CONFIRMATION OF "UNIVERSALITY RULE" IN SOLUTE DISTRIBUTIONS: STUDIES OF SIMULTANEOUS EFFLUX OF Na+ AND D-ARABINOSE FROM SINGLE FROG EGGS LIVING, DYING, AND DEAD

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• *The effluxes of labeled Na+ and labeled ~arabinose from single frog ovarian eggs were simultaneously recorded from normal eggs and eggs in various stages of deterioration following exposure to the metabolic poison Na iodoacetate. Findings were:*

1. At least two fractions of Na+ occur in these egg cells: one fast-exchanging fraction and one slowly exchanging fraction. Also, at least two fractions of ~arabinose occur: one fast exchanging, the other slowly exchanging.

2. Apparently the fast fractions of both Na+ and D*-arabinose represent free Na+ or ~arabinose in the cell water with rates of efflux limited by passage through the cell surface. The slow fractions appear to represent Na+ or* D*-arabinose adsorbed on macromolecular sites; their rates of loss are limited by the rates of desorption.*

3. While the poisoned cells steadily gain Na+, neither the rates of efflux of the fast-exchanging Na+ nor the rates of loss of the slowly exchanging Na+ show evidence of a slow-down. This contradicts the Na-pump theory.

4. The most outstanding and consistent observation is a steady gain in concentration of both the fast-exchanging Na+ and the fast-exchanging ~arabinose as the poison takes effect. Maximum concentrations are reached in dead eggs.

5. A correlation coefficient of +0.92 is demonstrated between the concentrations of fast-exchanging Na+ and fast-exchanging ~arabinose in the eggs studied. This finding offers support for the "universality rule" according to which the changing levels of Na+ and D*-arabinose, as well as other solutes normally excluded from the same cells, all have a unitary origin—the changing physical state of the cell water. Therefore changes of all these solutes should proceed* pari passu *and show positive correlation.*

INTRODUCTION

In 1949 Abelson and Duryee demonstrated in single frog ovarian eggs the existence of two fractions of Na+ having different rates of exchange with extracellular labeled Na+.[1] Although Abelson and Duryee refrained from speculating on the mechanism involved, they did allude to binding as a possible cause of the slow exchanging fraction.

In 1956 Ling and Schmolinske suggested that a similar slow exchanging fraction of Na+ from frog sartorius muscles is rate-limited by desorption from protein

sites primarily within the cells.[2] Comparing the Na+ efflux of single frog eggs with that of single frog muscle cells, in which two fractions of intracellular Na+ with different exchange rates were also demonstrated, Ling suggested in 1962 that in both muscle and egg cells the fast exchanging fraction represents free Na+ in the cell water and the slow exchanging fraction represents Na+ adsorbed on cytoplasmic macromolecular sites.[3] Later Dick and Lea[4] as well as Horowitz and Fenichel[5] confirmed the existence of two fractions of Na+ in frog and toad eggs. The latter team[6] also produced evidence that the exchange of the fast fraction was rate-limited by passage through the cell membrane.

The molar concentration of Na^+ in amphibian ovarian eggs is considerably lower than that in the external bathing solution. One explanation for this low level of cellular Na+ is provided by conventional membrane-pump theory: that is, this low level is maintained by continual outward pumping of Na^+.[6] Another explanation, based on the association-induction hypothesis, is that the cell water exists in a state of polarized multilayers[7–10] and that in this state water has reduced solubility for Na+ and other solutes (such as sugars).

In this paper we report experiments designed to test these opposed theories on the basis of the following two fundamental criteria:

(1) Slow-down of Na+ Efflux in Dying Cells

According to the pump theory, the outward Na+ flux rate is maintained to a large degree by an active transport process. Thus a mandatory prediction of the theory is that poisons which effectively raise the level of Na+ in the cell do so by slowing down the outward pumping of Na^+ and so diminishing the efflux rate of Na^+.

According to the association-induction hypothesis, the physical state of water in a resting cell is maintained by ATP, considered in this context not as a fuel but rather as a "cardinal adsorbent" allosterically controlling the conformation of certain key proteins.[9–11] In turn, the conformation of those cell proteins determines the physical state of water adsorbed in multilayers on the exposed NH and C = O groups of the protein "backbones." When metabolism is effectively blocked, as by cell poisoning, ATP concentration declines. With diminishing ATP concentration, the level of intracellular Na^+ rises because of a return of the cell water from its state of polarized multilayers with low Na+ solubility to a state closer to that of "normal" liquid water with high Na+ solubility. Therefore, in this model, the level of Na+ reflects an equilibrium property of the bulk of intracellular water, so that the rate at which Na+ travels across the cell surface should be found not directly related to the level of Na+ in healthy or in poisoned cells. This theoretical expectation was experimentally confirmed recently in studies of frog muscles.[12]

(2) "The Universality Rule"--Synchrony in Rise of Na^+ and D-Arabinose Concentrations in Poisoned Eggs

To our knowledge, a clearly enunciated Na-pump hypothesis still does not exist (see ref. 13, p. 13), much less a consistent and rigorous theory. A number of ad-

vocates of the pumping approach believe that the pump is intimately associated with the Na-K activated cellular ATPase.[14,15] Like most living cells, microbes such as E. *coli* maintain a low intracellular concentration of Na^+. Since these microbes do not possess Na-K activated ATPase, an altogether different (and as yet unspecified) mechanism for its Na-pump must be postulated. Hence some investigators have attributed the asymmetrical distribution of sugars in E. *coli* to pumps driven by the cellular resting potential. Other investigators, studying sugar distribution in mammalian cells, believe that the low concentrations of non-metabolized sugars in these cells are not the result of pumps at all but are due to a postulated absolute impermeability of mitochondria1 membranes to these sugars.[16] In any case, study of such suggestions and purported supporting evidence only emphasizes that the pump proposal has not yet been developed into a unified theory and therefore is unable to predict any simple relation between the levels of pairs of dissimilar solutes such as Na^+ and D-arabinose in living cells.

A totally different explanation is presented by the association-induction hypothesis. It attributes maintenance of a low concentration of any non-metabolized free solute dissolved in the water of a resting living cell to one and only one mechanism; to wit, reduced solubility in cell water existing in the state of polarized multilayers. Thus when the physical state of water in the cell changes, as for example in response to decreased ATP concentration, the relative solubilities of all the normally excluded solutes should change *pari passu*. This theoretical prediction is referred to as the "universality rule."[10] It follows that in the experiments discussed here, there should be observed a positive correlation between the level of Na^+ and the level of non-metabolized D-arabinose in the cell water at different times in the same dying poisoned cells. (For evidence that D-arabinose is generally not metabolized, see ref. 17, p. 795, and ref. 18.) It should be emphasized that the universality rule applies only to normally excluded solutes that exist as free solutes in the cell water; the rule does not apply to solutes in the adsorbed state.

MATERIAL§ AND METHODS

Fully mature ovarian eggs were obtained from North American leopard frogs (*Rana pipiens pipiens,* Schreber). Single eggs were dissected free from the ovarian lobe in cold Ringer's solution. Most of the connective tissue attached to the eggs was removed, leaving just enough to permit handling with fine forceps. The following procedures were then followed:

Efflux studies. Eggs were incubated at 25°C in a modified Ringer's solution called the Ringer-GIB medium (for basic composition, see ref. 19), containing 100 mM Na^+, 5 mM D-arabinose, 40 to 80 μCi/ml of ^{22}Na (ICN Pharmaceutical Inc., Irvine, California, Lots 35, 37, and 39), and 90 to 200 μCi/ml ^{3}H-labeled D-arabinose (ICN, Lot 619625). Sodium iodoacetate (IAA) (Sigma Chemical Company, St. Louis, Missouri, Lot 102C-0540) was included in the incubation solu-

tion at a concentration varying from 0.1 to 1.0 mM for those experiments in which egg cells were to be poisoned.

Incubation time varied between 19 and 20 h for normal eggs and between 18 and 66 h for IAA-treated eggs. After incubation each single egg was picked up by its remaining tag of connective tissue. Adhering solution was removed by placing the egg on filter paper moistened with incubation solution, then gently blotting. The egg was next tied to a fine wire and rinsed for carefully timed intervals in successive tubes containing 1.5 ml portions of Ringer-phosphate solution (for basic composition, see ref. 20). This modified Ringer's solution contained 100 mM Na^+ and 5 mM D-arabinose but no isotope labels.

The washing solution, maintained at 25°C(a 0.1°C), was gently bubbled with air through a (Bardic) 22-gauge catheter. To prevent adherent fluid from being carried over from one solution to the next, the egg was dragged gently along the inner wall of the tube before each transfer and the wire was touched to filter paper.

