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MAINTENANCE OF LOW SODIUM AND HIGH POTASSIUM LEVELS IN RESTING MUSCLE CELLS

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SUMMARY

1. Previous work has shown that a frog sartorius muscle consists of parallel cells running all the way from one end of the muscle to the other and that amputation of one end of the muscle is not followed by regeneration of a new cell membrane. If now only the cut end of the amputated muscle is exposed to a Ringer solution in which the solutes ^{42}K and ^{22}Na act as radioactive labels and the rest of the cell is suspended in air, we have what is described as an effectively membraneless open-ended cell or EMOC preparation. In this case the only remaining anatomically intact plasma membrane and pumps are made nonfunctional by the removal of 'sources' for inward pumps and 'sinks' for outward pumps.

2. The healthy region of a frog sartorius muscle EMOC preparation continues to accumulate labelled K^+ to a level higher than that in the Ringer solution and to exclude labelled Na^+ to a level below that in the Ringer solution, much as a normal uncut muscle does in its normal environment. The differences were reduced by inclusion of ouabain in the medium.

3. The diffusion coefficient of Na^+ in the normal muscle cytoplasm at 25 °C was measured using two methods. The average diffusion coefficient measured was $2{\cdot}07 \times 10^{-6}$ cm^2/sec, roughly 1/6 that of the diffusion coefficient of Na^+ in a 0·1 N-NaCl solution.

4. The data obtained are discussed in terms of the association-induction hypothesis. In this theory asymmetrical solute distribution, basically an expression of a non-energy consuming metastable equilibrium state, is the result of specific combinations of two opposing mechanisms: adsorption which raises the level of the intracellular solute; and exclusion from cell water which tends to lower it.

INTRODUCTION

Virtually all living cells selectively accumulate K^+ and exclude Na^+ from an environment poor in K^+ but rich in Na^+. Fundamentally, there are only three types of mechanisms whereby a finite amount of chemical substance can be distributed and maintained indefinitely in adjoining spaces at different concentrations. These mechanisms are (*a*) the presence of an insurmountable energy barrier between the spaces (thermodynamic isolation mechanisms), (*b*) continuous operation of an energy-consuming pumping mechanism (the steady-state mechanism), and (*c*) a difference in the solubility of the substance in the two spaces (the equilibrium mechanism).

The theory of a cell membrane barrier insurmountable to Na^+ but not to K^+ has long since been proposed (Mond & Amson, 1928; Boyle & Conway, 1941) and disproved (Heppel, 1940; Steinbach, 1940). Only two alternative categories of theories now remain; the steady-state model, currently synonymous with the membrane-pump theory; and the equilibrium models, including the sorption theory of Troschin (1951, 1966) and the association-induction hypothesis (Ling, 1951, 1952, 1962, 1964, 1969, 1973).

There is experimental evidence against the membrane-pump theory for the maintenance of high K^+ and low Na^+ concentrations in resting cells. Ling has shown (1962) that under specified conditions, the Na pump alone would consume 15–30 times the total energy available to the cell. This objection to the steady-state model, presented in detail fifteen years ago (Ling, 1962), has been confirmed in general principle by Jones (1965) and Minkoff & Damadian (1973, 1974).

A second example of experimental evidence against the pump model consists of the demonstration that the levels of Na^+ and K^+ in the cell do *not* depend on the rate of outward Na^+ flux, as demanded by the pump theory (Ling & Ochsenfeld, 1976).

In the membrane-pump model, there must be an intact, functional cell membrane in order to maintain asymmetrical K^+ and Na^+ distribution since it serves as the barrier to isolate the cell interior from its external environment, and as the seat of the pumps which this theory postulates. On the other hand, an intact and functional cell membrane is not indispensable in the equilibrium models, in which the mechanism of the asymmetrical distribution resides in the entire substance of the cell, and hence primarily in the cytoplasm. In this report, I shall present a third example of experimental evidence against the pump theory, utilizing a recently introduced technique called the *effectively membraneless open-ended cell* technique (Ling, 1973). With this technique, one can render inoperative the postulated pumps in a portion of the cell membrane left intact. One can then study the distribution of ions in muscle cytoplasm directly exposed to external solutions.

