HOW DOES OUABAIN CONTROL THE LEVELS OF CELL K^+ AND Na^+? BY INTERFERENCE WITH A Na PUMP OR BY ALLOSTERIC CONTROL OF K^+-Na^+ ADSORPTION ON CYTOPLASMIC PROTEIN SITES?

GILBERT N. LING

Department of Molecular Biology, Pennsylvania Hospital, Eighth and Spruce Streets, Philadelphia, Pennsylvania 19107

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SUMMARY

1. A technique called the EMOC technique is described which permits the study of the concentration distribution of labeled sodium in a frog sartorius muscle after the cut tibial end of the muscle has been exposed to a solution containing labeled sodium.

2. Two types of experiments showed that there was no regeneration of cell membrane at the cut end or within the muscle cells after up to 44 hours of incubation at 25°C. There was no significant difference in the volume of extracellular space or total water content between the control and ouabain muscle.

3. Inclusion of ouabain (10^{-6} to 10^{-4} M) in the source solution increased the uptake of labeled sodium by the muscle. At the locus of maximum difference, the ouabain muscle could gain 30 mmoles/kg more labeled sodium than the control.

4. The accumulation of labeled sodium in a second pool of unlabeled Ringer solution bathing the intact end of the muscle was studied. The results contradict the theory that the lower sodium uptake in the control muscle results from a more effective unloading of labeled sodium into the source solution via the extracellular space. Were this the case, more labeled sodium would also have been unloaded into the second pool of the control muscle than that of the ouabain muscle. The reverse was observed.

5. The concentrations of labeled sodium in the extracellular spaces of the ouabain and control muscles were assayed with a new centrifugation method. The results showed that the level of labeled sodium in the extracellular space of the control muscles was far beneath that required by the pump model to explain the lower level of sodium in the control than that in the ouabain muscle.

6. These experimental findings and a theoretical inconsistency contradict the theory that the low level of sodium found in frog muscle cells is maintained by a sodium pump and that ouabain increases cell sodium by hampering the pumping.

7. The data agree with the alternative concept that the low sodium level in normal cells reflects a low solubility of sodium in the cell water and a preference of anionic ad-

sorption sites ror potassium over sodium. uuabaln Increases cell sodium because it d e creases the bias against sodium in the adsorption.

INTRODUCTION

Three types of explanations have been offered to interpret the maintenance of a high concentration of K^+ and a low concentration of Na^+ in virtually all living cells: (a) the absolute impermeability of the cell membrane to Na^+ but not to K^+;[1] (b) continued, energy-consuming concentration of K^+ in, and extrusion of Na^+ from the cell by coupled Na-K pumps located in the cell membrane;[2–4] and (c) the selective preference of protoplasmic adsorption sites for K^+ over Na^+ and the low solubility of Na^+ in cell water, which exists in a state of polarized multilayers.[5–10] In the first explanation, Na^+ in the cell and in its external environment are thought to be thermodynamically isolated from each other; in the second explanation, intracellular and extracellular K^+ and Na^+ are thought to be in a steady state. In the third explanation, they are thought to reflect a state of metastable equilibrium. There is no other known mechanism to cause an unequal distribution of a finite amount of a chemical substance in contiguous spaces.

Experimental evidence has long invalidated the first explanation.[11,12] A clearcut choice between the equilibrium-state model and the steady-state model, which are fundamentally opposed to each other and irreconcilable, is urgent.[13] The adoption of a fundamentally incorrect model will almost certainly doom many promising biomedical investigations.

In recent years, growing attention has been focused on the effect of ouabain in increasing intracellular Na^+ concentration at the expense of K^+.[14] Proponents of the membrane theory emphasize evidence that ouabain increases intracellular Na^+ concentration by inhibiting the postulated Na-K pump.[15–17] However, this evidence can be interpreted in other ways. According to the association-induction hypothesis, ouabain causes a displacement of adsorbed K^+ in the cell by Na^+ because this drug acts as a "cardinal adsorbent," increasing the relative preference for Na^+ on cooperatively linked protein sites in the cell.[8,18–22] In the present paper we report decisive experimental results.

METHODS

A voluntary muscle cell is much longer than it is wide. If we amputate one end of the cell and bring only this cut end into contact with an aqueous medium while the intact part of the cell is kept in air or petroleum jelly, the cell membrane and its postulated pumps, although anatomically intact, are physiologically no longer functional because the surrounding air or petroleum jelly, unlike an aqueous solution, cannot serve as source or

sink for solutes pumped. Such a preparation will be referred to as an Effectively Membraneless Open-ended Cell, or in brief, as an EMOC Preparation. Theoretically speaking, an isolated single muscle fiber of the giant barnacle would be better for this purpose. Unfortunately, we have not yet succeeded in developing a method that will enable an amputated barnacle muscle cell to survive long enough for the present purpose. Therefore, we chose instead the sartorius muscles of northern American leopard frogs. There is a drawback in this choice, because a sartorius muscle does not represent pure muscle cells but also contains a sizable extracellular space. As indicated below, however, the complexity introduced by the extracellular space is resolvable.