When removed from the final tube of washing solution, the egg was once more gently blotted, then weighed and extracted in 1.5 ml of 5% trichloroacetic acid (TCA) for residual ^{22}Na and ^{3}H-D-arabinose. ^{22}Na activities in the washing solutions and in the TCA extract of the eggs were measured either directly in a Nuclear Chicago automatic γ-scintillation counter or together with ^{3}H activity of labeled D-arabinose measured in Bray's scintillation fluid in a Packard β-scintillation spectrophotometer. Model 3330.

To measure the effluxes of labeled Na^+ and labeled D-arabinose from connective tissues of the same frog from which the eggs had been removed, we combined a piece of tissue from the "stalk" of the ovary with another piece from the ovarian lobe tissue after removal of all eggs. These tissues were then incubated in labeled solutions and washed in exactly the same manner as the eggs.

Time course studies. 10 to *15* eggs were incubated in a solution of the same composition as that used for the efflux studies. Incubation time varied from 0 to 140 h. After incubation and removal of adhering fluid, the eggs were placed in a polypropylene tube containing 0.1 N HCI, that had been heated in a boiling water bath.[19] The total sodium and potassium content of the sample was determined by means of atomic absorption spectrophotometry. ^{22}Na activity was measured in the Nuclear Chicago y-scintillation system. Chloride was analyzed with a Buchler-Cotlove chloridometer.

Resolution of efflux curves into fractions. The different fractions of labeled Na^+ and D-arabinose emerging from the eggs were resolved in part by the standard procedure of successively peeling off the slowest exponential fraction, then the next slowest, etc.[3] This method is adequate when dealing with slower fractions. However, it may incur error in obtaining the magnitude of the fast fractions because the small amount of labeled solution adhering to the outside of the egg tends to exaggerate the value. For that reason we chose to obtain the amount of fast-exchanging labeled Na^+ by extrapolating to zero time from the second point (taken 15 sec

after washing began), the third point (taken **30 sec** after washing began), and immediately following experimental points after correction for isotope in extracellular tissue contents. We believe that the latter procedure yields a more correct estimation of the fast fraction than simple extrapolation from all initial points including the first point (at zero time), since the first point includes the radioactivity in a thin film of adherent Ringer's solution. This film is washed away in less than a second.

RESULTS

Percentage of Extracellular Tissue

Surrounding each egg cell is a thin layer of ovarian tissue. By exhaustive squeezing, blotting, and washing away of egg cytoplasm we obtained a weight of 1.1 ± .083% for this extracellular tissue. That finding agrees well with Neville's result of 1.5%[21] for fixed tissue using histological sectioning and staining techniques.

Time Course of *Labeled Na+ Uptake*

Figure 1 shows that exposure of frog eggs to a Ringer's solution containing ^{22}Na leads to a rapid accumulation of labeled Na+ in the cell in agreement with earlier reports. A steady level of 10 μmoles/g fresh weight was then maintained by the cell for at least 24 h. After that, the level of labeled Na^+ began to rise again until it

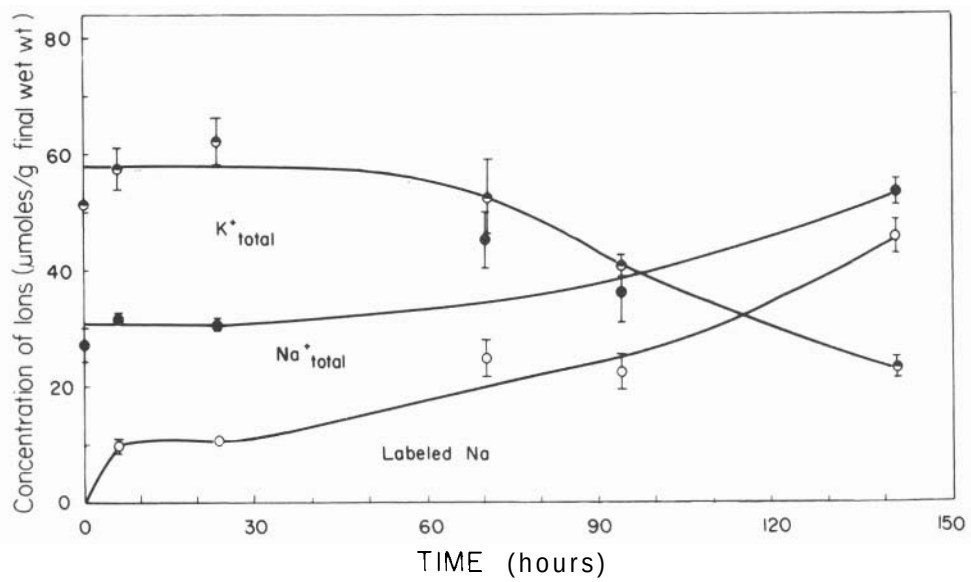


FIGURE 1. Time course of uptake of labeled Na^+, and of change of total K^+ and Na^+, in normal frog eggs (25°C). Each point is the average of four determinations. In this and in all following graphs the distance between the two horizontal bars represents twice the standard error. The experiment was conducted in late fall.

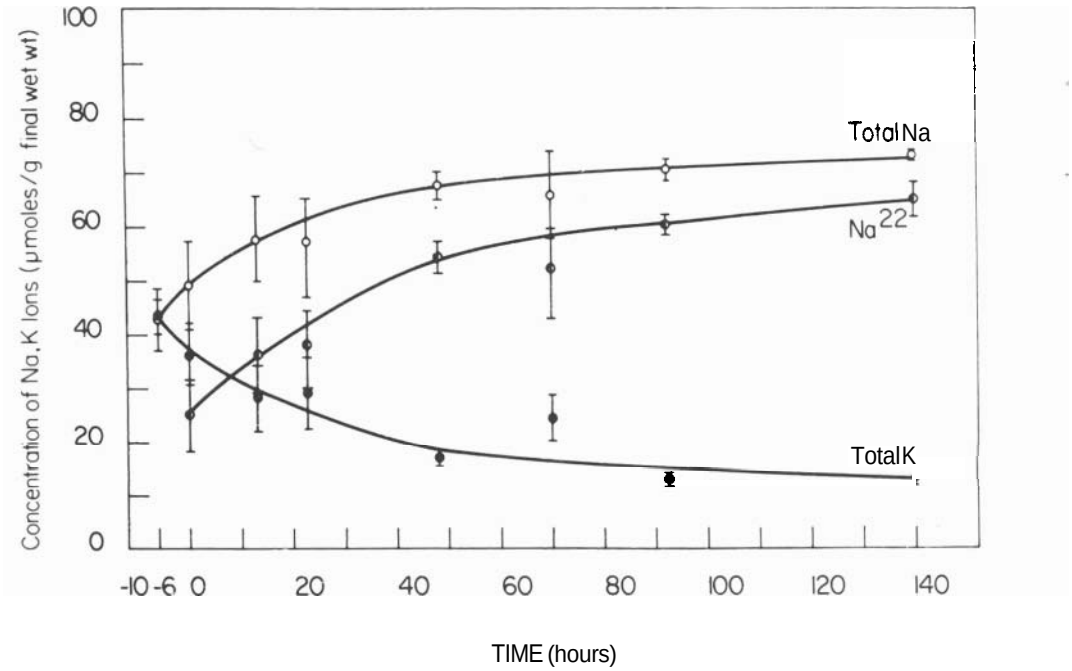


FIGURE 2. Time course of uptake of labeled Na^+, and of change of total K and Na^+, in late-season frogs (25°C). Each point in this graph and the following graph (Fig. 3) is the average of four determinations. The experiment was conducted in late spring. Note the much lower initial concentration of K' and higher concentration of Na^+ in this set of experiments. Incubation of "Na-labeled Ringer's solution began at minus 6 h.

reached a level of 45 μmoles/g fresh weight after **140** h of incubation. During all that time, the total Na+ in the cell was higher than the labeled Na+ by a substantial factor, in agreement with the. observations of Abelson and Duryee.[1] But their reported period of observation lasted only 4 h; in that period the level of labeled Na+ in the cell reached **12%** of the total Na^+. During our much longer period of observation, the exchangeable fraction reached about 33% of total cell Na^+ after **24** h of incubation, rising to about 80% by the end of **140** h of incubation. It should be pointed out that the gain of total Na+ at the end of the experiment was largely due to a gain of the exchangeable fraction of Na^+. The nonexchangeable fractions decreased very slowly.

Figure 2 shows a similar experiment in which mature eggs in late spring were used. The initial Na+ is much higher in these eggs than in the eggs of Fig. **1,** and the initial K^+ is much lower. Nevertheless, the gain of exchangeable Na^+ and the maintenance of a nonexchangeable fraction of Na+ are quite parallel for the two experiments.

