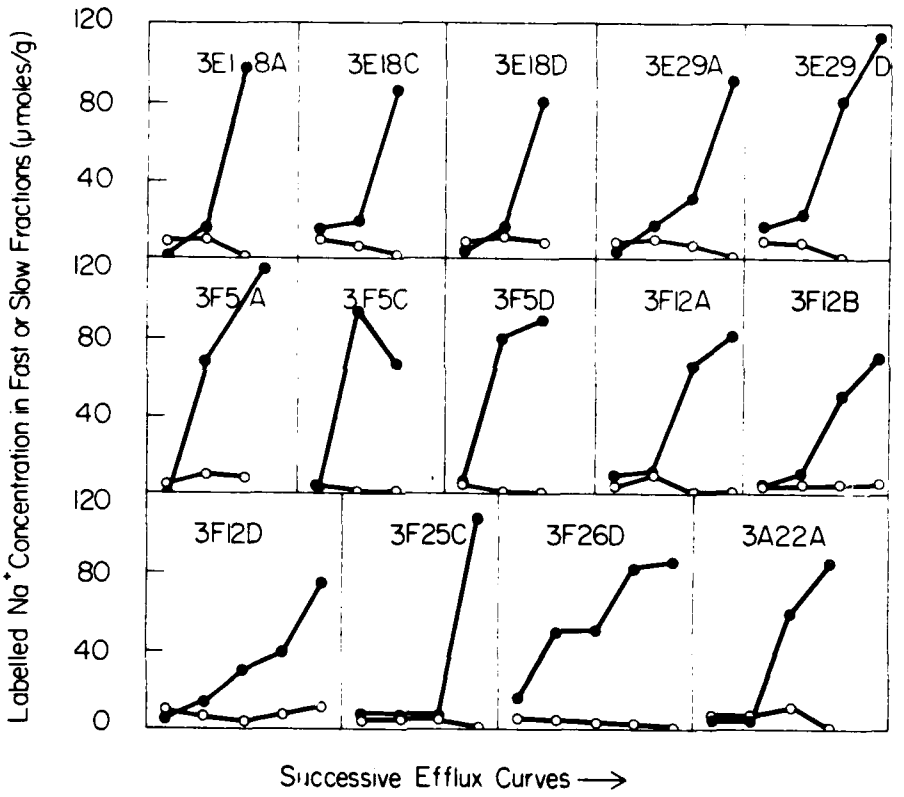

Labelled Na+ Concentration in Fast or Slow Fractions (μmoles/g)
120
80
40
120
80
40
120
80
40
0
3E18A
3E18C
3E18D
3E29A
3E29D
3F5A
3F5C
3F5D
3F12A
3F12B
3F12D
3F25C
3F26D
3A22A
Successive Efflux Curves ⟶