Figure 1 shows two variations of the EMOC setups. The key feature is the silicone rubber seal (RTV 118 translucent sealant, General Electric) at the bottom of the glass tube. A narrow slit in this seal snugly fits the sartorius muscle partially drawn through it. To make the close fitting slits I found it convenient to use templates of parafin strips of the same dimension as the sartorius muscles.

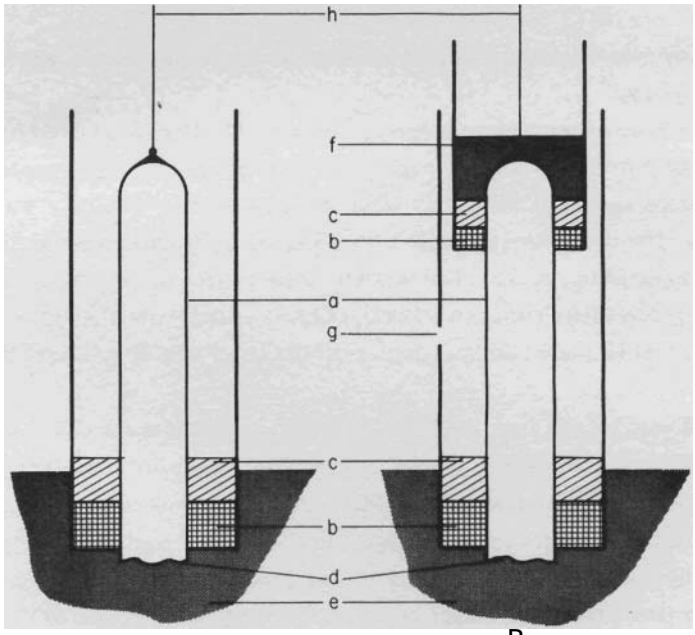

Figure 1. Diagrams of plain EMOC tube (A) and of doubledeck EMOC tubes (B). (a) sartorius muscle with intact pelvic end fixed with thread (h) and cut end (d) exposed to labeled solution (e); (b) silicone rubber with slit; (c) vaseline seal; (f) auxiliary pool of unlabeled Ringer-GIB medium; (g) air vent.

Before filling the tube partially with vaseline (usually to a depth of about 5 mm above the top of the silicon rubber seal), a piece of No. 6 Deknatel surgical silk thread was drawn through the slit. This piece of thread was later tied to one end (in most cases the tibial end) of the muscle and used to guide it through the slit. Another piece of thread tied to the other end of the muscle used to position the muscle in the tube so that it would remain straight at its natural length but not touching the wall of the tube.

After the tibial protruding end of the muscle was cut about 1.0 mm from the outer edge of the silicone rubber seal, the EMOC tube was lowered into a vial containing the Ringer solution labeled with ^{22}Na until the cut end of the muscle was immersed in the solution. The entire assembly was then placed in an air tight jar filled with humidified 95% O_2 and 5% CO_2 and shaken gently in a constant temperature room maintained at 25°C ± 1°C.

Figure 1B shows a double-decker EMOC setup. In this case a constriction in the wider lower tube held in place a narrower tube with a similar silicone-rubber vaseline seal. Ringer solution was added on top of the vaseline layer to serve as an "auxiliary pool." In this arrangement, the intact end of the sartorius muscle was in contact with the auxiliary pool on the top, while the cut end of the muscle was in contact with the source solution at the bottom.

Strict sterility was maintained in all operations as a sartorius muscle, when contaminated, dies rapidly at 25°C.

At the end of the experiments the muscle, without blotting, was placed on a piece of cardboard covered with glazed graph paper. The mm ruling of the graph paper permitted sections of 2 mm to be cut accurately after the muscle-paper assembly was frozen in liquid nitrogen. The cut sections usually 5 to 10 mg in weight were then stored in liquid nitrogen before weighing. A 5% trichloroacetic acid extract of the muscle section was assayed for its radioactivity either on a Nuclear Chicago Automatic 7-scintillation counter or on a Packard 314E β-scintillation counter. In the latter case Bray's scintillation fluid was used.

The method used for the analysis of the labeled Na^+ content in the extracellular space, to be described in full elsewhere as part of a new technique for measuring the size of extracellular spaces and "extractible" water from cells etc., involved centrifugation of the muscle tissue resting on filter paper in a humidity-controlled packet. It was shown that over a wide range of centrifugal forces, as well as duration of spinning, a healthy muscle will lose a constant amount of weight which, in quantity, corresponds to that obtained by three other newly introduced methods for the assay of extracellular space in frog muscles.[23-25] The labeled Na^+ concentration in this extracellular fluid was then calculated from the ^{22}Na collected on the filter paper assembly and from the weight loss of the muscle. The extracellular space volume varied between 5 and 10% of the muscle and the labeled Na^+ concentration in the extracellular space was, within an error of 6%, the Na^+

concentration in the bathing solution (103% ± 3.4%, n = 16).