Inclusion of 0.1 mM **IAA** was found to accelerate the increase of cell Na^+ as shown in Fig. **3.** As in the .case of the unpoisoned cells, the gradual gain of Na+ accompanying the loss of K^+ was also primarily a gain in exchangeable Na^+.

Simultaneous Effluxes from Normal Eggs

Figure 4 shows four sets of simultaneous effluxes of labeled Na+ and labeled D-arabinose from four different and normal ovarian eggs. Efflux curves of treated extracellular tissue were also constructed and are shown in Fig. 5. For Fig. 4 as well as for subsequent groups of the efflux curves of frog eggs, the lines were obtained from the best-fitting curves after correction for contribution by extracellular tissue.

Given in Figs. 4A to 4D, the semilogarithmic plots of D-arabinose efflux curves in all cases were essentially straight lines. In contrast, three of the simultaneously measured Na+ efflux curves from the same cells appear quite curved and are resolvable into two exponential fractions with different slopes. The Na+ efflux curve from one egg shown in Fig. 4C, however, appears as a simple straight line similar to the D-arabinose efflux curves.

As indicated in Table I, the eggs of Fig. 4 were incubated in labeled Ringer's solution at 25°C for approximately 20 h. All the labeled Na+ observed thus belongs to the exchangeable fraction shown in Fig. 1. The data also demonstrate the existence of two (or more) fractions of Na^+ within the exchangeable fractions. The average half-time exchange ($t_{1/2}$) of the slow-exchanging fraction is around 1,000 min or about 17 h (Table I). At that rate of exchange, nearly all of the fraction (> 99%) should have become labeled with ^{22}Na after 140 h of incubation. Yet Fig. 1 quite

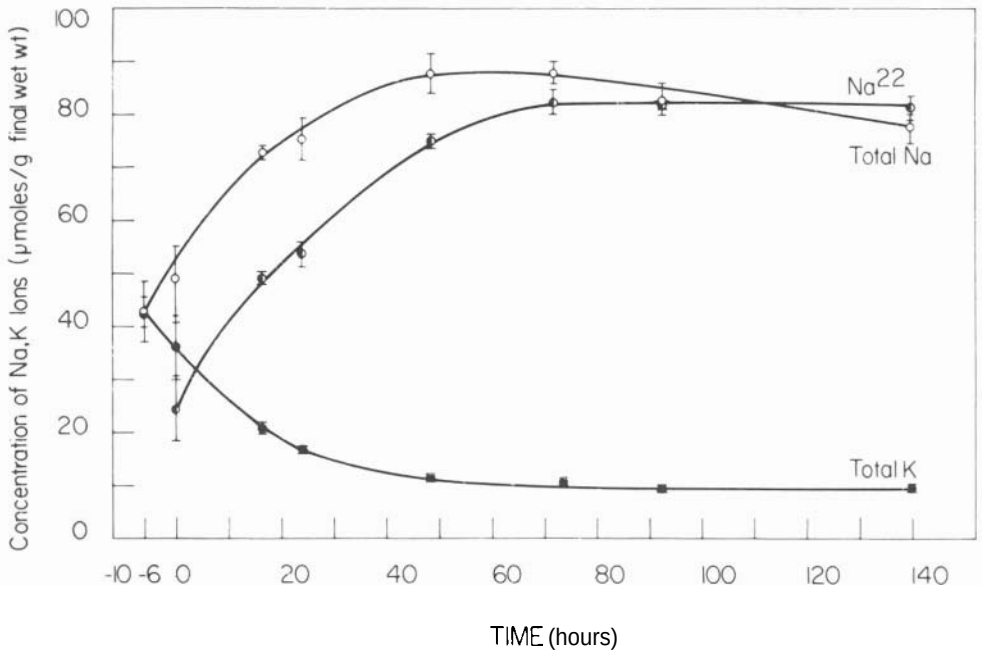
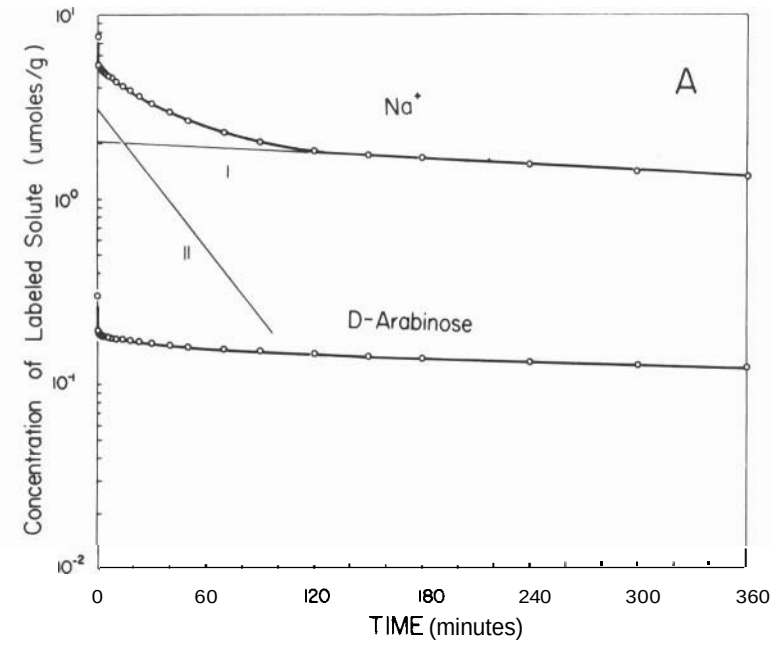


FIGURE 3. Time course of uptake of labeled Na^+, and of change in concentrations of K^+ and Na^+ in IAA-poisoned eggs. Eggs were preincubated for 6 h in ^{22}Na-labeled Ringer's solution before introduction of iodoacetic acid (IAA). Water content was 58.6% at 96 h and 60.5% at 140 h.



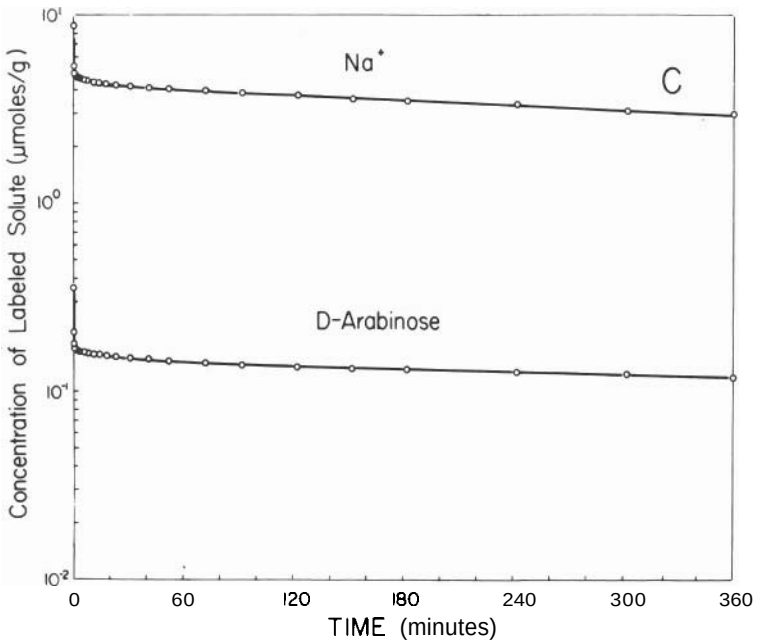
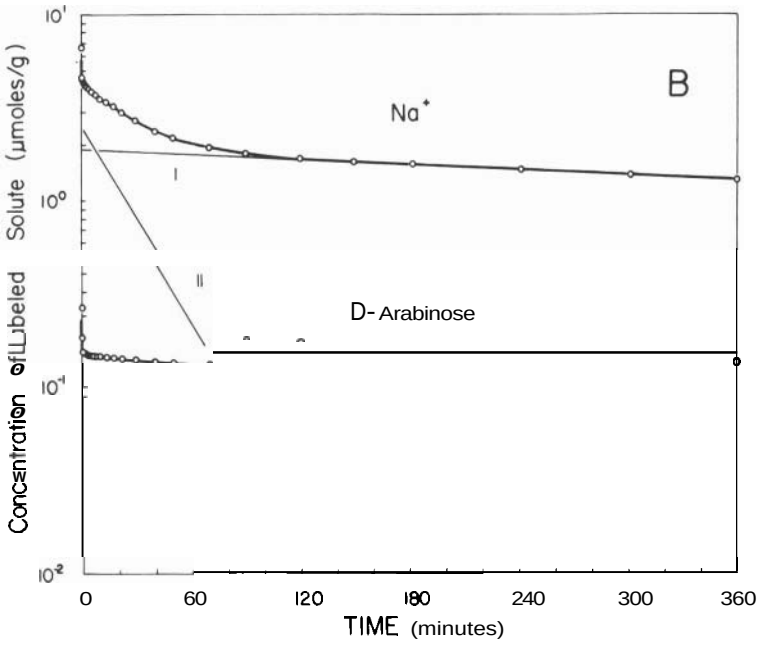
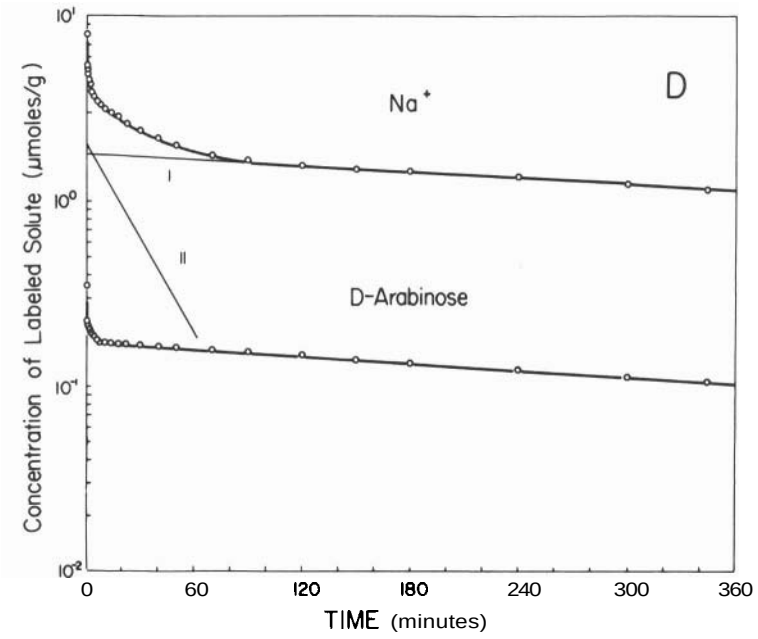


