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UNDERESTIMATION OF Na PERMEABILITY IN MUSCLE CELLS: IMPLICATIONS FOR THE THEORY OF CELL POTENTIAL AND FOR ENERGY REQUIREMENT OF THE Na PUMP

GILBERT N. **LING**
Department of Molecular Biology, Pennsylania Hospital, 8th and **Spruce** Streets, Philadelphia, Pennsylvania 19107

• *A new method permits, in a complex efflux curve from living cells, identification of the fraction of labeled solute rate-limited by intracellular-extracellular exchange. Using this method, it was found that in Na^+ efflux from frog voluntary muscle, the slow fraction conventionally described as rate-limited by membrane permeability is wrongly assigned. Instead, a much faster fraction, usually camouflaged by the efflux of labeled Na^+ in the extracellular space, represents the exchange of intra- and extracellular Na^+ rate-limited by cell surface permeation. Quantitatively, the result shows that the Na^+ permeability constant has up to now been underestimated by a factor of 10. Implications of that finding for the theory of the resting potential, and the minimum energy required of resting frog muscles, are discussed.*

INTRODUCTION

Levi and Ussing[1] first studied the labeled Na^+ efflux from frog sartorius muscles and noted that the efflux curve could be resolved into two fractions. They assigned the slower fraction, with a half-time of exchange ($t_{½}$) of about 30 min. to intracellular-extracellular exchange and the faster fraction to the efflux of Na^+ trapped in extracellular space. Most experimental data reported since then have been interpreted on the basis of these assumptions, although some investigators have suggested that part of the fast fraction may not be due to the extracellular space. For example, Carey and Conway[2] proposed a "special Na+ region." One proposed site is the sarcolemma; however, electron microscopic examination shows that the sarcolemma is too thin (0.1 μ) to account for the observed fast-exchanging Na^+.[3] Keynes and Steinhardt,[4] and later Rogus and Zierler,[5,6] postulated that the sarcoplasmic reticulum (SR) is "open" to the outside of the cell and that Na^+ trapped in the SR is the source of the fast-exchanging Na^+. But that explanation is negated by a considerable body of evidence (see DISCUSSION below).

Though concerned about the origin of the fast fraction, none of the cited authors questioned the validity of the equally important assumption made by Levi and Ussing—that the *slow* fraction represents the exchange of intracellular and extracellular Na^+, rate-limited by passage through the surface membrane.

On the other hand, my own studies have led me to suspect (a) that the slow fraction is rate-limited not by surface permeation but by desorption of labeled Na+ from intracellular adsorption sites, and (b) that a part of the fast fraction is what represents intracellular-extracellular exchange.[7-11]

Additional experimental studies are presented here to test the alternative concepts concerning the origins of the slow- and fast-exchanging Na+ from frog muscle cells. These studies utilize a new methodology that permits identification within a multiple-component efflux curve, of the fraction rate-limited by surface permeation.

THEORY

Identification in composite *efflux* curves of the *efflux* fraction rate-limited by surface permeation. If cells are briefly exposed to a labeled solute in a normal Ringer solution, the inward flux rate (F_{iw} in units of micromoles per ml of cell water/min) can be obtained by dividing the number of micromoles of labeled Na+ gained by the cells by the product of the duration of exposure to the labeled Na^+ and the volume of water in the cells. This method can yield correct data only when the intracellular-extracellular exchange of the labeled solute is described by a single rate constant corresponding to that determined by the surface permeability. More often, however, the flux curve is complex, as in the case of Na+ efflux from frog muscle cells. Here a method is needed that can objectively and clearly provide recognition in a complex efflux curve of the fraction rate-limited by surface permeation. It is to fill this need that I present the following technique:

Consider a single cell (or a small group of similar cells) bathed in a Ringer solution. Constant exchange of Na+ occurs between the cell and the external medium. Let us assume that the rate-limiting step in this exchange is indeed the cell surface permeability. In that case the outward flux rate (F_{ow}) in units of μmoles/ml of cell water/min is

$$F_{ow} = d[Na^+]_{in}/dt = (A/V)P_{ow}[Na^+]_{in} \quad (1)$$

where A and V are the surface area and the volume of the cells in cm^2 and cm^3 respectively. P_{ow}, the outward permeability constant for Na^+, is expressed in units of cm/sec. $[Na^+]_{in}$ is the intracellular Na+ concentration in units of μmoles/ml of cell water. One may define

$$k_1 = (A/V)P_{ow} \quad (2)$$

and then write Eq. 1 as

$$F_{ow} = d[Na^+]_{in}/dt = k_1[Na^+]_{in}. \quad (3)$$

Similarly, for the inward flux rate,

$$F_{iw} = d[Na^+]_{ex}/dt = (A/V)P_{iw}[Na^+]_{ex} \quad (4)$$

$$k_2 = (A/P)\,P_{iw} \quad (5)$$

and

$$F_{iw} = d[Na^+]_{ex}/dt = k_2[Na^+]_{ex} \quad (6)$$

where $[Na^+]_{ex}$ is the Na+ concentration in μmoles/ml in the external Ringer solution and P_{iw} is the inward permeability constant, also expressed in units of cm/sec. With these basic relations one can devise a simple procedure yielding two values of F_{iw} from a single efflux study (and also the value of the equilibrium intracellular Na+ concentration). The two F_{iw} values must agree if the particular efflux fraction at issue is indeed limited by surface permeation.

Basically this technique is similar to that used in my previous efflux studies but with certain further requirements. First, the chemical composition of the Ringer solution, the temperature, and the other conditions in the isotope-loading step must be identical with those in the washing step. Second, the cells must be in equilibrium with this Ringer solution throughout the entire efflux study (with the exception of the radioactive tracer). Third, exposure of the tissue to the Ringer solution labeled with radioactive Na+ must be as brief as possible. With these requirements satisfied, the efflux study follows the conventional procedure and arrives at two values for F_{iw} by the following methods:

(1) THE INTERCEPT METHOD. In the usual manner, the logarithm of the remaining labeled Na+ in the muscle t min after washing began is plotted as the ordinate against t as the abscissa. After correction has been made for extraneous contributions of labeled Na+ from connective tissue elements, extracellular-space fluid, etc., the efflux curve is resolved into several exponential fractions by the "ex-

ponential peeling" method. The intercept on the ordinate of the particular fraction under investigation is then divided by the time of exposure of the cell to the labeled Na+ in minutes and the water content of the cell in ml to yield one value for F_{iw}.

(2) THE SLOPE METHOD. As mentioned above, with the exception of the minute amount of radioactive Na+ the system is in a state of equilibrium throughout the experiment. Therefore

$$F_{iw} = F_{ow} \qquad (7)$$

or

$$k_2[Na^+]_{eq,ex} = k_1[Na^+]_{eq,in} \qquad (8)$$

where $[Na^+]_{eq,in}$ and $[Na^+]_{eq,ex}$ are the equilibrium concentration of Na+ inside and outside the cells, respectively, under the specified conditions. From Eqs. 6 and 8, then, it follows that

$$F_{iw} = k_1[Na^+]_{eq,in} \; . \qquad (9)$$

One can obtain k_1 from the slope of the same efflux curve (which has already provided one value of F_{iw} by the intercept method) or more conveniently from the half-time of exchange ($t_{½}$) since

$$k_1 = \ln 2/t_{½}. \qquad (10)$$

From Eqs. 9 and 10 it follows that

$$F_{iw} = (0.693/t_{½})[Na^+]_{eq,in} \qquad (11)$$

providing a second method to determine F_{iw}.

Intracellular ion concentration. The equilibrium concentration of Na+ is obtained separately. Cells similar to those used above, under conditions otherwise identical to those in the short-exposure experiments, are exposed to a similar labeled solution but long enough to insure equilibrium. Efflux curve resolution is then carried out in the usual manner. From the zero time intercept of each of the different fractions one obtains a value for $[Na^+]_{eq,in}$ corresponding to that fraction. In the case of Na+ efflux from frog muscles there are two intercepts, one from the fast fraction and the other from the slow fraction; thus there will be two values for $[Na^+]_{eq,in}$.