FIGURE 4 A

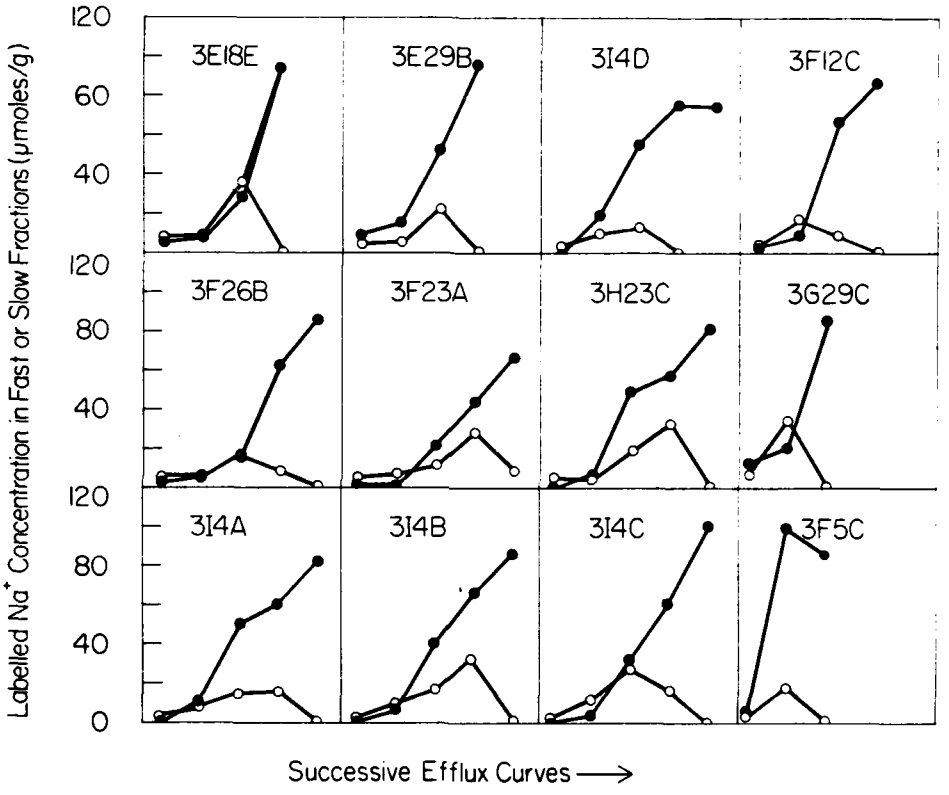

3E18E
3E29B
3I4D
3F12C
3F26B
3F23A
3H23C
3G29C
3I4A
3I4B
3I4C
3F5C
120
60
40
120
80
40
120
80
40
0
Labelled Na+ Concentration in Fast or Slow Fractions (μmoles/g)
Successive Efflux Curves ⟶