METHODS

All the experiments to be described were performed on isolated sartorius muscles of north American leopard frogs (*Rana pipiens, pipiens*, Schreber). Unless otherwise specified, the experiments were conducted in a room with a constant temperature of 25 ± 1 °C. ^{22}Na and ^{14}C-labelled inulin (lot no. 591163) were from ICN, Isotope and Nuclear Division, Cleveland, Ohio; ^{32}P-free ^{35}S-labelled SO_4 (lot no. 4731) was from New England Nuclear Corporation, Boston; and ^{42}K came from Cambridge Nuclear Corporation, Princeton, New Jersey.

The modified Ringer solution called the Ringer-GIB medium 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; twenty amino acids; fourteen vitamins; and reduced gluthione (Ling & Bohr, 1969). As a rule, no antibiotics were included in the Ringer-GIB solution used in both influx and efflux experiments. Special caution was exercised to preserve sterility in experiments lasting several days at 25 °C.

The basic EMOC technique

The basic technique for monitoring inward movement of labelled ions into cut muscles was similar to those described earlier (Ling, 1973) and is illustrated in Fig. 1. In experiments with

both ^{42}K and ^{22}Na, the radioactivity of the samples was twice assayed, once immediately after the experiments to determine both ^{42}K and ^{22}Na and a second time after virtually all ^{42}K had decayed.

Intracellular diffusion coefficient measurements

The longitudinal diffusion coefficient of labelled Na^+ in the cytoplasm of frog sartorius muscles was measured in two different ways: in outward diffusion studies, the movements of

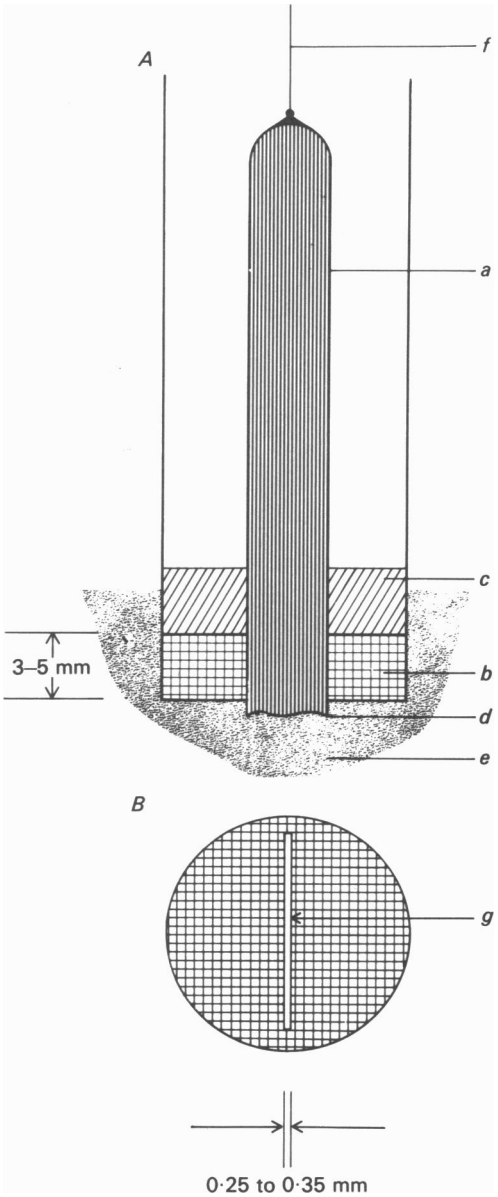

Fig. 1. Diagram of the EMOC tube. *A*, side view. *B*, bottom view. Only the cut end of the muscle is in direct contact with the labelled Ringer solution. *a*, sartorius muscle; *b*, silicone rubber gasket; *c*, Vaseline; *d*, cut end of muscle; *e*, bathing solution; *f*, anchoring string; *g*, slit in silicone rubber gasket.

labelled Na^+ from a muscle previously loaded with ^{22}Na were examined; in inward diffusion studies, the movements of labelled Na^+ into a non-labelled muscle were examined.