If the slow fraction is considered to represent intracellular-extracellular exchange, $[Na^+]_{eq,in}$ is obtained by extrapolating only the slow fraction to zero time. In testing the alternative hypothesis—i.e., that the fast fraction is rate-limited by surface permeation—the intercept of the fast fraction is considered as $[Na^+]_{eq,in}$. A similar distinction exists in evaluating F_{iw} by the intercept method, in which, in the first case, F_{iw} refers only to the intercept of the slow fraction; in the second case, F_{iw} refers to the sum of the slow- and fast-fraction intercepts.

Exponential Peeling: Basic Method for *Complex Efflux* Curve Resolution

The efflux curves of the muscle-fiber bundles, after correction for fluid in the extracellular space and for connective tissue contributions, are resolved into their components by the exponential peeling method conventionally used. However, despite the wide usage of this method, to the best of my knowledge, the significance of the values obtained has not been carefully evaluated. Since accuracy of quantitative parameters estimated are critical in the present study, I have undertaken a simple model evaluation to be described next.

The rate of efflux of an isotope-labeled solute can be determined by the rate of surface permeation if the surface permeation rate is considerably slower than (a) the rate of solute diffusion within the cell, (b) the rate of solute passage through the surface of subcellular compartments, as well as (c) the rate of solute desorption from macromolecular adsorption sites. Under these conditions and after removal of or corrections for isotope trapped in the extracellular space and in the connective tissue elements, the rate of efflux can be described by a single exponential

curve. But when the rate of surface permeation is faster than one or more of the rate-limiting steps mentioned above, a more complex picture emerges.

Let us consider, for example, the case in which surface permeation is faster than desorption and in which this desorption from macromolecular sites is the only source of the (single) slow fraction. (The same conclusions would apply to the case in which the slower fraction is rate-limited not by desorption but by permeation through the surface of subcellular particles.) The solute to be discussed will again be Na^+.

If a living cell is incubated for a sufficiently long period in a Ringer solution containing radioactively labeled Na^+, an equilibrium will be reached between labeled Na^+ in the external solution and labeled Na+ in the intracellular (interstitial) water at concentration $[Na^*]_{int}$ as well as between the latter and adsorbed labeled Na+ at concentration $[Na^*]_{ad}$. Thus

$$[Na^*]_{int} \underset{k_4}{\overset{k_3}{\rightleftarrows}} [Na^*]_{ad} \qquad (12)$$

where k_3 and k_4 are the rate constants of adsorption and desorption within the cells and are expressed in units of min^{-1}.

If the labeled cell is then washed in a stream of non-radioactive Ringer solution, the rate of loss of interstitial labeled Na+ is

$$-d[Na^*]_{int}/dt = k_5[Na^*]_{int} \qquad (13)$$

where k_5 is the outward rate constant of surface permeation, and

$$-d[Na^*]_{ad}/dt = k_4[Na^*]_{ad} - k_3[Na^*]_{int} \qquad (14)$$

The solutions to Eqs. 13 and 14 are respectively

$$[Na^*]_{int,t} = [Na^*]_{int,t=0}\exp(-k_5t) \qquad (15)$$

and

$$[Na^*]_{ad,t} = [Na^*]_{ad,t=0}$$
$$\{[k_4/(k_3 - k_5)]\exp(-k_5t) - [k_5/(k_4 - k_5)]\exp(-k_4t)\} \qquad (16)$$

where $[Na^*]_{int,t}$ and $[Na^*]_{ad,t}$ are the interstitial and adsorbed labeled Na+ concentrations in the cell t min after washing began. $[Na^*]_{int,t=0}$ and $[Na^*]_{ad,t=0}$ are the initial interstitial and adsorbed labeled Na+ concentrations. Representing the total labeled Na+ concentration in the cell at time t as $[Na^*]_{in,t}$, one obtains

$$[Na^*]_{in,t} = [Na^*]_{in,t=0}\exp(-k_5t) + [Na^*]_{ad,t=0}\{[k_4/(k_3 - k_5)]\exp(-k_5t) + [k_5/(k_5 - k_4)]\exp(-k_4t)\}. \qquad (17)$$

If k_5 is much larger than k_4, then some time after washing begins the first two terms involving $-\exp(-k_5t)$ on the right-hand side of Eq. 17 become negligible when compared to the last term. At this time Eq. 17 is reduced to

$$[Na^*]_{in,t} = [Na^*]_{ad,t=0}\exp(-k_4t) \qquad (18)$$

or

$$\log[Na^*]_{in,t} = -k_4t + \log[Na^*]_{ad,t=0}. \qquad (19)$$

That is, in a plot of the logarithm of the concentration of labeled Na+ remaining in the cell at time t, against the time of washing, t_w, the curve becomes linear, with a slope equal to the desorption rate constant, $-k_4$. By extrapolating from the straight line to zero time ($t=0$), one obtains an estimate of the initial concentration of adsorbed labeled Na, $[Na^*]_{ad,t=0}$. By subtracting the slower fraction from the efflux curve, one obtains the interstitial labeled Na+ efflux curve, $[Na^*]_{int,t=0}$ as well as k_5.

If k_5 is, say, 100 times larger than k_4 the method of analysis can without doubt yield accurate estimates of all four parameters: k_4, k_5, $[Na^*]_{int,t=0}$, and $[Na^*]_{ad,t=0}$. However, of greater importance is the question of how accurate this type of analysis is when k_4 and k_5 are much closer together in value.

To answer, I calculated and plotted three

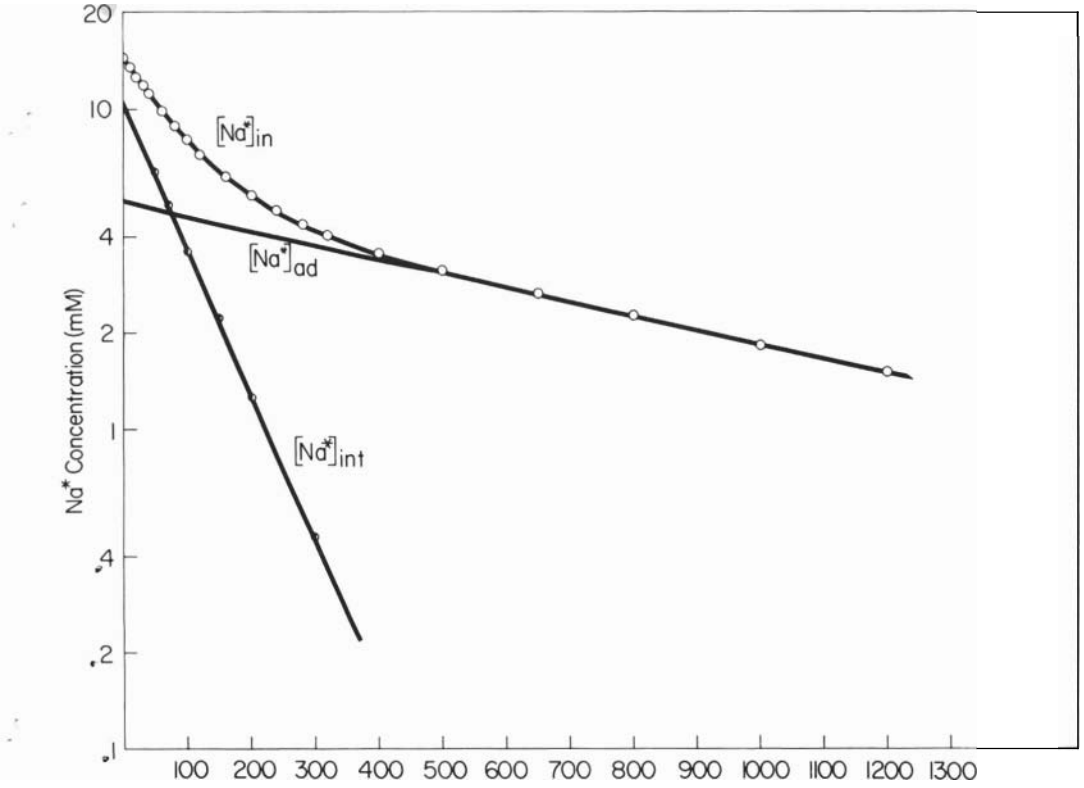


FIGURE 1. A theoretical two-component complex Na+ efflux curve from a living cell in which the rate of surface-limited intracellular-extracellular exchange (k_5) is 10 times faster than the rate of Na+ desorption (k_4): $k_5 = 10^{-2}$ min^{-1}; $k_4 = 10^{-3}$ min^{-1}. Theoretical curves were computed according to Eq. 17. Equilibrium concentration of adsorbed Na^+ is 5 mM; that of the free interstitial Na+ is 10 mM (i.e., 10 millimoles/liter of cell water). As the figure illustrates, recovery of both rate constants and initial concentration of interstitial and adsorbed Na+ are within 1% accuracy.