All experiments were performed on the isolated sartorius muscle from well-fed northern American leopard frogs (Rana *pipiens pipiens*, Schreber). The Ringer solution used contained Na (100 mM), K (2.5 mM), Ca (1.0 mM), Mg (1.2 mM), PO_4 (2.7 mM), HCO_3 (15.7 mM), Cl (88.7 mM), SO_4 (0.8 mM), NO_3 (0.1 mM), glucose (23.5 mM), 20 amino acids, 14 vitamins, and reduced glutathione (50 μM).[26] No antibiotic was added. In the high magnesium Ringer solution used, the concentrations for Na^+, Mg^{++}, and Cl^- were 19.6, 72.0, and 149.7 mM respectively.

[22]Na was from International Chemical and Nuclear Corporation, Irvine, California; ouabain octahydrate and collagenase (*Clostridium* Welchii) from Sigma Chemical Co., St. Louis, Missouri.

RESULTS

Are All Muscle Fibers of Equal Length in a Frog Sartorius EMOC Preparation?

The muscle fibers in a frog sartorius muscle are not all equal in length because the tibial end of the muscle (unlike the pelvic end) tapers. Difference in length of the muscle fibers due to the uneven tibial end, however, is insignificant because in the EMOC preparation the tibial end of the muscle is amputated at about 1 cm from the extreme tip. This amputation transforms the shape of the muscle from a trapezoid to that of a rectangle, since the cut is located more proximally than the tapering tibial end.

However, it is conceivable that some muscle fibers may not reach all the way from one end of the muscle to the other. This would be the case, if for example, these fibers have long tendons. To find out whether this is the case, I have dissected sartorius muscles, fiber by fiber and measured the lengths of all fibers with both ends intact. This operation was greatly facilitated by prior incubation of the muscle in a Ringer solution containing collagenase (100 units/ml) and by dissecting the muscle so treated in a high Mg^{++} Ringer solution, in which the muscle fibers are not excitable. Using this method, I have dissected a total of five sartorius muscles in the course of which, I did not encounter a single fiber that was significantly shorter than its immediate neighbors.

Paired Sartorius Muscle as Controls

Figure 2 shows the labeled Na^+ distribution in four pairs of sartorius muscles after the cut ends of these muscles were exposed to similar ^{22}Na-labeled, Ringer-GIB medium for 20 hours at 25°C.[26] There is some variability among different pairs of muscles (determined primarily by the health of the frog); more so than between pairs of muscles from the same frog. For this reason, each experiment to be described here had as its control the paired sartorius muscle from the same frog; though to economize, the data are often pooled, as in Figures 3 and 4.

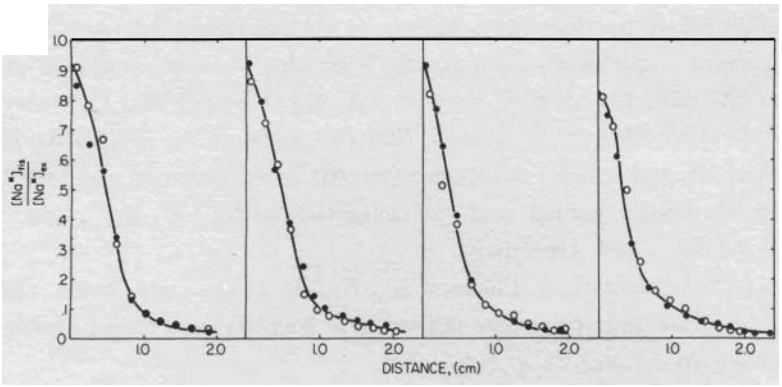

Figure 2. Concentration distribution of labeled Na^+ in paired sartorius muscles. Exposure was 20 hours at 25°C. Data show agreement in uptake between paired muscles. Abscissa represents distance from cut end; ordinate, the ratio of labeled Na^+ in cell water as a fraction of final equilibrium concentration of labeled Na^+ in the source solution (ca. 100 mM).

Effect of Ouabain on the Movement of Labeled Na^+ into Muscles Through the Cut Ends

Figure 3 shows that after 5 hours (25°C) of exposure, the presence of 10^{-4} M ouabain in the solution bathing the cut end of the muscle enhanced uptake of labeled Na^+ from the solution. At the region 6 mm from the cut end, the ouabain-treated muscle took up some 30 mmoles/kg more of labeled Na^+ than did the control muscle. Figure 4 shows that exposure to 10^{-5} M ouabain for 44 hours at 25°C brought about still greater extra uptake in the normal muscle in areas farther away from the cut end. Other experiments showed that exposure to 10^{-6} M ouabain produced similar results.