FIGURE 4 B

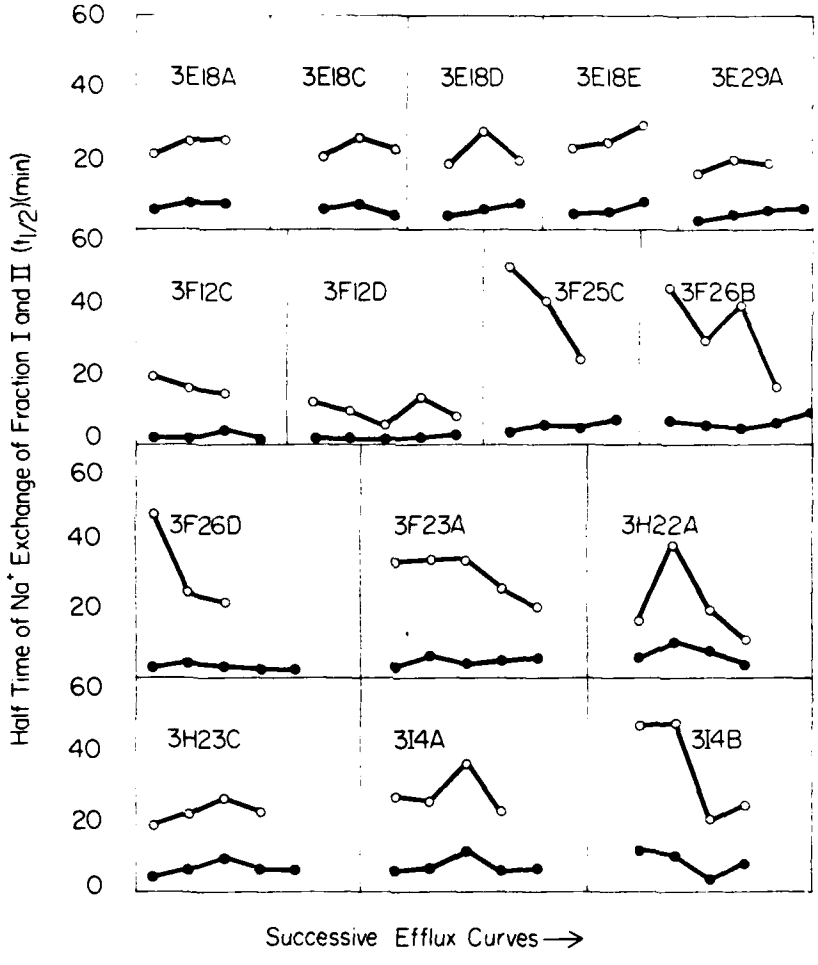

60
40
20
0
3E18A
3E18C
3E18D
3E18E
3E29A
3F12C
3F12D
3F25C
3F26B
3F26D
3F23A
3H22A
3H23C
314A
314B
Half Time of Na⁺ Exchange of Fraction I and II ($t_{1/2}$)(min)
Successive Efflux Curves ⟶

FIGURE 5

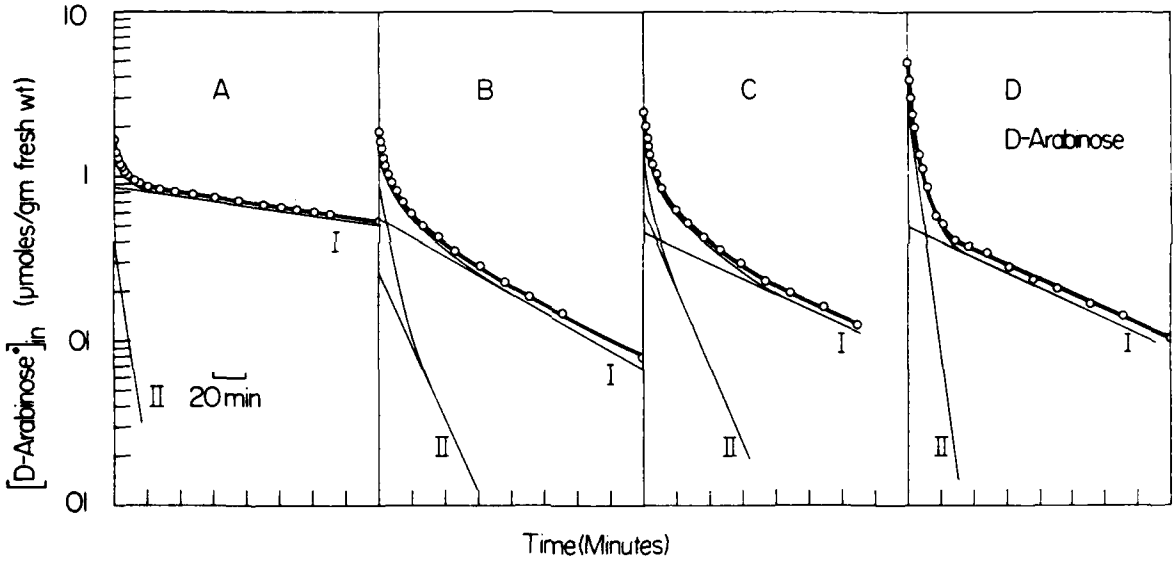

10
1
01
01
A
B
C
D
D-Arabinose
I
II
20min
[D-Arabinose*]in (μmoles/gm fresh wt)
Time(Minutes)