sets of theoretical curves with $[Na^*]_{int,t=0}$ ($= 10$ mM), $[Na^*]_{ad,t=0}$ ($= 5$ mM), and k_5 ($= 10^{-2}$) remaining constant but with k_4 equal to 1/10, 1/5, or 1/2 of the value of k_5. Fig. 1 shows that when k_4 and k_5 differ by a factor of 10 very accurate results are obtained for all parameters. When k_4 and k_5 differ by a factor of 5 (Fig. 2), reasonably accurate values (10%) are still obtainable for k_4 and k_5, but the values of $[Na^*]_{int,t=0}$ and $[Na^*]_{ad,t=0}$ are accurate only to within 20%, with $[Na^*]_{ad,t=0}$ artificially exaggerated at the expense of $[Na^*]_{int,t=0}$. When k_4 and k_5 differ by a factor of 2, the time constants k_4 and k_5 estimated are still surprisingly accurate, but the initial concentrations of adsorbed and interstitial Na* become seriously distorted—$[Na^*]_{ad,t=0}$ is now really twice what it should be and $[Na^*]_{int,t=0}$ is reduced to nearly ½ its true value.

This analysis was based on the adsorbed fraction being ½ the concentration of interstitial Na*. However, in the case where the adsorbed fraction is equal to the concentration of interstitial fraction, roughly the same accuracy obtains as shown in Fig. 3. Whereas in Fig. 2, k_5 is 5 times faster than k_4, the extrapolated initial concentrations are accurate to within 20%; the k_4 and k_5 within 10%.

MATERIALS AND METHODS

Materials. All experiments were performed on voluntary muscle-fiber bundles isolated from the semitendinosus muscles of North American leopard frogs (Rana *pipiens pipiens,* Schreber). The method of isolating these small bundles from the semitendinosus muscles was essentially the same as that used for isolating single muscle fibers. Care was taken to remove large pieces of tendon, leaving only a minimum amount for handling and for tying with a piece of silk thread (see below), and to isolate the fiber bundles only from an area of the muscle not entered by nerve fibers and blood vessels. This selection of muscle fibers serves, first, to minimize the amount of extraneous tissues involved and, second, to avoid inclusion of muscle fibers innervated by small nerve fibers.[12] Connective tissues containing small nerves and blood vessels resembling those present in the muscle were isolated from the outer surface of the skinned legs of the same frog that provided the muscle.[11]

The glucose-free Ringer-phosphate solution used throughout this investigation (except as otherwise described) contained the following: NaCl (104.7 mM), $NaHCO_3$ (6.6 mM), KC1 (2.5 mM), NaH_2PO_4 (2.0 mM), $MgSO_4$ (1.2 mM), Na_2HPO_4 (1.2 mM), and $CaCl_2$ (1.0 mM). The total Na+ concentration in this solute is 113 mM. Ringer-GIB medium, a complete incubation medium containing vitamins and amino acids, was used

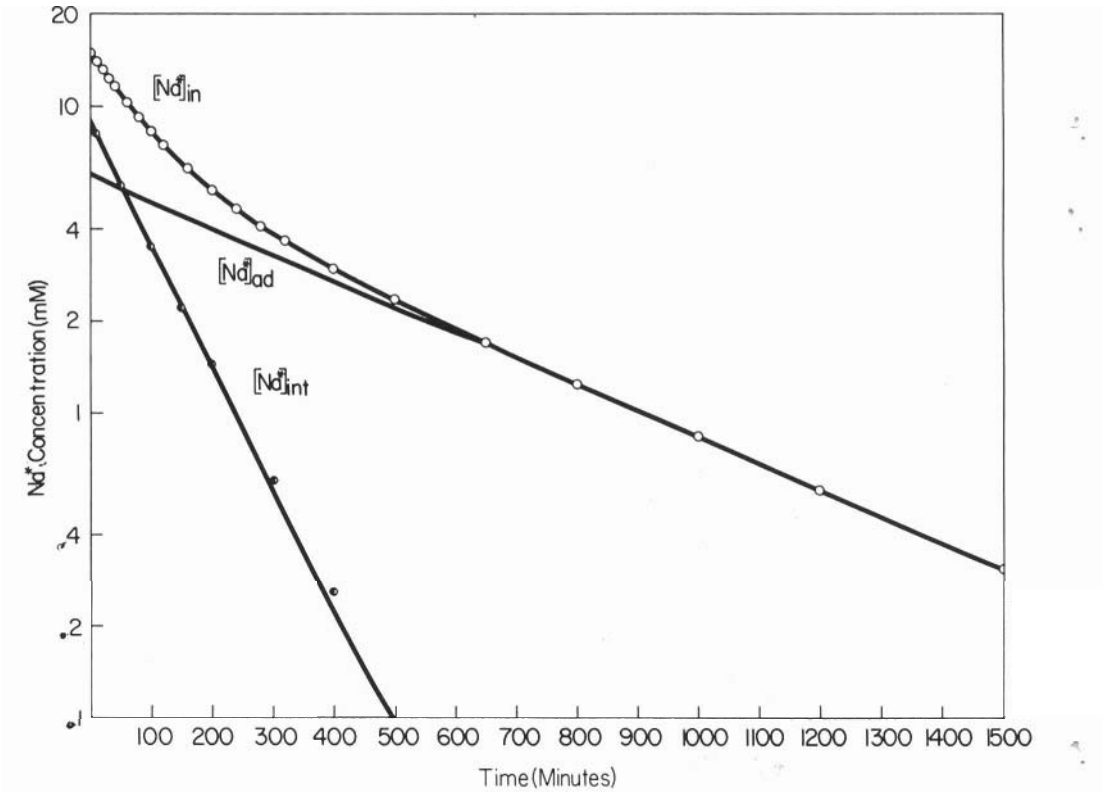


FIGURE 2. **A theoretical two-component complex Na+ efflux curve from a living cell in which the rate of surface-limited intracellular-extracellular exchange (ka) is 5 times faster than the rate of Na^+ desorption (k_4): $k_5 = 10^{-2}$ min^{-1}; $k_4 = 2 \times 10^{-3}$ min^{-1}. The equilibrium concentration of adsorbed Na+ is 5 mM; that of the free interstitial Na+ is 10 mM. Recovery of the rate constants is still accurate to within 10%. However, the concentration of the two fractions recovered are accurate to only 20%.**