Membrane Regeneration and Other Possible "Trivial" Causes for Ouabain-Induced Enhancement of Labeled Na^+ Uptake

Enhanced uptake of labeled Na in ouabain-treated muscles could be due to the following trivial causes: (1) membrane regeneration in the cut normal muscle but not in the cut ouabain-treated muscle; (2) ouabain causes an increase of extracellular space; and (3) ouabain causes cell swelling. To test each of these possible causes, I carried out the following experiments:

1. Membrane Regeneration in Normal Cut Muscle

a. The cut end of a sartorius muscle in an EMOC tube was exposed to an unlabeled Ringer-GIB solution for 24 hours. The cut end was then exposed to 10 mM labeled sucrose for 2 hours (25°C) and compared to its pair, which was exposed to a similar solution immediately after the tibial end was cut. In four sets of experiments, the control uncut muscles took up 0.073 ± 0.013 μmoles/g; freshly cut muscles took up 0.174 ± 0.031

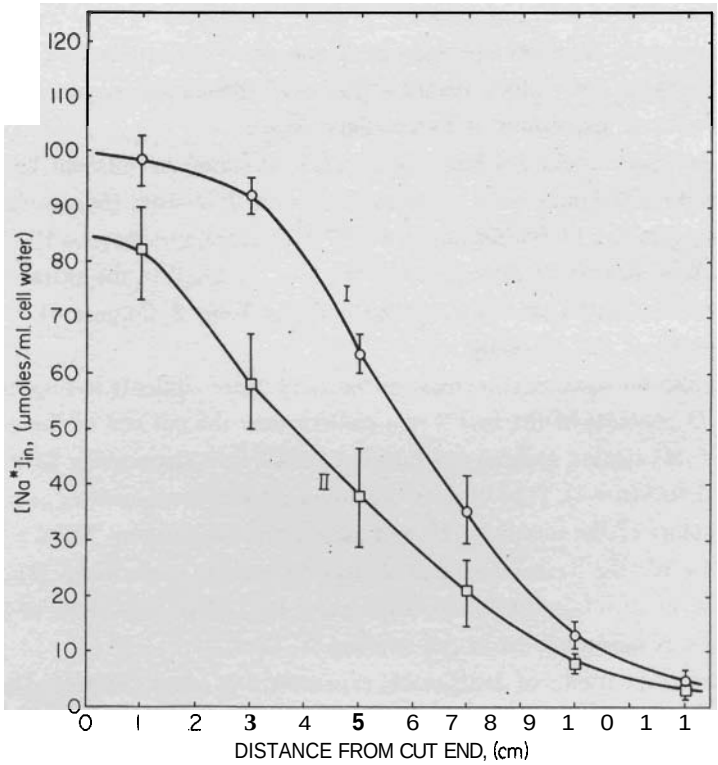

Figure 3. Concentration distribution of labeled Na^+ in muscles exposed to 10^{-4} M ouabain (Curve I) and control muscle (Curve II). Exposure lasted 5-6 hours (25°C). Muscles were 2 cm in length. Ordinate is in units of μmoles labeled Na^+ per gram of cell water. Abscissa, same as in Figure 2. Average of four sets of experiments. Distance between horizontal bars around each point represents 2x standard error. Concentrations of labeled Na^+ in sections beyond 1.1 cm from the cut end are roughly equal in the control and experimental muscle and are not shown.

μmoles/g; experimental cut and incubated muscles took up 0.169 ± 0.016 μmoles/g.

b. In another four sets of experiments, the experimental muscles were mounted in the EMOC tubes, the tibial ends amputated and exposed to unlabeled Ringer solution for 51 hours at 25°C. These experimental muscles and their freshly cut pairs were both exposed to ^{22}Na-labeled Ringer solution for 28 hours at 25°C. The uptake concentrations of labeled Na^+ were 22.3 ± 5.1 μmoles/g; those of their controls were 22.7 ± 2.5 μmoles/g.

c. In another series of studies the experimental muscle, after incubation for 43 hours at 25°C, was partly pulled out and a fresh cut was made at 3 mm below the region of the muscle where the greatest drop of labeled Na^+ concentration 'occurred (at about 1 cm from the cut end in Figure 4). These muscles and their freshly amputated pairs were exposed for 27 hours at 25°C to Ringer-GIB solution containing 10 mM labeled D-arabin-

ose. Five experimental muscles took up 3.53 ± 0.20 μmoles/g while their five control muscles took up 3.28 ± 0.20 μmoles/g.

These experiments show no regeneration of cell membrane, either at the cut end, or within the cell, which could block inward migration of labeled Na^+ in the control muscle.