FIGURE 4. Time course of simultaneous efflux of labeled Na^+-ion and labeled D-arabinose from single egg. All eggs were incubated at 25°C in modified Ringer's solution [10] containing 100 mM labeled Na^+-ion and 5 mM labeled D-arabinose. Eggs were washed at 25°C in a Ringer-phosphate solu-





tion containing Na^+ and D-arabinose at the same concentrations as in the incubation media. Line represents values after correction for ovarian tissue contribution. Other pertinent data can be obtained from Table I: (A) 3–J–24–B; (B) 3–J–24–E; (C) 3–J–24–C; (D) 3–J–24–A.

TABLE I (a). Exchangeable Fractions of Na^+ and D-Arabinose and Their Efflux Rates [a]

Expt. no.	Duration of incubation (h)	IAA concentration (mM)	Final wt. (mg)	% H_2O	$[Na]_{ex}$ (final)	$[D\text{-Arabinose}]_{ex}$ (final)
3J24A	19.0	0	2.49	51.3	100	4.62
3J24B	19.0	0	2.55	51.3	100	4.62
3J24C	19.0	0	2.71	52.3	100	4.62
3J24E	19.0	0	2.47	51.3	100	6.62
3J24J	41.0	0.5	3.20	57.4	100	4.64
3J24K	41.5	0.5	3.20	57.4	100	4.64
3J24N	42.0	0.5	2.99	57.4	100	4.64
4K25B	18.3	1.0	3.69	60.5 [b]	100	4.96
4K25C	18.5	1.0	4.23	60.5 [b]	100	4.96
4K25E	19.0	1.0	3.91	60.5 [b]	100	4.96
4L10A	18.0	1.0	3.99	62.5	100	4.78
4L10B	18.3	1.0	3.53	62.5	100	4.78
4L10C	18.5	1.0	3.89	62.5	100	4.78
4L10D	18.7	1.0	3.57	62.5	100	4.78

clearly shows that such was not the case. The incomplete exchange beyond 140 h of incubation showed that more than one slow exchanging fraction of Na+ exists in normal eggs.

Having established that the Na+ in all egg cells is not exchanging at an equal rate we see that nevertheless the data of Fig. 4C indicate that the efflux rate for Na+ is described by a single rate constant. Our tentative explanation is that in this case, low Na+ permeability of the cell surface camouflages the fast- and slow-exchanging fractions of intracellular Na^+.

Effect of IAA on the Sintultaneous Effluxes

Figure 6 shows that when frog eggs were exposed to the metabolic poison, iodoacetate, the Na+ and D-arabinose efflux curves both assumed the two- (or multiple-) component profiles. Indeed, in Fig. 7 we appear to have caught in action the transition from the single. logarithmic surface-limited exchange for both Na^+ and D-arabinose, as illustrated in Fig. 4C, to the two-component effluxes seen in Figures 4A, 4B, and 4D.

Figures 6A, 6B, 6C, and 6D are examples of simultaneous efflux curves of Na+ and D-arabinose in eggs at different states of deterioration as the cells were succumbing to the poisoning effect of IAA and the levels of cell Na+ and cell D-arabinose were gradually and steadily rising until they reached and exceeded those in the surrounding medium.

TABLE I, *continued* (b). Labeled Na Concentration (μmoles/g Wet Weight)

Expt. no.	Total	Fraction I (slow) Intercept	Fraction I (slow) $t_{1/2}$ (min)	Fraction IA (intermediate) Intercept	Fraction IA (intermediate) $t_{1/2}$ (min)	Fraction II (fast) Amount	Fraction II (fast) $t_{1/2}$ (min)	q_{Na}
3J24A	4.63	1.900	660			2.70	19.0	0.053
3J24B	5.45	2.050	560			3.40	24.5	0.066
3J24C	5.70	4.220	658			1.50	11.5	0.029
3J24E	5.35	1.800	550			3.55	18.0	0.069
3J24J	41.30	9.780	317	1.81	16.0	29.70	2.5	0.519
3J24K	37.00	4.490	302			32.50	3.5	0.565
3J24N	40.50	7.350	295	1.98	37.5	33.1	2.0	0.540
4K25B	67.00	13.700	626			53.3	4.5	0.880
4K25C	37.00	3.150	528			33.8	10.0	0.560
4K25E	3.05	0.584	986			2.47	14.0	0.040
4L10A	79.00	0.033	750			79.0	3.0	1.260
4L10B	78.00	14.20	323	2.10	34.5	42.8	6.5	0.690
4L10C	61.20	6.350	155	3.82	34.5	51.0	4.0	0.820
4L10D	63.00	11.20	225	2.17	25.0	50.0	2.0	0.800

TABLE I, ***continued*** (c). Labeled **D-Arabinose** Concentration (μmoles/g Wet Weight)

Expt. no.	Total	Fraction I (slow) Intercept	Fraction I (slow) $t_{1/2}$ (min)	Fraction IA (intermediate) Intercept	Fraction IA (intermediate) $t_{1/2}$ (min)	Fraction II (fast) Amount	Fraction II (fast) $t_{1/2}$ (min)	q_{arab}
3J24A	0.286	0.160	1360				66.0	
3J24B	0.206	0.149	950				42.5	
3J24C	0.240	0.145	1130				23.5	
3J24E	0.238	0.172	465				1.0	
3J24J	1.870	0.365	139	0.153	31.0	1.36	3.0	0.51
3J24K	0.710	0.144	132			0.57	4.5	0.22
3J24N	1.380	0.265	138	0.139	33.0		3.0	0.52
4K25B	3.140	0.632	187			2.51	11.0	0.84
4K25C	0.960	0.167	168			0.79	25.0	0.26
4K25E	0.187	0.0522	156			0.06	26.0	0.02
4L10A	2.850	0.0406	262			2.85	7.0	0.96
4L10B	2.140	0.517	211	0.414	35.5	1.48	7.0	0.49
4L10C	2.850	0.444	156	0.251	26.0	2.16	6.5	0.72
4L10D	2.770	0.570	187	0.40	10.0	1.80	1.0	0.60

Initial [D-arabinose] = 5mM. Initial [Na] = 100 mM. "Solute concentration values include correction for connective tissues and are expressed as μM/g final wet weight. [b] These values are averages from the other determinations.