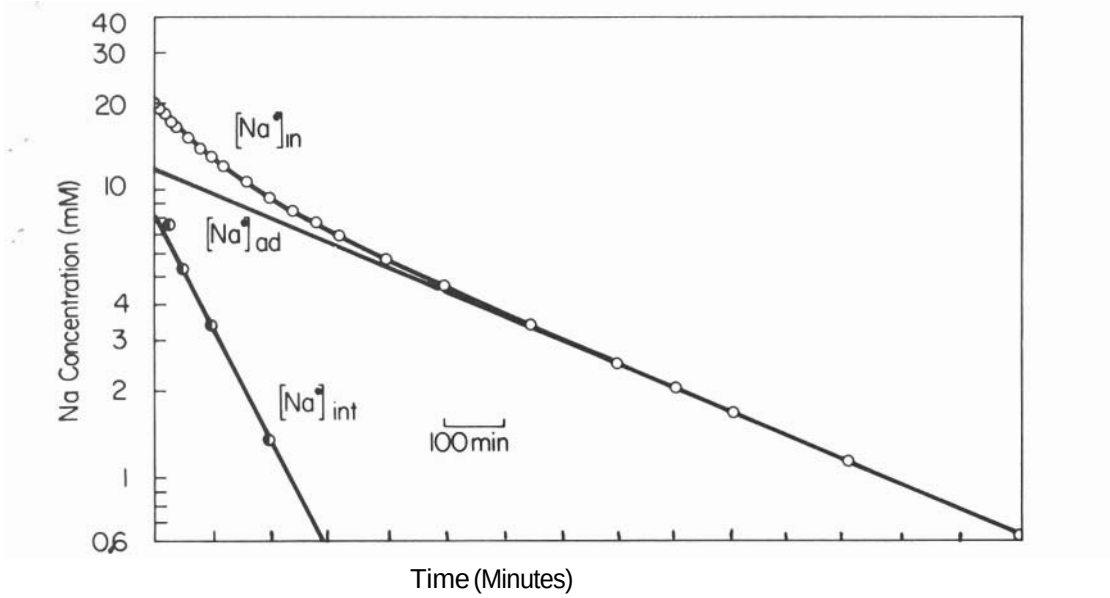


FIGURE 3. A theoretical two-component complex Na^+ efflux curve from a living cell in which the rate of surface-limited intracellular-extracellular exchange (ka) is 5 times faster than the rate of Na^+ desorption (k_4): $k_5 = 10^{-2}$ min^{-1}; $k_4 = 2 \times 10^{-3}$ min^{-1}. The equilibrium concentration of adsorbed Na+ is 10 mM; that of the free interstitial Na+ is 10 mM. Recovery of the rate constants is still accurate to within 10% but the initial concentration of adsorbed and free fraction are much distorted: the lower fraction is spuriously increased by 90% at the expense of the faster fraction, off by about 20%.

only for certain stated purposes; its detailed composition has been fully described elsewhere.[13]

All chemicals used were of reagent grade. ^{22}Na (Lot No. 4771CG1) was obtained from New England Nuclear Corporation, Boston, Massachusetts.

Tissue incubation. Isolated muscle-fiber bundles and connective tissues were first incubated at 4°C in a Ringer-GIB medium equilibrated with a mixture of 5% CO_2, 45% O_2, and 55% N_2 for about a week. This prolonged incubation permitted full disintegration of injured muscle fibers while leaving normal muscle fibers intact and healthy.[13] The disintegrated fibers were easily recognizable and were dissected away before exposure to labeled Na^+.

Isotope loading. The muscle-fiber bundles freed from all injured and dead cells and companion connective tissue elements isolated from the same animal were incubated at room temperature in normal Ringer-phosphate solution containing 20 to 40 μCi/ml of ^{22}Na either briefly (1 to 2 min) or long enough to insure equilibration (i.e., 16 h or longer).

Removal of extracellular-space fluid. To remove the radioactive extracellular-space fluid, the incubated muscle was first placed upon a deck of filter paper wetted with the ^{22}Na-labeled incubating Ringer-phosphate solution. The filter paper with its muscle bundles (or connective tissues) was then hermetically sealed in Parafilm and spun at 1000g for 4 min.[14]

Efflux study. The isotope-loaded muscle bundle (or connective tissue) was then removed from its Parafilm wrap and one end of the bundle tied with Deknatel silk thread. The thread was then pulled through a glass U-tube (i.d. = 4 mm), and the muscle bundle was anchored at the bottom part of the U, which sat in the "well" of a γ-scintillation counter. A steady stream of non-radioactive

Ringer solution was drawn through the U-tube at the rate of 1 ml/sec while the remaining radioactivity was being monitored on a scaler.[7,15] The efflux experiments were conducted at the same room temperature (22° to 25°C) as that of incubation and isotope loading.

At the end of the experiment, the muscle-fiber bundle was taken out of the U-tube and, after blotting, was weighed on a torsion balance.[13] The number of muscle fibers was estimated with the aid of a dissecting microscope, although no attempt was made to be precise. To determine the specific activity of the labeled Na^+, a weighed amount of the original bathing solution was introduced into the bottom of a U-tube similar to those used for irrigating the muscle bundles, and the radioactivity was assayed with the same γ-scintillation counter.

Correction for labeled Na^+ taken up by connective tissue elements. Loose connective tissues from the same frog have been shown to have composition and ion-uptake characteristics similar to those of connective tissues within a muscle. These tissues were treated in a manner identical to the muscle bundles. More specifically, for each series of studies of tissues provided by a single frog, two pieces of connective tissue were isolated: one piece was equilibrated for at least 24 h in thelabeled Ringer solution to which the muscle bundles were exposed; the other piece was equilibrated for the same brief period of time as the companion muscle-fiber bundles.

Using several different methods, I and my co-workers concluded that frog sartorius muscle contains about 9% connective tissues (see ref. 7, p. 210; also refs. 14, 16, 17). No study has been made on semitendinosus muscle-fiber bundles, but differences are likely to be small if care is taken not to include heavy pieces of tendons. To minimize the amount of tendon required, muscle bundles were tied with the thread at only one end; this permits complete removal of all tendon tissue at the other end of the muscle. However, from time to time, the connective tissue content of a fiber bundle was below the average figure of 9%.[10] In these cases, the correction made on the basis of a 9% contribution led to negative vaiues for the concentration of labeled Na+ in the cells, seen most conspicuously in that portion of the efflux curve after more than 2 h of washing. Thus the tail ends of the efflux curves could provide a guideline for adjustment in the percentage of connective tissue when the need arises. As a rule, however, changes in the connective tissue correction caused little difference in the significant values of the intercepts and the slopes, which are largely determined by the earlier part of the efflux curves.

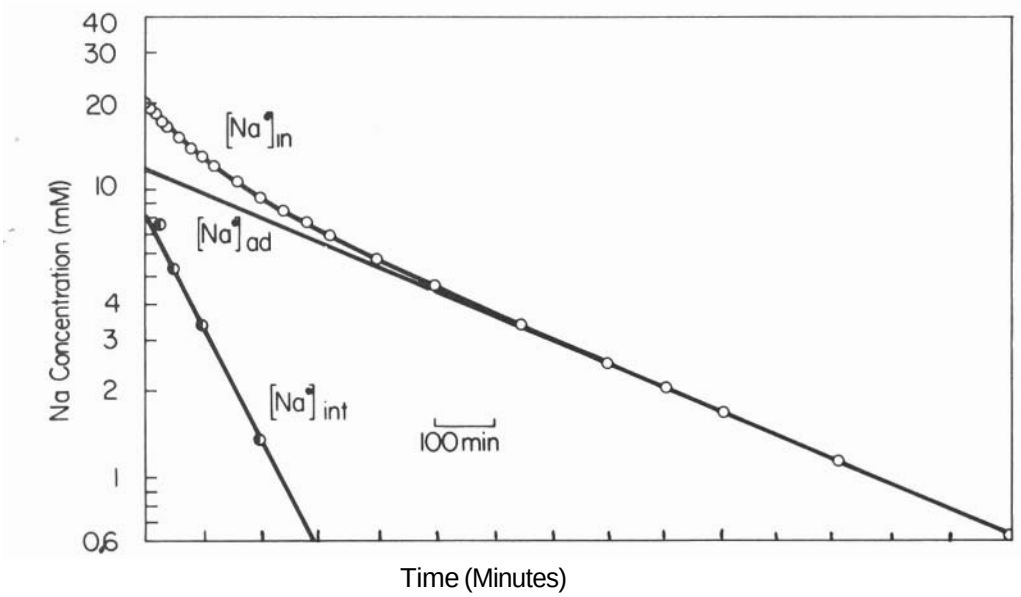
Calculations. To determine F_{1w} by the intercept method, one must remember that if the fast fraction is assumed to be the fraction rate-limited by surface permeation, then the amount of labeled Na+ that has entered the cell will include this fraction as well as the slow fraction. If, on the other hand, one assumes it is the slow fraction that is rate-limited by surface exchange, the intercept of the slow fraction alone yields the amount of Na+ that has entered the cell.

To calculate F_{1w} from the slopes, as discussed above, one needs the value of equilibrium concentration of labeled Na^+, $[Na^+]_{eq,in}$ (see Eq. 11). This value was obtained from a separate muscle bundle from the same semitendinosus muscle after long incubation in the labeled Ringer solution.