2. Ouabain-Induced Expansion of Extracellular Space

Extracellular space could not have significantly increased by ouabain because there was (a) no increase of inulin space in muscles treated with ouabain [four control muscles gave inulin space 9.8% ± 1.0% while ouabain (10^{-4} M) treated muscles gave 10.6% ± 1.8%], and (b) a lack of significant difference (at the p = 0.1 level) in the extracellular fluid volume determined by the centrifugation method (see Table 2, Column 4).

3. Ouabain-Induced Cell Swelling

There was also no significant increase in the total water contents in response to ouabain. The H_2O contents of the first 3 mm sections near the cut end of the control and ouabain (10^{-5} M) treated muscles (44 hours at 25°C) were respectively 83.9% ± 0.25% and 83.2% ± 0.70% (n = 4). Thus ouabain did not cause additional swelling at the cut end. The H_2O contents of the remaining 17 mm away from the cut were 79.0% ± 0.68% and 78.0% ± 0.84% for the normal and ouabain-treated muscles respectively. Since ouabain does not cause an expansion of extracellular space, the lack of an increase of total water indicates a lack of ouabain-induced cell swelling.

Figure 4 shows the results of double-label experiments in which ^{3}H-labeled D-arabinose was included in the source solution containing ^{22}Na. From the locus where the maximum difference in labeled Na^+ uptake is seen to the intact end of the muscle, the labeled D-arabinose intake in the control and that in the ouabain-treated muscles are not distinguishable. In the region closer to the cut end, the D-arabinose uptake is actually higher in the control muscle. These findings reaffirmed the above conclusions; they emphasized that the ouabain-effect is specific to Na". The enhanced uptake of labeled Na^+ is not due to any one of the trivial causes.

Testing the Mandatory Predictions of the Membrane Pump Theory

According to the membrane pump theory, the cytoplasm is, in essence, a protein solution, indifferent to ouabain. Ouabain increases intracellular Na^+ by interferring with the Na pump in the cell membrane. These basic assumptions of the membrane theory demand that the smaller uptake of labeled Na^+ in the control muscle be due to more effective pumping, while the ouabain-poisoned muscle with its pumps slowed down retain more of the labeled Na^+ that has entered the cut end.

Shifting intracellular labeled Na^+ into the extracellular space—the only thing the membrane pump can do in the EMOC—is in itself insufficient to account for the observed difference in labeled Na^+ uptake, for the labeled Na^+ concentrations presented in Figures 3 and 4 refer not exclusively to intracellular Na^+, but to the sum of intracellular and

extracellular labeled Na^+. In order to explain the observed difference of total labeled Na^+ uptake shown in Figures 3 and 4, a second mandatory requirement is that the Na^+, thus pumped into the extracellular space, must be able to diffuse back into the source solution where it originally came from.

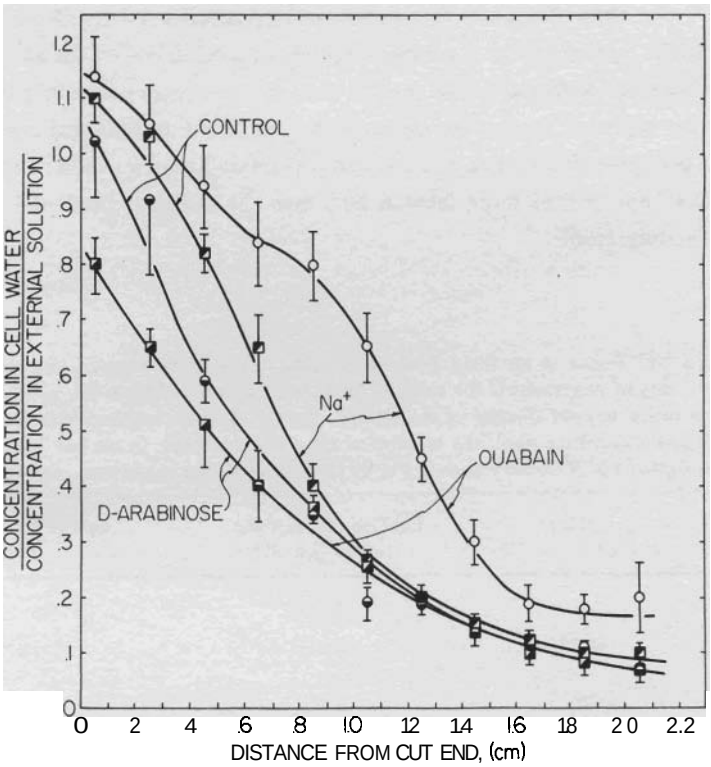

Figure 4. Concentration distribution of labeled Na^+ and D-arabinose in muscle exposed to 10^{-5} M ouabain and their controls. Exposure lasted 44 hours (25°C). Data are average of four sets of muscles. Ordinate is concentration in cell water as a fraction of final external concentrations, which were 100 mM for Na^+ and 9 mM for D-arabinose.