In the outward diffusion studies, a modification of the method described by Ling & Ochsenfeld (1973*a*) on K^+ diffusion in muscle cytoplasm was used. The modification was twofold: (1) intact rather than cut muscles were studied and (2) the extracellular fluid of the muscle was removed by prior centrifugation. The reasons for these changes are as follows.

Amputation of one end of the muscle elicits alterations in the adjacent muscle cytoplasm (Ling & Ochsenfeld, 1973*a*; also see Discussion below). To obtain the diffusion coefficient of a solute in normal cytoplasm, it would be preferable not to amputate. However, intact muscle can be used only in the case where the time for the intra-extracellular exchange of the solute involved is much shorter than the duration of the diffusion experiment. This is so in the case of Na^+: the time for 99% intra-extracellular exchange of Na^+ at 25 °C is $1\frac{1}{2}$ hr (Ling *et al.* 1973) while durations of the diffusion experiments were more than ten times that long (i.e. 19 hr). The maximum error introduced by leaving the membrane intact was thus less than 8%. Furthermore since the error makes the measured value somewhat slower, it weighs against the argument we will advance in the Discussion, thus strengthening the conclusion.

A substantial portion of the Na^+ in a normal sartorius muscle is found in the extracellular space. To study the diffusion of Na^+ in muscle cytoplasm, removal of this extracellular Na^+ was essential. This removal was routinely carried out by the centrifugation method to be described below.

When the sartorius muscle was inserted into the EMOC tube, a portion of the intact tibial end of the muscle was kept outside the gasket end of the EMOC tube, so that the outer edge of the silicone rubber gasket was about 2 mm away from the point at which the muscle began to taper. This procedure insured that the diffusion process studied would take place in an unchanging number of muscle cells which, in a previous report, I have shown to extend without exception, from one end of the muscle to the other (Ling, 1973; see also Lockhart & Brandt, 1938).

At the conclusion of the experiment, the washed tibial end of the muscle was first blotted before being cut off. The remaining muscle was then frozen in liquid nitrogen and cut into sections of two different sizes. The first section, adjacent to the amputated end, was 1 mm in length whereas all others were 2 mm. While still frozen, the sections were weighed and projected into counting tubes containing 1 ml. 0·1 N HNO_3, and their radioactivity was assayed on a γ-scintillation counter.

In the inward diffusion study, the EMOC technique was the same as that employed in the outward diffusion study with two exceptions. First, the muscle was not previously equilibrated in labelled Na^+; secondly, that the Ringer-GIB solution bathing the exposed intact end was labelled with ^{22}Na and was only 1 ml. in volume. As in the case of outward diffusion studies, the muscles were centrifuged to remove extracellular fluid before installation in the EMOC tubes.

Estimation of the effectiveness of the silicone rubber gasket in reducing diffusion through the extracellular space

In the EMOC tube, the sartorius muscle was held in place on one end by an anchoring thread, and on the other by a snugly fitting slit in the silicone rubber plug used to close the end of the glass tube (Fig. 1). The slit width (usually 0·25–0·35 mm) was chosen so as to fit the muscle sizes. An experiment was designed to find out just how effective this gasket was in reducing solute movement through this region of compressed extracellular space.