To calculate $[Na^*]_{in}$ from labeled Na+ concentration in centrifuged muscle, $[Na^*]_{cell+ct}$ in units of μmoles per gram of fresh weight

$$[Na^*]_{in} = \{[Na^*]_{cell+ct} - 0.09[Na^*]_{ct}\}/\{0.80 - 0.09 - 0.09(0.85 - 0.30)\} = 1.52\{[Na^*]_{in} - 0.09[Na^*]_{ct}\}. \quad (20)$$

$[Na^*]_{in}$ is the labeled Na+ concentration in the cells in μmoles/g of fresh cells. $[Na^*]_{ct}$ is the concentration of labeled Na+ in one gram of fresh connective tissues; 0.09, the



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percentage weight of the extracellular-space fluid is also the percentage weight of the wet connective tissues; *0.85* is the water content of fresh connective tissues; and *0.30* is the percentage of "extracellular space" of the connective tissue that is accessible to the extracellular-space probe, poly-L-glutamate,[3] and is thus already included in the 9% extracellular space assayed with poly-L-glutamate[17] among other methods.

RESULTS

Figure 4 shows a typical ^{22}Na efflux curve of a small muscle-fiber bundle after prolonged exposure to ^{22}Na-labeled normal Ringer-phosphate solution to insure equilibrium and after removal of extracellular-space fluid by centrifugation. This efflux is not distinguishable from those reported earlier for frog sartorius muscles after removal of extracellular-space fluid.[11] After correction for the connective tissue contribution, the curve is resolvable into a slow fraction (Fraction I) and a fast fraction (Fraction II). The intercepts of the straight lines of each of these fractions in the semilogarithmic plot provide the equilibrium concentrations of Na+ in the respective fractions.

Figure *5* shows three examples of the labeled Na+ efflux curves from small muscle-fiber bundles that were exposed to labeled normal Ringer-phosphate solution for 3 to 5 min. Comparing Fig. *5* with Fig. 4, one notices how closely the total amount of intracellular labeled Na+, accumulated in such a brief period of time, approached the equilibrium concentrations. Since all labeled Na+ in the extracellular space has already been removed and the contribution of connective tissue (including small blood vessels, nerves, and blood cells) has also been corrected for, the speed of uptake of labeled Na+ gives a clear indication of the high rate of ^{22}Na exchange. It is stressed that the **3** to *5* min of exposure to labeled Na+ includes not only the time of incubation (1 to 2 min) but also the time spent placing the fiber bundle in the hermetically sealed package as well as the first minute of centrifugation, since at the end of that minute

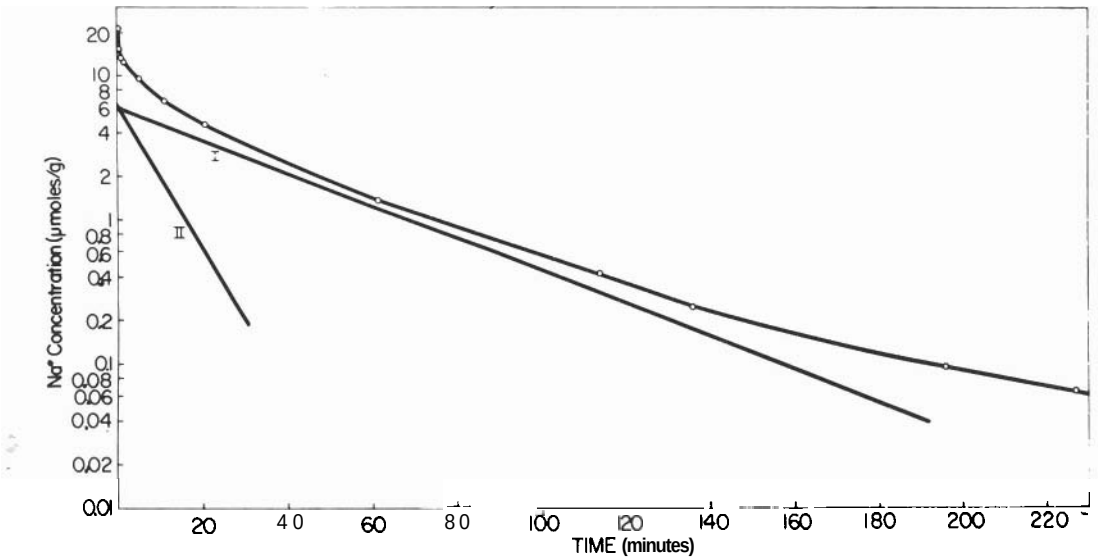


FIGURE 4. Na+ efflux curve of small muscle-fiber bundles freed of extracellular-space fluid after 20 h of incubation in ^{22}Na tagged with Ringer-phosphate solution (16 h at 4°C followed by 4 h at room temperature). Correction had been made for labeled Na+ in connective tissues from the same frog after identical treatments. (Experiment of 11/18/77.)