"Auxiliary Sink" Experiment: We introduced our auxiliary pool or sink containing no labeled Na^+ at the intact end of the muscle (Fig. 1B) in order to determine whether there is indeed more effective unloading of labeled Na^+ in the control muscle than in the ouabain-treated muscle via the extracellular space.

As Figure 4 shows, after 44 hours of exposure to 10^{-5} M ouabain, the largest difference in labeled Na^+ concentration occurs in the middle of the muscle preparation. Thus, pumps

in the middle of the control muscle must have been effectively engaged in the pumping if the membrane pump model is correct. Once the labeled Na" reaches the extracellular space, part of it returns to the muscle cells. The remaining labeled Na^+ in the extracellular space diffuses both toward the cut end (bathed in the source solution) and toward the intact end. The presence of 100 mM of labeled Na^+ in the source solution hampers the unloading of labeled Na^+, a disadvantage not shared by the auxiliary pool, which contains no labeled Na^+. One thus anticipates that a mandatory prediction of the membrane pump model would be an accumulation of a larger quantity of labeled Na^+ in the auxiliary pool of the control muscles, with its healthy pumps, than in its ouabain-poisoned pair. Typical results from two types of studies (using somewhat different techniques) are shown in Table 1. Both demonstrated that it was the ouabain-treated muscles rather than the normal muscles that transported more labeled Na^+ into the auxiliary pool—the difference sometimes exceeding 100%.

Table 1. Labeled Na^+ found in auxiliary pool of muscles treated with ouabain and their control pair. In the first 3 sets of experiments the simple EMOC tube shown in Figure 1A was used, but the vaseline layer was raised to near the top of the muscle. The vaseline was covered with 0.2 ml Ringer solution to serve as the auxiliary pool; the incubation time was 24 hours. In the last 3 sets of experiments the double-decker EMOC tubes shown in Figure 1B were used; the incubation time was 69 hours.

Experiment Number	Ouabain Concentration (M)	Labeled Na^+ Uptake in Muscles (μmoles/g)	Labeled Na^+ found in Auxiliary Pool (μmoles)
2D12I	0	31.1	0.5
2D12II	10^{-}	43.1	1.18
2D12III	0	33.3	0.50
2D12IV	10^{-4}	37.1	1.42
2D12V	0	25.0	0.48
2D12VI	10^{-4}	40.1	1.51
2K131C	0	30.6	1.90
2K131D	10^{-5}	39.4	3.12
2K131E	0	32.9	2.14
2K131F	10^{-5}	44.7	2.33
2K131G	0	32.9	2.03
2K131H	10^{-5}	42.5	3.22

Labeled Na^+ Concentration in the Extracellular Space: Requirement According to Theory and Experimental Results: Since there are no longitudinal oriented pumps in the extracellular space, labeled Na^+ can only return via the extracellular space by diffusion. To move significant amounts of Na^+ back into the source solution, however, the concentration of labeled Na^+ in the extracellular space must be built up to a level higher than that in the source solution, which contains approximately 100 mM of labeled Na^+.

The first four columns of Table 2 present some details of the experiment graphically described in Figure 3. Here we found a difference in uptake between the labeled Na^+ in the ouabain-treated muscle and that in the control muscle. We may ask, "How high must the labeled Na^+ concentration in the extracellular space of the control muscle be built up in order to return by diffusion into the source solution these extra amounts of labeled Na^+?"

To answer this in the simplest manner, we make use of a well-known equation (Eq. 1)[27] which describes the relation between the (extracellular space) diffusant concentration (C), the self-diffusion coefficient of the diffusant (D), and the total amount of the diffusant (M_t) that has diffused out of the system after time t:

$$M_t = 2C\sqrt{\frac{Dt}{\pi}} \qquad (1)$$

Now D for Na^+ at 25°C is 1.28 x 10^{-5} $cm^2/sec.$[28] Remembering that C, the concentration, is expressed in moles/cc, one can readily calculate that for the first four sets of data given in Table 1, the extracellular space would have to be maintained at a labeled Na^+ concentration of 192, 730, 520, and 248 mM in order to unload the extra labeled Na^+ observed. These figures were calculated, however, from Eq. 1, describing diffusion, into a pool containing no labeled Na^+. In reality, the labeled Na^+ had to diffuse into a pool already containing 100 mM of labeled Na^+. The required concentration in the extracellular space must thus be still higher than those cited. A minimum requirement is that they must each be 100 mM higher—i.e., 292, 830, 620, and 348 mM respectively for the four muscles, with an average of about 500 mM. The corresponding average for the last four sets of data in Table 2 is 395 mM.

Table 2 shows that after five hours of incubation, the actual concentrations of labeled Na^+ measured in the extracellular spaces of the normal control muscles were not higher than that found in their pairs treated with 10^{-4} M ouabain; and, in the control muscles, the labeled Na^+ concentrations in the extracellular spaces were far below the minimum required concentration to bring about a return of the extra labeled Na^+ "missing" in the control muscle as compared with the ouabain-treated muscle.