The top diagram in Fig. 2 illustrates how in principle the EMOC set-up resembles a classic arrangement for measuring diffusion coefficients (D) in liquids (Jost, 1960, p. 437). The amount of one or several substances diffusing through the narrow capillary connexion of length l and cross-sectional area A from a source solution at concentration C_1 in compartment 1 to a sink solution at concentration C_2 in compartment 2 is related to D by the equation

$$s = Dt \frac{A}{l} (C_1 - C_2). \quad (1)$$

In this experiment we used this equation not to measure D but rather to estimate the total cross-sectional area (A) of the extracellular space in that part of the muscle within the gasket of the EMOC preparation as shown in the bottom diagram of Fig. 2. To obtain A, we first

had to determine s. Both ^{14}C-labelled inulin and ^{35}S-labelled SO_4 are known to be confined primarily to the extracellular space in muscle tissues. I measured the amount of each which travels in a time interval t from the source solution bathing the intact tibial end of the sartorius muscle (compartment 1) to the extracellular space beyond the inner edge of the gasket (compartment 2).

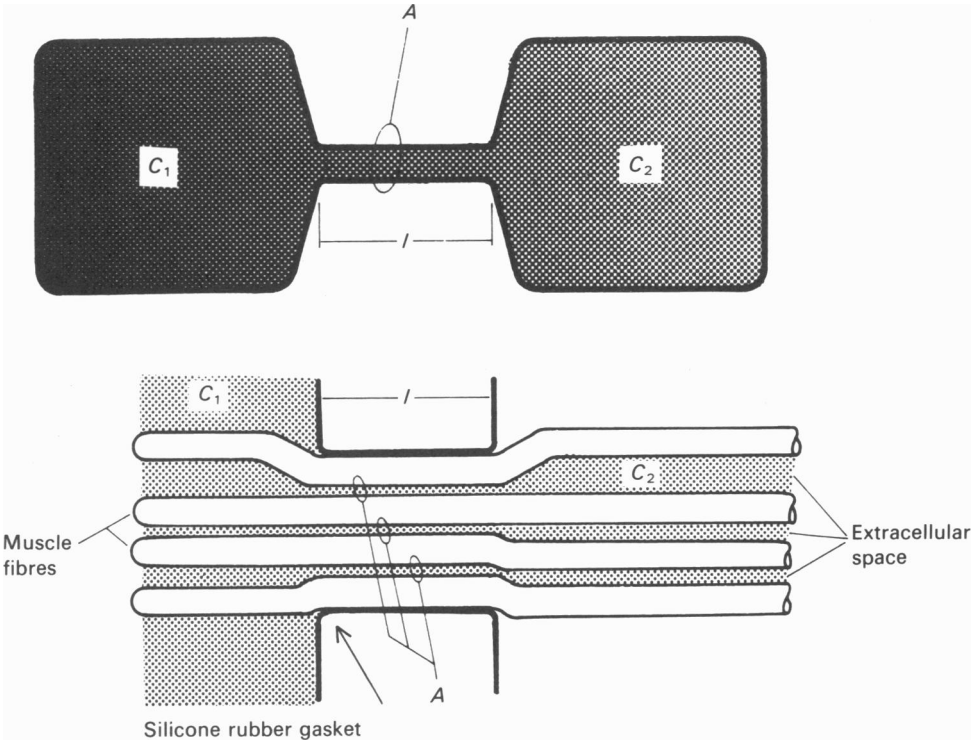

Fig. 2. Diagrammatic illustration of the functions of the snug-fitting silicone rubber gasket in reducing solute and solvent movement into the intact end of the muscle. The top part shows the classic set-up for measuring diffusion coefficients. The narrow capillary bridge linking the two compartments 1 and 2 is of cross-sectional area A and length l and determines the rate of diffusion from the compartment containing the solute at concentration C_1 to compartment 2 containing solute at concentration C_2. In the bottom part the compressed region of the extracellular spaces serves the same purpose as the narrow capillary; it determines the rate of diffusion of solute from the source solution (C_1) bathing the left end of the muscle to the 'sink' in the form of the uncompressed extracellular space of the right portion of the muscle (C_2).