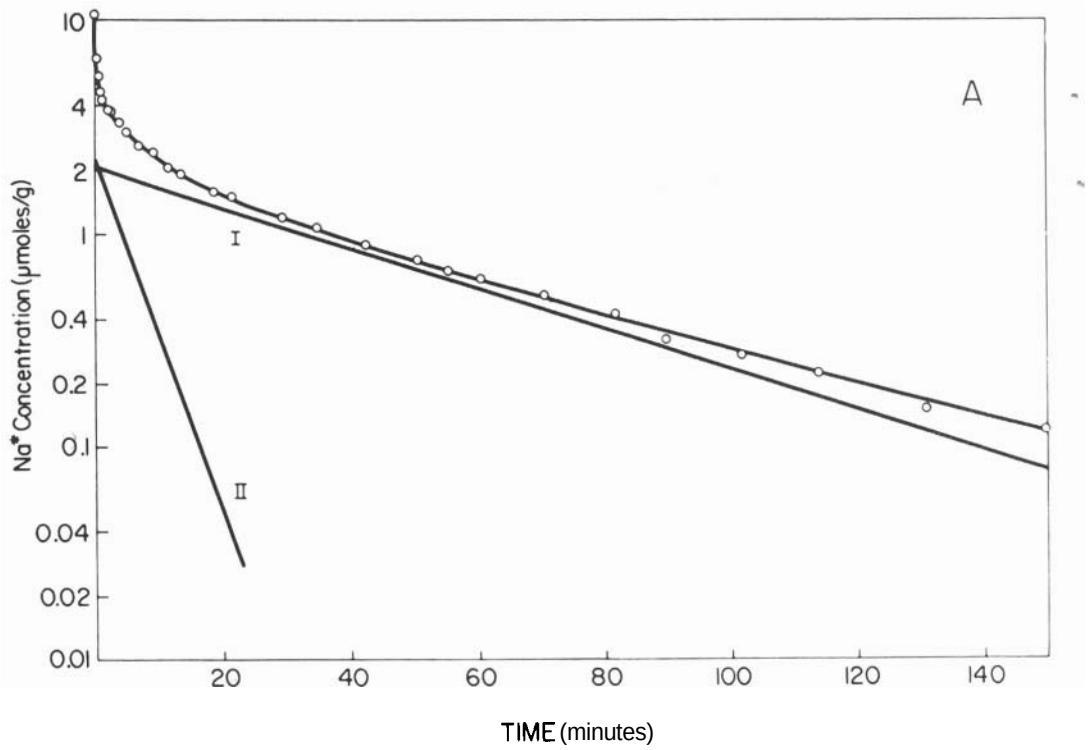


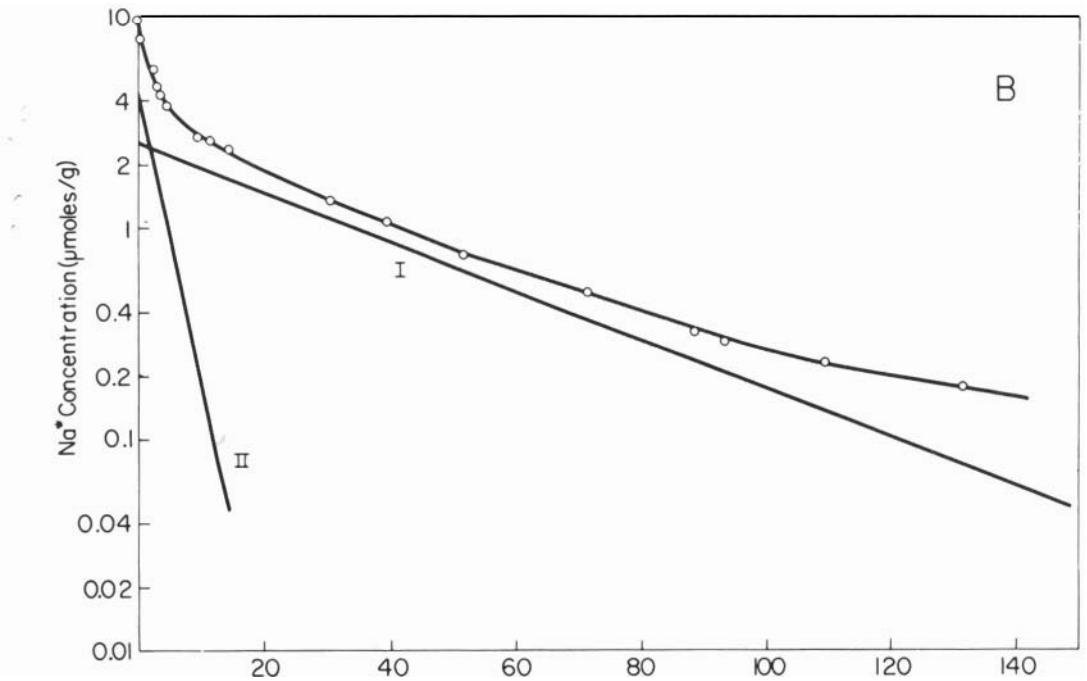
FIGURE 5. Na+ efflux curve of small muscle-fiber bundles freed of extracellular-space fluid after **20** h of incubation in tagged normal Ringer-phosphate solution **(16** h at **4°C** followed by approximately 4 h at room temperature) and then brief exposure of ^{22}Na-labeled normal Ringer-phosphate solution. Correction had been made for labeled Na+ in connective tissue from the same frog after identical treatment. ***A:*** Muscle 1 of **11/18/77.** B: Muscle E_0 of **11/24/77.** C: Muscle D_4 of **11/23/77.** (For detailed information on these experiments see Table I.)

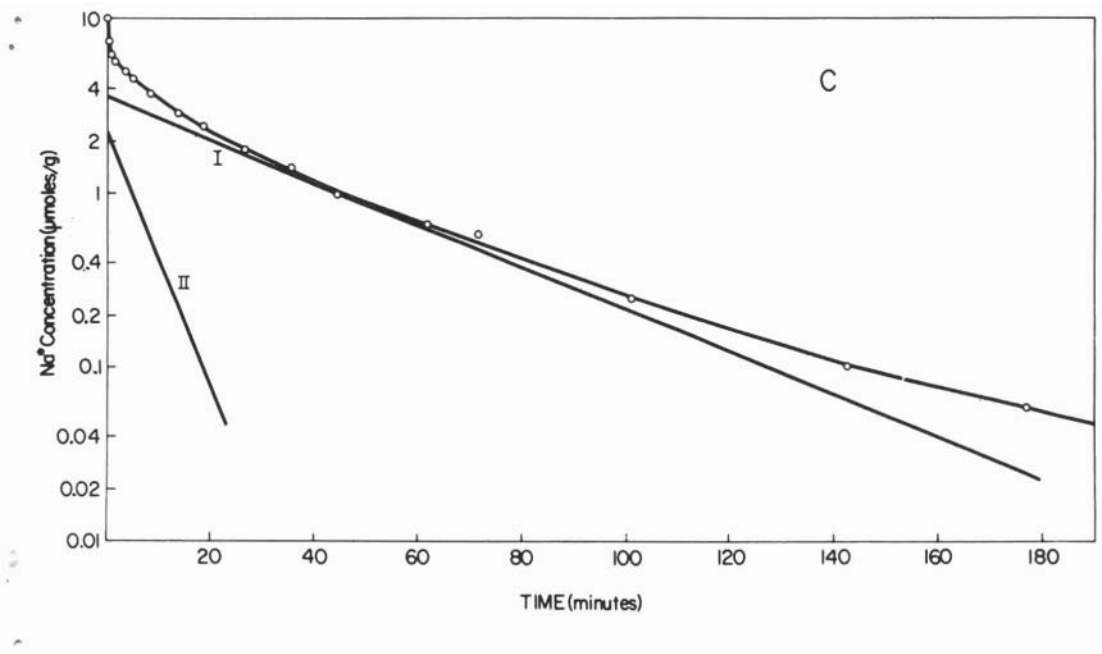
the extracellular-space fluid is nearly totally removed.[14]

Table I presents the equilibrium concentration of labeled Na+ in Fractions I and II obtained from efflux studies such as those shown in Fig. 4. In addition, Table I presents muscle-fiber bundle weight and the approximate number of muscle fibers, as well as the zero-time intercepts and the $t_{½}$, of both fractions. The data permit the calculation for each fraction of the influx rate from $t_{½}$ according to Eq. 11 (referred to here as A) and that from the zero-time intercepts (referred to here as B).

It is to be noted that the efflux rate constants of the fast and slow fractions in all cases differ by a factor of 5 or more, averaging about 8. Furthermore, in 19 out of a total of 22 experiments, the slow fraction is smaller than the fast fraction. Thus the error introduced in the resolution of fractions is even smaller than the theoretical examples calculated and shown in Figs. 2 and **3,** where the slow fraction manifests 1/5 the rate of the fast fraction. The method of efflux component resolution in these experiments therefore suffers no serious distortion in consequence of the use of the exponential peeling method.

As pointed out earlier, the influx rate determined by $t_{½}$ (A) and by the intercept (B) should agree for the fraction truly representing the one rate-limited by intracellular-extracellular exchange. In other words the ratio





B/A, expressed as a percentage, should be close to 100.

From all 22 sets of data obtained the B/A ratio, calculated on the basis of the original Levi-Ussing contention that it is the slow fraction (Fraction I) which is rate-limited by intracellular-extracellular exchange, is 481% ± 39.5% and is therefore self-contradictory,

On the other hand, the B/A ratio, calculated on the basis of the assumption that it is

TABLE I. Summary of Experimental Data Leading to Identification of Na+ Fast Fraction as Representing Surface-Limited Intracellular-Extracellular Exchange. (Identification is indicated by agreements of two influx rates–one from the slopes, the other from the intercepts. Virtually all experiments were performed on small muscle-fiber bundles isolated from semitendinosus muscle; the lone experiment of 10/31/77 was on a whole sartorius muscle.