Table 2. Comparison of experimentally found labeled Na^+ concentrations in the extracellular space ($[Na^+]_{e.c.s.}$) in the control muscle with minimum $[Na^+]_{e.c.s.}$ theoretically required by the membrane-pump theory. First four sets of experiments were the same as in Figure 3. **Duration** of all experiments listed in Table, ***5-6*** hours. Minimum theoretically required $[Na^+]_{e.c.s.}$ were calculated from Eq. 1 plus 100 **mM.** Average values of ***395*** **mM** refer only to last four sets of data. Only $[Na^+]_{e.c.s.}$ in the control muscles were included in the average of the last column.

Exp. Number	**Ouabain** Concentration (M)	Labeled Na^+ Uptake (μmoles)	Extracellular Space (%)	Ouabain-Induced Extra Uptake of Labeled Na^+ (μmoles)	$[Na^+]_{e.c.s.}$ (mM) Theoretical Minimum	$[Na^+]_{e.c.s.}$ (mM) Experimentally Found
2C17A	0	0.96			292	
2C17B	10^{-4}	1.20		0.24		
2C17C	0	0.81			830	
2C17D	10^{-4}	2.14		1.33		
2C17E	0	0.62			620	
2C17F	10^{-4}	1.51		0.89		
2K20C	0	1.09			348	
2K20D	10^{-4}	1.54		0.45		
2K201G	0	0.79	6.3		350	52.8
2K201H	10^{-4}	1.28	3.4	0.49		(113)
2K201I	0	0.74	8.3		298	51.3
2K201J	10^{-4}	1.05	6.5	0.31		(43.2)
2K201K	0	0.86	8.7		245	50.9
2K201L	10^{-4}	1.10	5.2	0.24		(95.2)
2K201M	0	0.63	9.6		685	38.6
2K201N	10^{-4}	1.48	8.4	0.85		(37.4)
Mean ± S.E.					395 ± 99	48.4 ± 3.29

DISCUSSION

The last two sets of experiments show that in these EMOC preparations the smaller uptake of labeled Na^+ in the control muscle is not due to a more vigorous pumping of labeled Na^+ into the extracellular space, from where it is then unloaded back into the original source solution.

There is, in addition, a theoretical difficulty which appears in and of itself, to represent evidence against the pump interpretation. The membrane-pump model demands that a high concentration of labeled Na^+ must be built up in the extracellular space before a significant quantity of labeled Na^+ could be unloaded into the source solution. However, a solution containing Na^+ at a concentration significantly higher than that in a Ringer solution is hypertonic. In such a hypertonic solution, muscle cells rapidly lose water to re-establish osmotic balance. This water movement, therefore, tends to reduce the level of Na^+ concentration in the extracellular space to the same (isotonic) concentration as that in the source solution. At this concentration there can be no effective delivery of labeled Na^+ from the extracellular space into the source. The only effect of this futile pumping would be a gain in extracellular space at the expense of the cell volume. However, even this prediction is not substantiated by the data on D-arabinose distribution and other experiments described above. These showed that the volume of the extracellular space is indifferent to ouabain.

This basic theoretical inconsistency in conjunction with the two negative experiments throw serious doubts on the theory that ouabain controls cell K^+ and Na^+ by poisoning the postulated outward Na pump.

One may, however, offer another alternative interpretation without abandoning the fundamental concepts of the membrane theory. Let us assume that the mobilities of Na^+ in the cytoplasm of ouabain-treated as well as in normal muscles are similar and that both are lower than that in the extracellular space. We then postulate that more labeled Na^+ accumulates in the ouabain muscle as a result of an ouabain-induced increase of inward Na^+ permeability of the cell membrane. However, experiments described above ruled out this interpretation also.

If the extra gain of labeled Na^+ in the ouabain muscle is due to more labeled Na^+ entering into the muscle cells via the extracellular space, we then anticipate the following: (a) the level of labeled Na^+ in the extracellular space would be lower in the ouabain-treated muscle than in its normal control; (b) in the ouabain-treated muscle less labeled Na^+ will be found in the auxiliary pool placed at the opposite end of the muscle.

In fact the data of Column 7 of Table 2 shows that the level of labeled Na^+ in the extracellular space is, if anything, higher in the ouabain muscle than in the control muscle. The data of Table 1 show that less, not more, labeled Na'' reached the auxiliary pool of the ouabain-treated muscle.

INFERENCES

Steady-State Model

According to the membrane theory the level of Na^+ (and K^+) are maintained at a low steady-state level by a balance of passive inward permeability of the cell membrane and active outward extrusion by postulated pumps also in the cell membrane. The cytoplasm is considered to play no significant role. In the confine of these tenets, there can be only two ways for ouabain to bring about a reversible rise of cell Na^+ level at the expense of cell K^+: (1) to increase the rate of passive inward Na^+ permeability and (2) to decrease the rate of outward pumping.