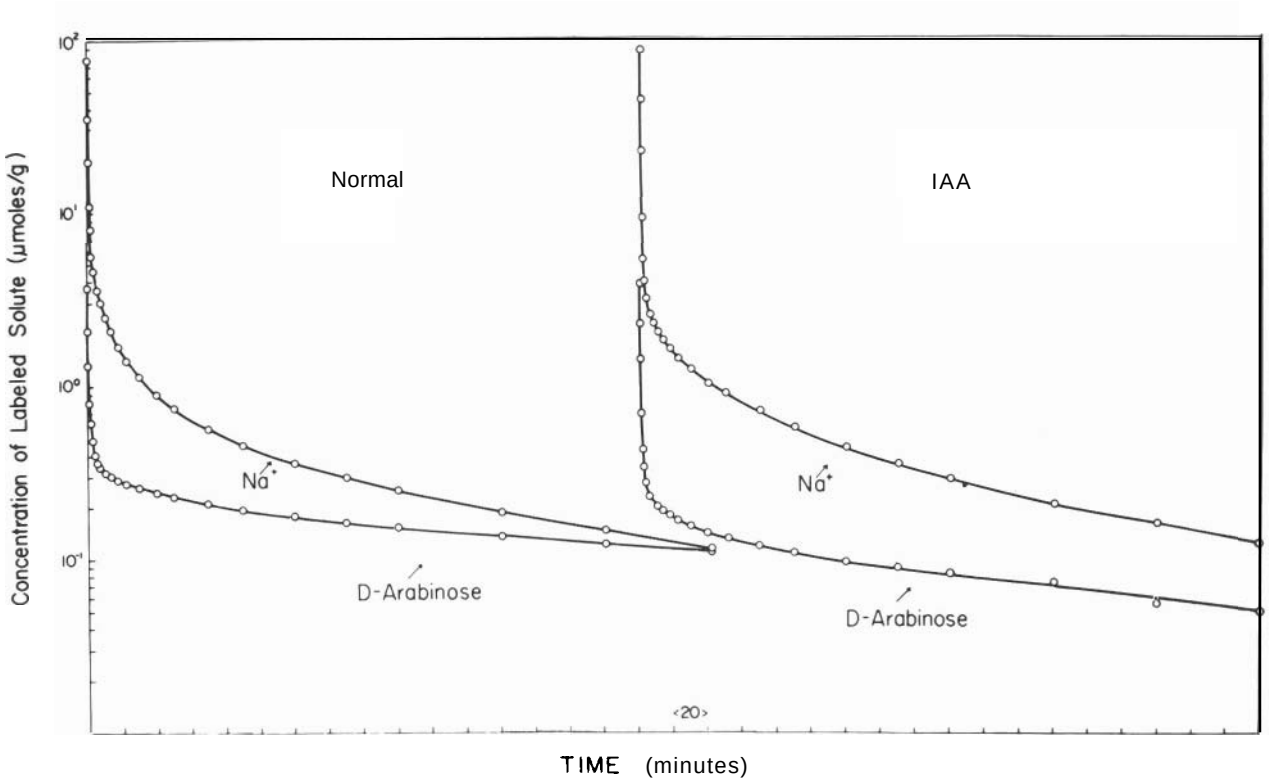


FIGURE 5. Time course of simultaneous efflux of labeled Na^+-ion and labeled D-arabinose from ovarian tissue. Tissue was obtained from the helium of the ovary and from the ovarian lobe after removal of all eggs. Tissues were incubated in labeled solutions and washed in exactly the same manner as were the eggs in Fig. 4. Points represent average of 6 efflux curves.

Resolution of Fractions from their Efflux Curves

Fractions of labeled Na+ and D-arabinose in the cell were resolved by successively peeling off the slowest exponential fraction, then the next slowest, etc. Thus yielded for the majority of cases were two fractions: labeled fraction I (slow) and fraction II (fast). In a few cases a third fraction, intermediate in time constant between the slow and fast fractions, was observed. We shall refer to this intermediate fraction as fraction IA.

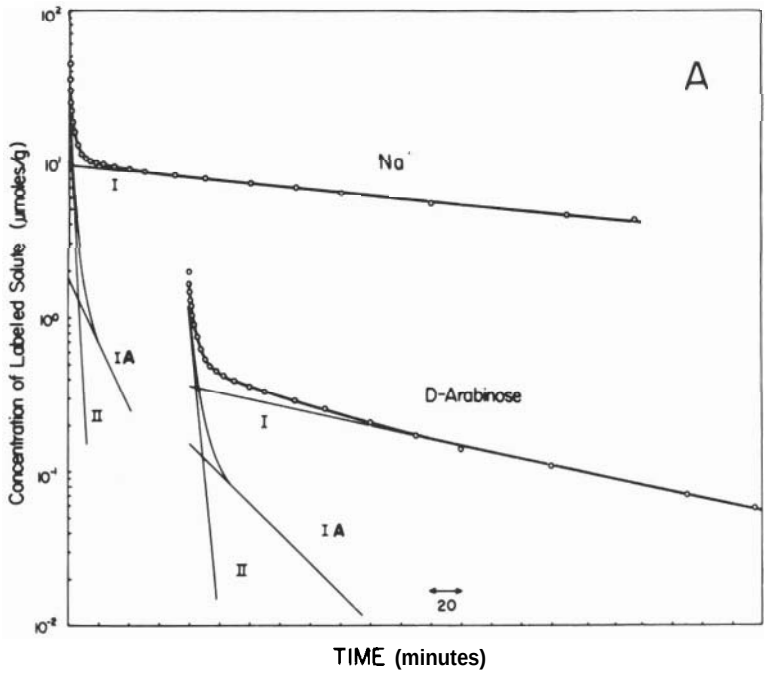
It is worth noting that in all IAA-treated eggs studied, if fraction IA occurred in the Na+ efflux curve, then the D-arabinose efflux curve from the same egg showed a similar intermediate fraction. Conversely, if the Na+ efflux showed no fraction IA, then the D-arabinose curve did not show a similar intermediate fraction. This correspondence suggests a common origin for the respective intermediate fractions of Na+ and D-arabinose.

Table I gives the data of experiments carried out over a 14-month period. The method used to determine the molar concentrations of Na+ belonging to the different fractions was as follows. In the cases of fractions I and IA, molar concentration could be estimated by simple extrapolation and by reading the intercept of the extrapolated line on the ordinate corresponding to zero time. But the magnitude of fraction II made a different procedure necessary. To obtain the total labeled Na^+ concentration of fraction II, if fraction IA did not exist, the total labeled Na^+ concentration of fraction I was subtracted from the total labeled Na+ concentration. In cases where IA did exist, the sum of fractions I and IA was subtracted from the total labeled Na^+. The major change in the Na+ efflux profiles in the dying muscle cells was observed to be a steady increase of the magnitude of fraction II for both Na+ and D-arabinose. The magnitude of fraction I was fairly constant before its final decline to low values in thoroughly dead cells.

DISCUSSION

Significance of Multiple Fraciions in Na+ Efflux

The first question to be settled is whether or not the observed fast fraction is an artifact — arising either from injury to the cell surface or entrapment of labeled Na+ in the complex cell surface invaginations.[22] For the following reasons, we believe that neither is the case. If the fast fraction of Na+ originates from either injury or entrapment, a similar fast fraction should exist for both labeled Na+ and labeled D-arabinose in the same egg. Figures 4A, 4B, and 4D show just the opposite: a fast fraction of labeled Na+ coexists with an absence of a similar fast fraction for D-arabinose. We therefore conclude that the two fractions of Na+ efflux reflect the properties of normal intact cells and that both fast and slow fractions originate within the cells. The basically linear efflux curves in the semilogarithmic plots for Na^+ (Fig. 4C) and D-arabinose (Figs. 4A-4D) also quite clearly show that the