		Muscle		Expo-	$[Na^+]_{in,eq}$		Slow fraction		Past fraction		Influx rate (mmoles/liter × min)					
											Slow fraction			Fast fraction		
												From			From	
Date	Mus-cle no.	Weight (mg)	Number of fibers	sure time (min)	Slow fraction (mM)	Fast fraction (mM)	Inter-cept (mM)	$t_{½}$ (min)	Inter-cept (mM)	$t_{½}$ (min)	From slope (A)	inter-cept (B)	B/A (%)	From slope (A)	inter-cept (B)	B/A (%)
10/27/77	I	8.6	54	4.0	5.9[a]	8.8[a]	2.1	33	4.2	6.0	0.12	0.44	252	1.02	1.05	103
10/31/77	I	75.5[b]		3.0	7.6	8.2	1.5	56	4.0	3.0	0.094	0.49	521	2.20	1.79	81
11/10/77	I	8.73	40	4.0	8.8	11.9	5.3	19.5	6.0	3.4	0.32	1.34	419	2.43	2.74	113
11/11/77	I	11.8	60	4.5	8.8	11.9	7.3	30	3.3	4.4	0.20	0.75	375	1.87	2.37	127
	II	10.1	60	4.5	8.8	11.9	3.5	21.5	4.9	3.7	0.28	0.52	186	2.57	2.19	85
	III	8.7	40	4.5	8.8	11.9	3.3	28	6.1	4.8	0.21	0.74	353	2.05	2.36	115
	IV	12.0	50	4.5	8.8	7.8	4.0	21.5	5.8	3.7	0.29	0.88	303	2.22	2.16	97
11/18/77	I	10.7	60	4.5	4.9	6.8	3.0	31	3.0	3.7	0.11	0.67	610	1.24	1.34	108
	II	4.6	28	4.5	4.9	6.8	2.4	17	5.8	1.5	0.20	1.11	555	3.19	2.61	82
11/22/77	I	6.4	40	4.0	5.0	6.7	2.4	32.5	3.3	4.1	0.11	0.61	555	1.12	1.44	129
	II	8.9	50	4.0	5.0	6.7	2.7	26	3.8	2.6	0.14	0.68	486	1.78	1.64	92
11/27/77	D1	10.3	35	5.0	6.1	10.6	4.9	23	10.6	2.9	0.11	0.97	882	2.54	3.10	122
	D2	10.1	35	5.0	6.1	10.6	6.1	22	7.3	3.2	0.20	1.21	605	2.30	2.68	117
	D3	12.2	50	5.0	6.1	10.6	6.2	26	7.0	2.6	0.17	1.25	735	2.84	2.64	93
	D4	13.0	60	5.0	6.1	10.6	5.3	24	3.5	4.3	0.15	1.06	707	1.72	1.76	102
	D5	4.1	15	5.0	6.1	10.6	6.4	23	6.4	3.1	0.18	1.28	711	2.46	2.55	104
11/23/77	E1	8.0	25	3.25	9.1	9.4	4.7	26	5.9	3.1	0.24	1.49	621	2.10	2.64	126
	E3	10.1	50	3.25	9.1	9.4	5.2	14	5.8	2.8	0.46	1.29	280	2.33	2.74	118
	E4	5.0	30	3.25	9.1	9.4	1.12	22	4.6	3.5	0.29	0.96	331	1.87	1.73	93
	E5	7.4	30	3.25	9.1	9.4	5.3	22	12.1	2.3	0.29	1.08	372	3.65	5.32	146
	E6	6.6	40	3.25	9.1	9.4	3.6	26	6.1	2.3	0.24	1.12	467	2.84	2.99	105
	E8	3.7	14	3.25	9.1	9.4	3.3	16	7.3	2.1	0.40	1.03	258	3.10	3.27	105
Mean								25.1		3.17	0.222	0.957	481	2.25	2.41	107
± SE								±1.86		±0.17	±0.02	±0.063	±39.5	±0.14	±0.19	±3.6

[a] Data in this column represent average of all corresponding values given in table. [b] A whole sartorius muscle was employed in this experiment.

the fast fraction (Fraction II) which represents intracellular-extracellular exchange, is 107% ± 3.6% and is therefore, within the experimental error, self-consistent.*

CONCLUSIONS AND DISCUSSION

In their original Na+ efflux study of frog sartorious muscles, Levi and Ussing contended that the fraction of Na+ with a half-time exchange of about 30 min (at room temperature) was rate-limited by membrane permeability. This contention was of critical importance because quantitative data supporting several major concepts of the membrane-pump theory have been interpreted on the basis of this conclusion.

Subsequently I presented evidence that the fast fraction may be derived only in part from the ^{22}Na trapped in the extracellular space, and the conclusion of Levi and Ussing may be mistaken. Indeed the extracellular Na^+ may have camouflaged a fast fraction that originates from within the cell and is in fact the Na+ exchange rate-limited by surface permeability. The evidence previously published in support of this view includes the following:

(1) The magnitude of Na+ belonging to the fast fraction (approximately 19%)[10] exceeds the extracellular-space fluid volume (i.e., 9%) determined by a variety of methods.[3,14,16,17]

(2) Single isolated frog muscle cell, which has no extracellular space in the usual sense (a thin film of adhering fluid is washed away within a second), has a similar fast fraction in its Na+ efflux (ref. 7, p. 293; see also ref. 9). I have shown in addition[10] that Hodgkin and Horowicz failed to observe this effect in their studies of Na+ efflux from single muscle fibers because of insufficient data points.

(3) The Na+ efflux from single frog ovarian eggs shows an entirely analogous complex curve resolvable into at least one fast fraction and two slower fractions. It is already widely agreed that in this case the fast fraction represents intracellular-extracellular exchange.[19-23]

(4) Removal of extracellular-space fluid by the recently introduced method of centrifugation and correction for contribution of connective tissue elements (including small blood vessels, some trapped blood cells, and small nerves) did not remove the fast fraction of the efflux curve of isolated sartorious muscles.[14]

(5) Efflux curves for ^{42}K and ^{22}Na simultaneously recorded from the same muscles freed of extracellular fluids showed a perfectly rectilinear slope for ^{42}K efflux but a curved, two-compartment slope for Na+ efflux.[11] This rules out the sarcoplasmic reticulum as the source of the fast fraction of Na+ efflux: otherwise, a similar fraction should be observed for K+, since hydrated K^+ is smaller than hydrated Na+ and thus would be able to enter through the pores of the bottom of the T tubules as postulated by Zierler et al.[5,6] Subsequently Somlyo et al., using X-ray microprobe analyses, suggested that muscle Na+ is not localized in the sarcoplasmic reticulum but evenly distributed.[24]

Taken as a whole, the above evidence argues strongly for a fast-exchanging fraction of Na+ originating within the cell and with

* **Too much emphasis should not be placed upon the closeness of B/A based on the fast-exchanging fraction as rate-limited by permeability to the predicted 100%. Rather, this agreement should be appraised in the relative sense when compared to B/A based on the slow exchanging fraction as rate-limited by permeability. Ideally the time of exposure to labeled Na^+, for example, should be a few seconds; in reality this exposure could not be much further reduced than those recorded (see MATERIALS AND METHODS). However, several factors, some tending to diminish, others to augment the value of B/A might have together produced the close agreement shown. Factors tending to decrease B/A include the backward diffusion due to long exposure time; factors tending to increase B/A include the reduced rate of influx during the first minutes of spinning and other operating steps leading to this step and the presence of a slow fraction which "traps" labeled Na^+, slowing its backward diffusion.**

an exchange rate-limited by the intracellular-extracellular exchange.

The significance of the slow fraction was also analyzed. On first consideration, this fraction could be regarded as either Na^+ adsorbed onto intracellular macromolecules or Na^+ trapped in subcellular particles. To refute the concept of subcellular compartment entrapment, one may cite the observation that with decreasing external K^+ concentration, Na^+ belonging to the slow fraction steadily multiplies until it equals the total concentration of $K^+ + Na^+$ in the resting cells.[25] Swelling of subcellular compartments to a magnitude closer to the total cell volume is unheard of and indeed is not possible, because deprivation of K^+ does not cause cell swelling; the bulk of muscle cell consisting of myosin and actin filaments do not vanish during (reversible) K^+ depletion and Na^+ accumulation. Recent autoradiographic work from our laboratory[26] and the remarkable transmission electron microscopic work of Edelmann[27,28] have left little doubt that intracellular K^+ is adsorbed in the A bands and Z line of muscle cells. Stoichiometic displacement of K^+ by Na^+ thus reasonably explains an increase in the adsorbed Na^+.

Hence the earlier evidence, as well as that presented here, clearly establishes that it is indeed the fast fraction, and not the slow fraction, that represents the intracellular-extracellular exchange, that the slow fraction represents Na^+ adsorbed, and that the rate-limiting step of the slow fraction is desorption.

Implications for Pump Theory

Let us now examine the implications of the above findings with respect to the current theory of the resting potential and the energy requirement of the postulated Na pump.

1. The permeability constants for *alkali*-metal ions for frog muscle. Table II lists some examples of the inward rate constants available in the literature for K^+, Rb^+, Cs^+, and Na^+ at room temperature.[7,29-31] The average of the four sets of earlier data (including my own) for Na^+ permeability was 0.13 h^{-1}. The new value from the present study (1.28 h^{-1}) is nearly 10 times higher. My old data were too low because at the time the centrifugation technique had not yet been developed. To remove the labeled ion in the extracellular space, the muscle had to be washed vigorously in a non-labeled Ringer solution for 5 min at O°C. As a result, much of the fast fraction was removed, spuriously reducing the influx rate.