Experiments described above, however, show that neither is the case. In addition, three possible "trivial" causes for the observed greater uptake of Na^+ in the ouabain-treated muscle were also considered: membrane regeneration at the cut surface or within the cytoplasm; ouabain-induced increase of extracellular space; and ouabain-induced cell swelling. Each of these was ruled out on the basis of at least two sets of independent evidence.

From these reasonings we must infer firstly that ouabain does not control the level of cell Na^+ by altering membrane functions. Since the basic tenets of the membrane theory dictate that ouabain must control membrane functions in order to increase cell Na^+ level, we must infer secondly that the basic tenets of the membrane theory are now seriously questionable.

Equilibrium Models

The experimental contradiction and theoretical inconsistency leave us with no other alternative but to reject the steady-state membrane model and to turn to the equilibrium models which on account of their not being dependent on a continuous energy supply, do not suffer from one of the serious difficulties that all the steady-state models confront: i.e., the insufficiency of energy supply as seen in amphibian voluntary muscle[6,9] and in the microbe, Escherichia *coli*.[29]

In general principles, the association-induction hypothesis, in harmony with the theory of Ernst[30] and the sorption theory of Troschin,[10] ascribes the seat of the asymmetrical distribution of K^+ and Na^+ to the cytoplasm. More specifically, it suggests that the increase in Na^+ uptake in the muscle brought about by ouabain reflects a change in the nature of the exposed cytoplasm from one permitting the entry of less Na^+, to one permitting the entry of more Na^+.[6-9]

In the association-induction hypothesis, low Na^+ concentration in the normal living cell reflects two distinct, though closely associated, features of the protoplasm: (1) the protein sites prefer K^+ over Na^+ by a factor of about 100—the intrinsic equilibrium con-

stant, $K^{\infty}_{Na \to K}$ ~ 100; (2) the cell water, existing as multilayers on the proteins, has a low solubility for solutes such as Na^+ and sugars. There are, a priori, two different ways to increase uptake of Na^+: (a) by returning cell water to a state closer to that of liquid water and/or (2) by decreasing the value of $K^{\infty}_{Na \to K}$.

Ouabain, at the concentration employed, apparently did not significantly change the resting state of cell water. Otherwise, there would have been a substantially greater uptake of D-arabinose, labeled Na^+, and sucrose through the cut end of the ouabain-treated muscle than that of the control muscle. Since this greater uptake did not occur, one must conclude that ouabain decreases $K^{\infty}_{Na \to K}$ and consequently causes the displacement of K^+ from protein sites by labeled Na^+.

One recalls that this is the conclusion drawn from other independent experiments by Ling," by Ling and Bohr,[18] and by Gulati and Jones[Z2] several years before the EMOC technique was developed. Thus, Ling showed that digoxin (lanoxin) at 6.6×10^{-6} M reduced $K^{\infty}_{Na \to K}$ in frog muscles from 100 to 5. Ling and Bohr showed that ouabain at 10^{-6} M reduced $K^{\infty}_{Na \to K}$ in frog muscles from 140 to 11. Gulati and Jones showed that ouabain at 10^{-6} M reduced $K^{\infty}_{Na \to K}$ in canine vascular smooth muscle from 90 to 3.

In the association-induction hypothesis, the entire living cell is seen as sharing, the properties of a proteinaceous fixed charge system. Ionic migration in such a system involves two basic steps: in one (the adsorption-desorption migration) the ion jumps from one adsorption site to another adsorption site; in the other (the saltatory migration) the ion jumps from one interstitial position (between the adsorption sites) to another interstitial position. An ion such as K^+, which is preferentially adsorbed on the bulk of anionic sites in normal muscle cells, occupies most of the adsorption sites and can migrate by both the adsorption-desorption route and the saltatory route. An ion such as Na^+ which is excluded from the bulk of adsorption sites, occupies far fewer adsorption sites and migrates primarily by the saltatory route.[6] This general principle applies both to migration across the cell surface and within the cell.

Migration via the adsorption-desorption route usually involves surmounting deeper energy barriers than migration via the saltatory route. So the mobility of a preferentially adsorbed ion such as K^+ may actually be lower than that of an excluded Na^+ ion. Yet the possible migrating paths are much more numerous for the more preferred ion than for the less preferred ion. This then, according to the association-induction hypothesis, explains why by decreasing the bias against Na^+ adsorption, ouabain increases the accumulation of labeled Na^+ in the cytoplasm (Figs. 3 and 4) as well as in the auxiliary pool (Table 1).

In conclusion, the present series of experiments, while presenting evidence against the membrane-pump model for the maintenance of intracellular K^+ and Na^+ concentration supports the association-induction hypothesis.

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