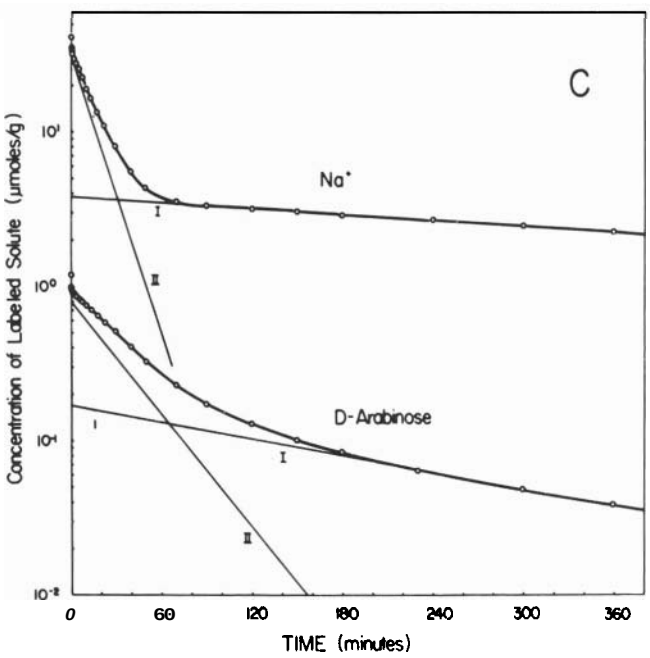
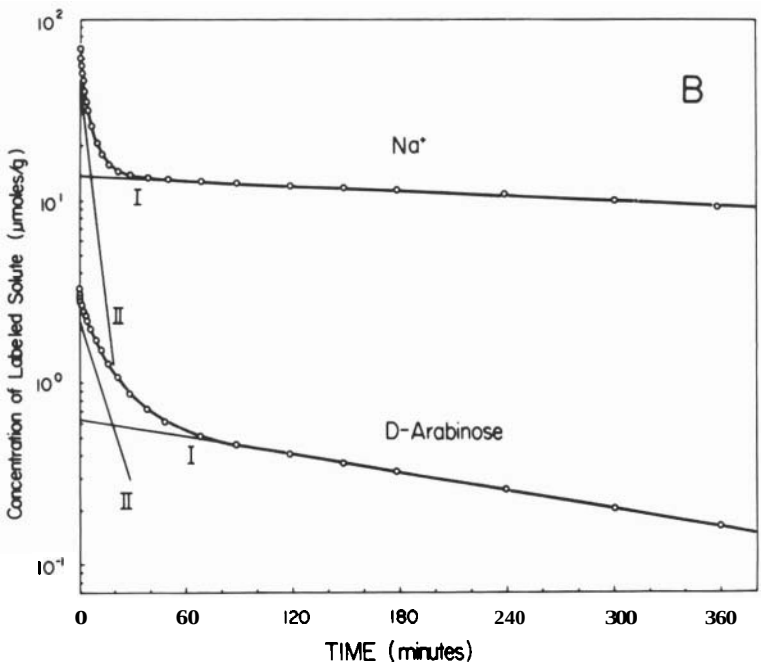
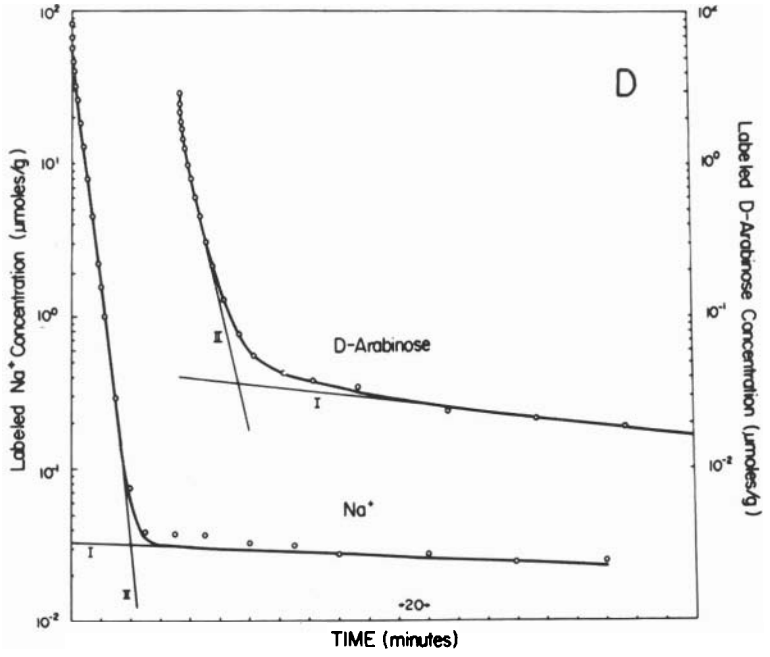


FIGURE 6. Time course of simultaneous efflux of labeld Na^+-ion and labeled D-arabinose from single IAA-poisoned eggs. Experimental procedure similar to that followed for Fig. 4





but with incubation solution containing iodoacetic acid. See Table I for other pertinent data: (A) 3–J–24–J; (B) 4–K–25–B; (C) 4–K–25–C; (D) 4-L–10–A.

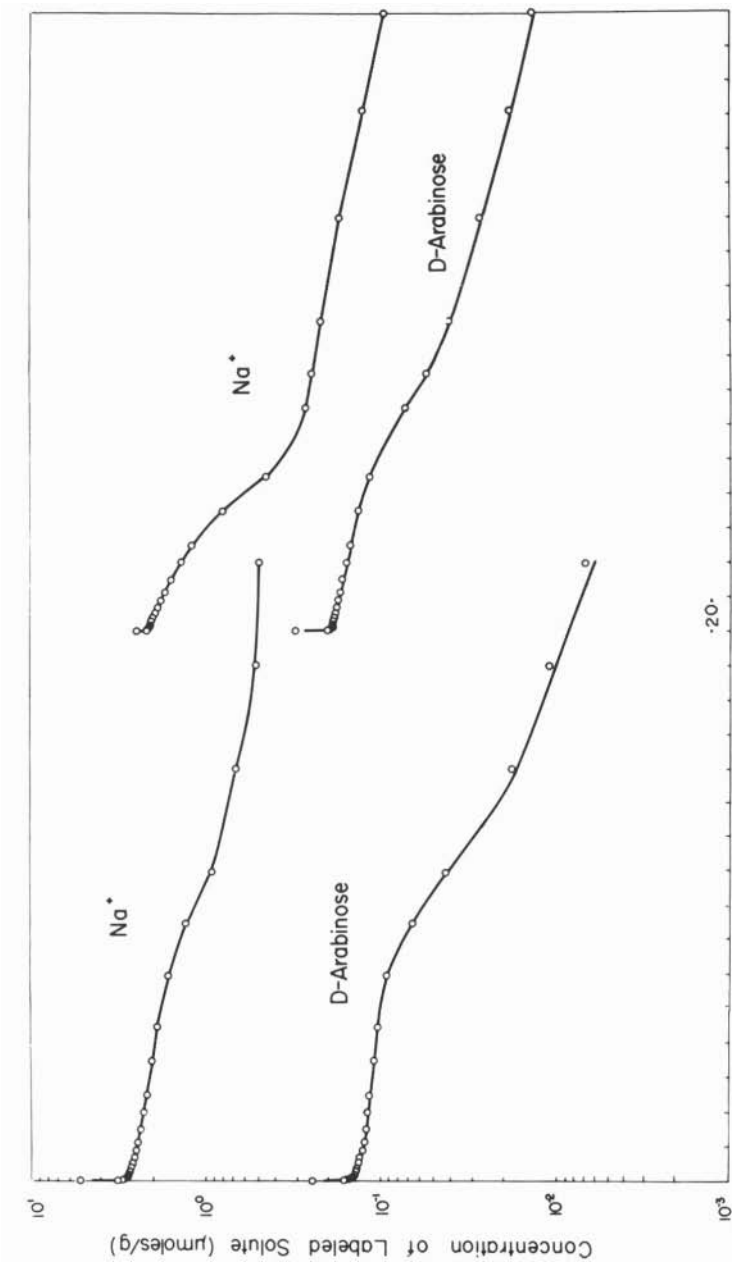


FIGURE 7. Time course of simultaneous efflux of labeled Na^+-ion and labeled D-arabinose from single IAA-poisoned eggs. Experimental procedure same as that followed for Fig. 4. Curves on left were obtained from a single egg incubated 18 h in a solution containing 1.0 mM iodoacetic acid. Wet weight was 3.86 mg. Concentration of labeled Na^+-ion was 3.90 μmoles/g wet weight. Curves on right were obtained from single egg incubated 41.5 h in a solution containing 0.5 mM iodoacetic acid. Wet weight was 3.42 mg. Concentration of labeled Na^+-ion was 21.7 μmoles/g wet weight.

surface invaginations are too shallow in depth and too small in total volume to produce any significant change as compared with the model of an egg cell with a smooth surface. This finding reaffirms similar conclusions based on autoradiographic studies.[23]

Significance of the Slow Fraction in Na^+ Efflux

A hitherto unresolved issue in Na^+ efflux studies of amphibian ovarian eggs is the nature of the rate-limiting mechanism for the slow fraction. Two opposed proposals have captured attention: (1) desorption from sites on proteins or other macromolecules, and (2) permeation through the membranes of subcellular particles. Dick and Fry[24] set out to test these alternatives. Their conclusion contradicted the macromolecular adsorption interpretation, because they found that when placed in cellophane bags and dialyzed, squashed egg cells soon lost all their Na^+. But the same experimental result contradicts equally well the subcellular compartmentalization interpretation.

Dick and Fry also showed that the bulk of the non-exchangeable Na^+ is in the cytoplasm rather than in the nucleus, a conclusion later confirmed by Century and Horowitz.[25] These findings ruled out one subcellular particle, the nucleus, as the seat of sequestration of the "non-exchangeable" Na^+.