FIGURE 6

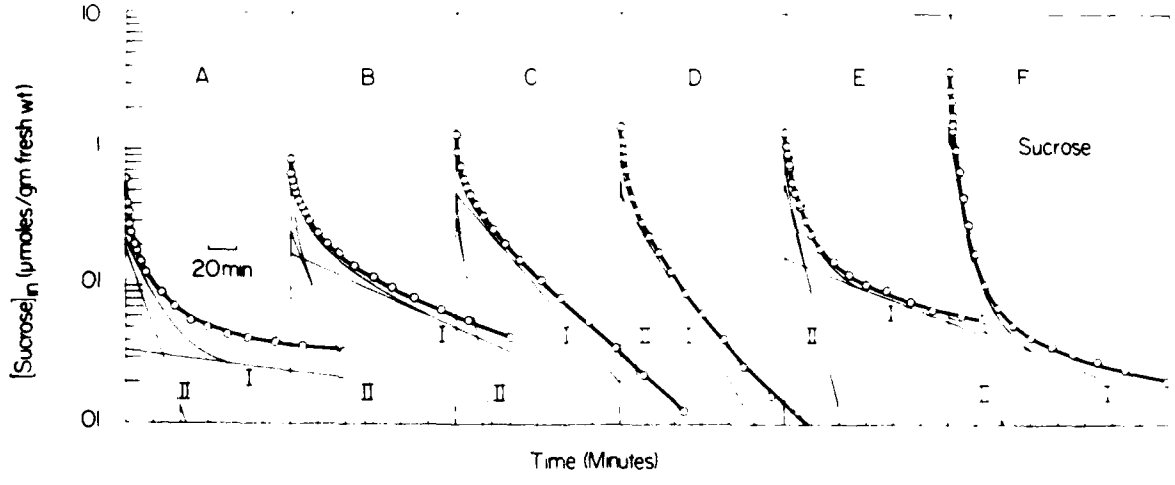

10
1
01
01
A
B
C
D
E
F
Sucrose
20min
I
II
[Sucrose]in (μmoles/gm fresh wt)
Time (Minutes)

FIGURE 7

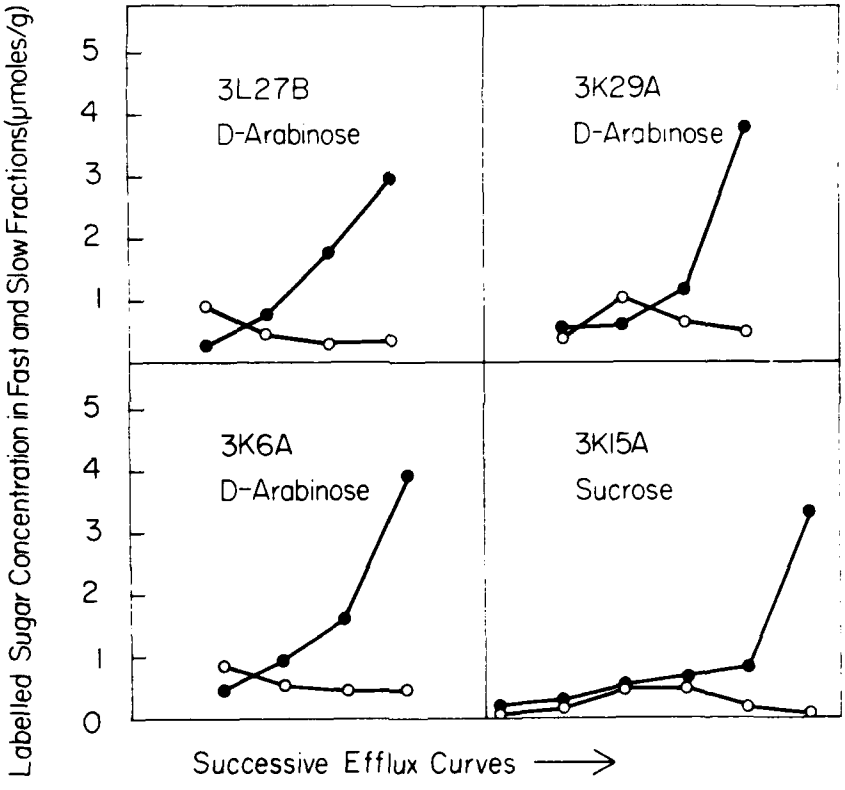

Labelled Sugar Concentration in Fast and Slow Fractions(μmoles/g)
3L27B
D-Arabinose
3K29A
D-Arabinose
3K6A
D-Arabinose
3K15A
Sucrose
0
1
2
3
4
5
Successive Efflux Curves

FIGURE 8

END

FILMED

1-84

DTIC