2. Theories of resting potential. According to the well-known Hodgkin-Katz equation, the cellular resting potential (ψ) depends on the absolute temperature (T); on the intra- and extracellular concentrations of K^+, Na^+, and Cl^-—respectively represented as $[K^+]_{in}$, $[K^+]_{ex}$, $[Na^+]_{in}$, $[Na^+]_{ex}$, $[Cl^-]_{in}$, and $[Cl^-]_{ex}$; and on their permeability constants P_K, P_{Na}, and P_{Cl}:[33]

$$\psi = RT/F \ln\{(P_K[K^+]_{in} + P_{Na}[Na^+]_{in} + P_{Cl}[Cl^-]_{ex})/(P_K[K^+]_{ex} + P_{Na}[Na^+]_{ex} + P_{Cl}[Cl^-]_{in})\}. \quad (21)$$

Subsequent studies by Hodgkin and Horowicz[18] revealed that the steady level of the potential is not influenced by external Cl^- concentration as predicted in Eq. 20. Katz, co-author of that equation, then elected to drop its chloride terms on the ground that "chloride ions are in equilibrium in resting muscle."[29] Reserving for a paper published elsewhere my comments on the validity of such a deletion,[34] I give here the Hodgkin-Katz equation in the revised form attributable to Katz:

$$\psi = RT/F \ln\{(P_K[K^+]_{in} + P_{Na}[Na^+]_{in})/(P_K[K^+]_{ex} + P_{Na}[Na^+]_{ex})\}. \quad (22)$$

The values of P_K and P_{Na} as given by Katz and cited in Table II are, respectively, 3.80 h^{-1} and 0.063 h^{-1}. The gas constant is equal to 8.614×10^{-2} mV-Faraday. At 25°C, T is 298. Therefore

$$\text{I}) = 8.614 \times 10^{-2} \times 298 \ln\{(3.8 \times 0.063 \times 16.9)/(3.8 \times 2.5 + 0.063 \times 104)\} = 87.6 \text{ mV}. \quad (23)$$

The values of ionic concentration used here are averages of all available data up to 1959:[7] $[K^+]_{in}$, 128 mM; $[Na^+]_{in}$, 16.9 mM; $[K^+]_{ex}$, 2.5 mM; and $[Na^+]_{ex}$, 104 mM.

Using the ***average*** value of P_K and P_{Na} from all old data in the literature collected in Table II, we have

$$\psi = 25.7 \ln\{(2.97 \times 128 + 0.13 \times 16.9)/(2.97 \times 2.5 + 0.13 \times 104)\} = 74.7 \text{ mV}. \quad (24)$$

Using the new value of P_{Na} from the present investigation, we find

$$\psi = 25.7 \ln\{2.97 \times 128 + 1.28 \times 16.9)/(2.97 \times 2.5 + 1.28 \times 104)) = 27.1 \text{ mV}. \quad (25)$$

The normal resting potential of frog muscle cells measured by a microelectrode filled with 3 M KCl and in the presence of 2.5 mM external K^+ and about 104 mM Na^+ at room temperature is in the range of 85 to 95 mV.[35,36] The revised version of the Hodgkin-Katz equation, when fitted with the correct values of the permeability constants for K^+ and Na^+, thus predicts a resting potential of only 27.1 mV!

This failure of the revised Hodgkin-Katz equation to explain the magnitude of the resting potential arises from a value of P_{Na} much larger than was once thought and indicates that the resting potential is not dependent on membrane permeability. Indeed, this is the third time that the conclusion of non-dependency has been reached (see refs. 36, 37). For an alternative theory of the resting potential according to the association-induction hypothesis, see refs. 7, 34, 37-41.

3. *Energy requirement* of *the Na pump.* Levi and Ussing[1] calculated the energy re-

TABLE II. A Comparison of the Present Data for the Inward Permeability Constant P_{in} and Inward Permeation Rate Constant k_2 With Representative Examples of Previously Published Data From This and Other Laboratories (For sources of data, see text.)

Ion	Author	k_2 (h^{-1})	Average	P_{iw} (cm/sec)	Relative permeability
K^+	Mullins, 1959	1.92	2.97	9.69×10^{-7}	1.
	Ling, 1962 (25°C)	3.06		15.4×10^{-7}	
	Ling and Ochsenfeld, 1965 (24°C)	3.10		15.6×10^{-7}	
	Katz, 1966	3.80		21.1×10^{-7}	
Rb+	Mullins, 1959	1.04	1.16	5.25×10^{-7}	0.39
	Ling, 1962 (25°C)	1.27		6.51×10^{-7}	
Cs^+	Mullins, 1959	0.11	0.24	0.556×10^{-7}	0.081
	Ling, 1962 (25°C)	0.36		1.81×10^{-7}	
Na^+	Mullins, 1959	0.085	0.13	0.429×10^{-7}	0.044
	Ling, 1962 (20°C)	0.15		0.758×10^{-7}	
	Harris, 1950 (18°C)	0.22		1.22×10^{-7}	
	Katz, 1966	0.063		0.35×10^{-7}	
Na+	(present paper)	1.28		6.47×10^{-7}	0.43

quirement of the Na pump based on their labeled Na^+ efflux data and the assumption that the slow fraction with a half-time exchange of 34 min represents the intracellular-extracellular exchange. The minimum energy consumption rate for the Na pump they obtained was 50 cal/kg/h. Harris[31] made a minor correction and arrived at an energy consumption rate of 45 cal/kg/h. Both sets of data are not incompatible with the resting output of frog muscles, estimated at 170 cal/kg/h.

The present investigation has shown that the slow fraction is not rate-limited by membrane permeability and that it is the fast fraction with an average half-time of exchange of 3.17 min that truly represents the rate of intracellular-extracellular exchange. This major revision alters the concept of the energy requirement for the pump substantially. Indeed the minimum energy requirement of resting frog muscle is not as previously calculated but equal to $45 \times 34/3.17 = 482$ cal/kg/h, which is 283% of the energy available!

This energy discrepancy, of course, is in accord with the energy need calculated for muscles under carefully controlled conditions whereby external energy sources were completely blocked. The minimum energy need under those conditions was found to be from 1500 to 3000% of the energy available.[7]

It may be pointed out that several attempts have been made to reduce the energy requirements of the Na pump. These include postulation of an exchange diffusion mechanism[42] and of sequestration of Na^+ in the sarcoplasmic reticulum.[5,6] Both have been contradicted by experiments.[11,24,43,44]

A more recent attempt to reduce the energy need involved a nonenergy-consuming pumping mechanism; i.e., the energy required to pump out Na^+ is fully recovered by the down-gradient movement of Na^+ into the cell.[45] This postulated mechanism is reminiscent of Maxwell's famous demon, which maintains concentration differences in two contiguous phases without energy consumption. Like all previously designed "perpetual motion" machines, it can at best be regarded as highly unlikely. Experimentally it has long been known that ouabain would poison an Na pump and render it nonfunctional; nevertheless ouabain has no effect on the inward Na^+ flux.[46] If Na^+ influx can indeed regenerate energy used in pumping, it must be mediated by the pump and thus would be sensitive to ouabain poisoning, contrary to fact.

Still a fourth possible remedy is worth considering (though to my knowledge it is not cited in the literature). That is, if Na^+ is pumped out not as a cation but accompanied by an equivalent concentration of the anion chloride, the outward pumping would no longer be against an electrical gradient. To some extent such a postulation is, I believe, theoretically more reasonable and could indeed reduce the energy requirement of the pump--although not by too much, because the chloride would then have to be pumped out against a concentration gradient. I have shown that the electrical gradient is 90 mV and the concentration gradient for Na^+ is equivalent to 58 mV. The chloride gradient for resting frog muscle in a normal Ringer solution is about equal to the Na gradient; by pumping Na^+ and Cl^- together, the energy requirement would be reduced by a factor of $1 - (58 + 58)/(90 + 58) = 20\%$. This energy saving is of modest significance when the discrepancy between energy demand and supply is in the realm of several hundred to several thousand percent, even if we totally disregard the concomitant energy needs of the long list of other pumps postulated along with nonpump energy requirements of the living cell.[10]

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