It should be noted that Dick and Fry[24] rejected the molecular adsorption interpretation on the basis of the assumption that marcromolecules in the cytoplasm exist in permanently stable states. However, there is no evidence for that assumption. We suggest that the adsorption of Na^+ on cytoplasmic macromolecules is not permanently stable. Rather, it is metastable and therefore sensitive to the inevitable disturbances during the destruction of the cells by squashing. The following factors support this desorption interpretation:

(A) Energy needs. In terms of the membrane-pump theory, the sequestration of Na^+ in subcellular particles implies installation of more energy-consuming pumps in their containing membrane—hardly a reasonable proposition since a single postulated Na^+ pump would consume 15 to 30 times more energy than the cell commands, and many other pumps have been postulated in addition to Na^+ pumps.[3] Recent attempts to suggest a reduction in the energy requirement of the Na^+ pump by relocating the bulk of cell Na^+ to within the sarcoplasmic reticulum have been experimentally shown to have no validity.[26]

(B) Osmotic demands. Figure 1 shows that in normal eggs the total concentration of K^+ is 60 μmoles/g of wet weight, and that of Na^+ is 30 μmoles/g of wet weight, their sum being 90 μmoles/g of wet weight. Table I shows that the water content of normal eggs is about 51%. Thus if both K^+ and Na^+ exist as free salt ions, the cytoplasmic salt content would be $90/0.51 = 176$ mM, milliosmolar.

Table II shows that normal surviving eggs contain 93 mM chloride. Even if other intracellular solutes are completely discounted (e.g., HCO_3^-, phosphates, and free amino acids) the total concentration of K^+, Na^+, and Cl^- alone would amount

to 269 milliosmolar, which is hypertonic by 269 − (2 × 118) = 33 milliosmolar. This would be an unstable situation, and water would move rapidly into the cell to lower the total free salt concentration in the cell. Since in fact no such water movement occurs, the cell content could not have been hypertonic.

Figure 3 shows that in response to IAA the egg cells soon lost nearly all K^+ and gained a roughly equimolar quantity of "exchangeable" Na^+. At 50 h, for example, total Na+ was 90 mmoles/kg of fresh weight and total K^+ was 10 mmoles/kg. At that point the water content was about 60%, the chloride content about 90 mM. Thus the total intracellular osmolarity of K+, Na^+, and Cl^- alone in this poisoned egg was, once more, 90 + (100/0.6) = 251 milliosmoles/1, again exceeding the osmolarity of Ringer's solution—even with other intracellular solutes completely disregarded.

The striking excess of osmotic activity in both surviving and dying eggs offers strong evidence that a substantial portion of cell Na+ is in an adsorbed state. This adsorbed Na+ is slow in exchanging because it is held tightly. It should be pointed out that the adsorption could be on proteins or other macromolecules in the cytoplasm, or within non-nuclear subcellular particles.

Significance of the Fast Fraction in Na+ Efflux

Since the work of Abelson and Duryee, there has been general agreement that the fast fraction of Na+ in amphibian eggs is largely found in the cytoplasm as free Na^+.[25] The main difference of opinion concerns the mechanism that maintains the fraction at the lower level observed. According to the membrane-pump theory, this mechanism is pumps; according to the association-induction hypothesis, it is exclusion from water existing in a state of polarized multilayers.

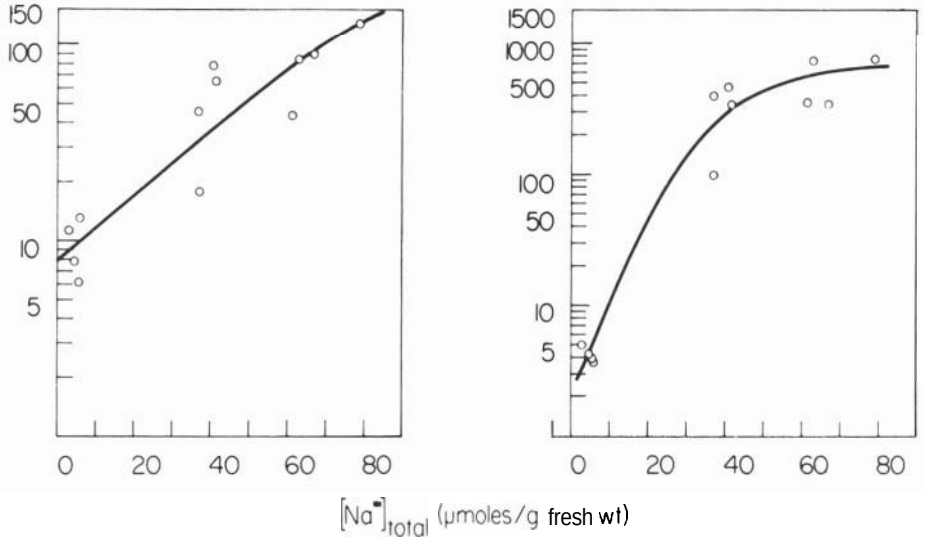


FIGURE 8. Relation between labeled Na^+ content of single egg and outward flux rate, F_{Na}^{out}, in mole/cm² sec; and permeability, κ, in cm/sec. Calculated from Eq. 1 as described in text. See Table II for other relative data.

TABLE II. Water and Chloride Contents of Surviving Isolated Eggs (Control) and IAA (1 mM) Poisoned Eggs

	Time of incubation (h)	H_2O (%)	Cl^- (μM/g cell H_2O)
Control	0	49.6	93.3 ± 0.93 (n = 8)
IAA	23	53.5	88.6 ± 0.27 (n = 4)
	93	60.9	101.7 ± 4.32 (n = 4)
	165	59.3	104.8 ± 3.36 (n = 4)

TABLE III. The Sodium Ion Flux and Permeability Constants of Poisoned Frog Eggs

Experiment Number	Total Na^+ (μmoles/g)	Weight (mg)	A/V_{av} $(cm)^{-1}$	$(t_{1/2})_I$ (min)	$(t_{1/2})_{II}$ (rnin)	κ_{out} (cm/sec)	$[Na^+]_{in}$ (mM)	F_{Na}^{out} (moles/cm² sec)
4 K 25 E	3.05	3.91	67.3	986	14.0	12.3×10^{-6}	4.08	5.00×10^{-8}
3 J 24 A	4.63	2.49	78.0	660	19.0	7.79×10^{-6}	5.26	4.10×10^{-8}
3 J 24 B	5.45	2.55	77.9	560	24.5	6.05×10^{-6}	6.23	3.72×10^{-8}
3 J 24 C	5.70	2.71	76.1	658	11.5	13.10×10^{-6}	2.93	3.86×10^{-8}
4 K 25 C	37.00	4.23	65.5	528	10.0	17.6×10^{-6}	56.00	98.70×10^{-8}
3 J 24 K	37.00	3.20	71.9	302	3.5	45.9×10^{-6}	56.60	397×10^{-8}
3 J 24 N	40.50	2.99	73.5	295	2.0	78.6×10^{-6}	57.40	451×10^{-8}
3 J 24 J	41.30	3.20	71.9	317	2.5	64.3×10^{-6}	51.70	332×10^{-8}
4 L 10 C	61.20	3.89	67.4	155	4.0	42.8×10^{-6}	81.60	349×10^{-8}
4 L 10 D	63.00	3.57	69.3	225	2.0	83.3×10^{-6}	80.00	721×10^{-8}
4 K 25 B	67.00	3.69	68.3	626	4.5	37.5×10^{-6}	88.00	325×10^{-8}
4 L 10 A	79.00	3.99	67.0	750	3.0	57.4×10^{-6}	126.00	724×10^{-8}

The membrane-pump approach envisages the interference with outward pumping of Na^+ as the main event in the faltering Na^+ exclusion of poisoned cells. The central phenomenon predicted for the Na^+ efflux of the poisoned and dying egg, therefore, is a slowing down of the outward Na^+ flux, revealed as a flattening of the slope of the semilogarithmic plot of remaining labeled Na^+ against time. This slowdown of Na^+ efflux rate is postulated as the cause of the rising level of Na^+ in the cell.

In the association-induction hypothesis, the normally low levels of excluded solutes are manifestations of the equilibrium properties of the bulk of cell water, which has no direct causal relation to the rate at which Na^+ crosses the cell surface barrier. According to this view, the main feature predicted for the Na^+ efflux from a poisoned and dying cell is a steadily increasing level of Na^+ in the cell water as revealed by a rising level of the fast fraction of Na^+ *pari passu* with a rising level of any other free solutes normally excluded from the cell water. These rising levels of the fast fraction are obtained from the fast fraction of the efflux curves by extrapolation to zero time.

Rate of Na^+ Efflux in Normal, Dying, and Dead Frog Eggs

If C_i is the concentration of a labeled solute (e.g., Na^+ or D-arabinose) in the cell and A and V are the total surface area and volume of the cell, respectively, the outward flux rate (F_i^{out}) in moles per unit surface area is

$$F_i^{out} = (V/A)(dC_i/dt) = \kappa_i^{out} C_i \qquad (1)$$

where κ_i^{out} is the permeability constant of the ith solute in units of centimeters per sec. In Table III, F_{Na}^{out} and other relevant parameters for the observed normal,

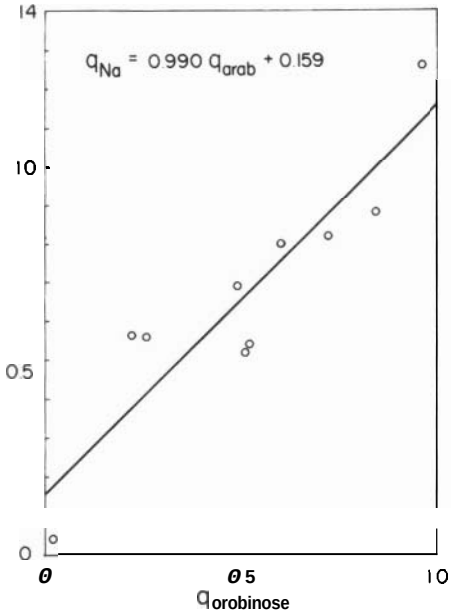


FIGURE 9. Correlation of the q-value of labeled Na^+ ion and the q-value of labeled D-arabinose in IAA-poisoned egg. The q-value was derived from the intercept of the fast-exchanging fraction (II) of the efflux curve in the course of deterioration of the egg in IAA.

dying, and dead cells are collected. In Fig. 8, κ_{Na}^{out} is plotted $[Na^+]_{total}$ in the left-hand figure and a similar plot of F_i^{out} against $[Na^+]_{total}$ is presented in the right-hand figure. Roughly speaking, an increase of total labeled Na+ in the egg cell can be used as a gauge of the state of deterioration brought on by the poison. Yet even as the total labeled Na+ was increasing steadily, there was no indication whatsoever of a slow-down of the outward Na+ flux or a decrease of κ_i^{out}. Quite the contrary, there was a steady gain of both.

The above finding falsifies the prediction of membrane-pump theory. On the other hand, the steady gain of both κ_i^{out} and F_i^{out} as a result of the poisoning is compatible with the association-induction hypothesis. (A recent experimental study of poisoned frog sartorius muscles showed a similar lack of slow-down in the Na+ efflux rate while the cells were rapidly gaining Na+ after poisoning.[12])

Correlation between Fast Exchanging Fractions

As mentioned earlier in connection with the universality rule, the association-induction hypothesis (but definitely not the membrane-pump theory) predicts the existence of a positive correlation between the levels of any pair of free, non-metabolized solutes normally excluded from the cell water. The reason is that their exclusion is governed by a common factor—the state of water in the cell. More precisely, the hypothesis predicts that the equilibrium distribution ratio, or q-value, of th ith free solute in the cell water divided by the concentration of the solute in the external solution should correlate with the q-value of any other normally excluded solute in the same cell at the same instant. Thus, if the hypothesis is basically correct and if, in addition, the intercept of the fast fraction II does indeed represent the concentration of the labeled solute in the cell water, as we have consistently contended,[3,9,26,27] a positive correlation should be found between the q-value derived from the intercept of fraction II of the Na+ efflux curve and the q-value derived from the intercept of the fraction II of the D-arabinose efflux curve in the same eggs in the course of their deterioration in IAA.

Figure 9 shows that such a positive correlation does indeed exist. The correlation coefficient is +0.91.

The present work was supported by NIH grants 2-ROl-CA16301-03 and 2-RO1-GM11422-13, and Office of Naval Research Contract Number NOOO14-71-C-0178. The John H. Hartford Foundation provided many of our basic facilities.

REFERENCES

1. **P. H. Abelson and W. R. Duryee, "Radioactive sodium permeability and exchange in frog eggs," *Biol. Bull.*, 96,205 (1949).**
2. **G. N. Ling and A. Schmolinske, "Further evidence for the fixed-charge hypothesis," *Fed. Proc., 15,* 120 (1956).**
3. ***G.* N. Ling, A *Physical Theory of the Living State: The Association-Induction Hypothesis,* Blaisdell Publishing Co., Waltham, Massachusetts, 1962.**

4. D. A. T. Dick and E. J. A. Lea, "Na fluxes in single toad oocytes with special reference to the effect of external and internal Na concentration on Na efflux," *J. Physiol.*, 174, 55 (1964).
5. S. B. Horowitz and I. R. Fenichel, "Analysis of sodium transport in the amphibian oocyte by extractive and radiographic techniques," J. *Cell Biol.*, 42, 120 (1970).
6. D. A. T. Dick and E. J. A. Lea, "The partition of sodium fluxes in isolated toad oocytes," *J. Physiol.*, 191,289 (1965).
7. G. N. Ling, "The physical state of water in living cell and model systems," *Ann.* NY *Acad. Sci.*, 125, 401 (1965).
8. ———, "Hydration of macromolecules," in *Water and Aqueous Solutions, R. A.* Horne, Ed., Wiley-Interscience, New York, 1972, pp. 663–700.
9. G. N. Ling, C. Miller and M. M. Ochsenfeld, "The physical state of solutes and water in living cells according to the association-induction hypothesis," *Ann.* NY *Acad. Sci.*, 204, 6 (1973).
10. G. N. Ling, "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 Physical State of Aqueous Cytoplasm, E.* Pollard, Ed., Marcel Dekker, in press.
11. G. N. Ling and C. L. Walton, "What retains water in living cells," *Science,* 191, 293 (1976).
12. G. N. Ling and M. M. Ochsenfeld, "Na^+ and K^+ in living cells: Do they depend on the rate of outward transport of Na^+?", *Physiol. Chem. Phys.*, *8*, 389 (1976).
13. I. M. Glynn and S. J. D. Karlish, "The sodium pump," *Annu. Rev. Physiol.*, 37, 13 (1975).
14. I. M. Glynn, "Membrane adenosine triphosphate and cation transport," *Br. Med. Bull.*, 24, 165 (1968).
15. S. L. Bonting, in *Membranes and Ion Transport,* Vol. 1, E. E. Bittar, Ed., Wiley-Interscience, New York, 1970, p. 257.
16. C. Miller, "Nonelectrolyte distribution in mouse diaphragm muscle. I. Pattern of nonelectrolyte distribution and reversal of the insulin effect," *Biochim. Biophys. Acta,* 339, 71 (1974).
17. W. Pigman, *The Carbohydrate,* Academic Press, New York, 1957.
18. M. Rangier, P. M. de Traverse and M. Bonville, "Utilisation des temtoses par l'organisme animal," *Bull. Soc. Chim. Biol.*, 30, 583 (1948).
19. G. N. Ling and G. Bohr, "Studies on ionic distribution in living cells. I. Long-term preservation of isolated frog muscles." *Physiol. Chem. Phys.*, 1,591 (1969).
20. G. N. Ling, "Studies of ion permeability. III. Diffusion of Br^- ion in the extracellular space of frog muscles," *ibid.*, 4, 199 (1972).
21. M. C. Neville, unpublished.
22. W. H. Massover, "Complex surface invaginations in frog oocytes," J. *Cell Biol., 58,* 485 (1973).
23. D. A. T. Dick and E. J. A. Lea, "Autoradiographic measurement of 22-sodium in the living oocyte," *J. Physiol.*, 180, 178 (1965).
24. D. A. Dick and D. J. Fry, "Location of ion exchangeable sodium in the nucleus and cytoplasm of oocytes of Bufo exposed to sodium-free solutes," *ibid.*, 231, 19 (1973).
25. T. C. Century and S. B. Horowitz, "Sodium exchange in the cytoplasm and nucleus of amphibian oocytes," *J. Cell Sci., 16,* 465 (1974).
26. G. N. Ling and C. L. Walton, "Simultaneous efflux of K^+ and Na^+ from frog sartorius muscle freed of extracellular fluids: evidence for rapidly exchanging Na^+ from the cells," *Physiol. Chem. Phys., 7,* 501 (1975).
27. G. N. Ling, "Studies on ion permeabiiity. I. What determines the rate of Na^+ ion efflux from frog muscle cells?" *ibid.*, 2, 242 (1970).

(Received July 27,1977)