In these calculations, the diffusion coefficient of labelled sulphate (at 25 °C) was $9{\cdot}40 \times 10^{-6}$ cm^2/sec (taken from Kushmerick & Podolsky, 1969); that of labelled inulin (at 25 °C) was $3{\cdot}06 \times 10^{-6}$ cm^2/sec. The latter was calculated from the data of Bunim, Smith & Smith (1937) for inulin at 37 °C, using the relation

$$D_1 = D_2 \frac{T_1}{T_2} \frac{\eta_1}{\eta_2}, \tag{2}$$

where D_1 and D_2 are the diffusion coefficients at temperatures T_1 and T_2, respectively, and η_1 and η_2 represent the viscosity of water at the two respective temperatures.

Removal and assay of extracellular space fluid

The method consists of first blotting an isolated sartorius muscle with the standardized procedure of Ling & Bohr (1969) and then spinning the blotted muscle at 1000 g for 4 min while the muscle sits on a deck of wetted filter paper in a hermetically sealed environment.

The weight loss after centrifugation was 9·30 ± 0·43 % (twenty-nine measurements). Variation of the centrifugal force between 400 and 1450 *g* and the time of spinning from 2 to 16 min (at 1000 *g*) produced essentially the same percentage weight loss. This basic procedure was also used in the assay of ^{22}Na content of the extracellular space fluid. In this case the ^{22}Na in extracellular fluid which collected on the filter paper was determined directly by counting the radioactivity of the filter paper deck in a well-type γ-scintillation counter.

Assay of total K^+

To assay the total K^+ content, the HNO_3 extract of the individual muscle sections after the second counting on a γ-scintillation counter was diluted with radiation buffers (Ling & Bohr, 1969) and assayed on a Perkin-Elmer atomic adsorption spectrophotometer, Model 103.

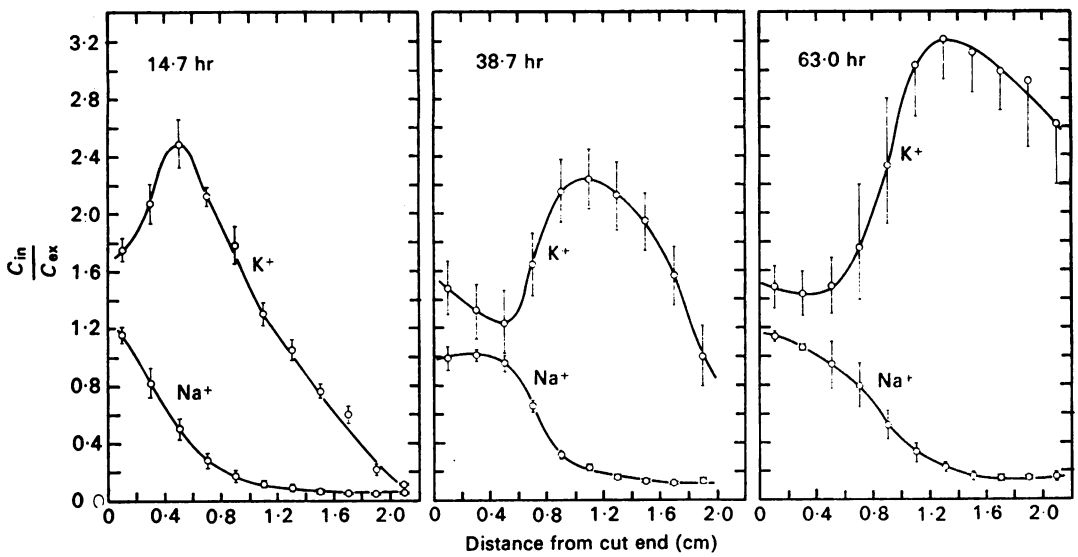

Fig. 3. The simultaneous influx of labelled K^+ and labelled Na^+ into sartorius muscles through their cut ends. The three groups of frog sartorius EMOC preparations were exposed to normal Ringer solutions labelled with both ^{42}K and ^{22}Na for 14·7, 38·7 and 63·0 hr, respectively. The abscissa represents the distance of the mid-point of each cut segment from the cut surface of the muscle fibres. The ordinate represents the ratio of the labelled ion concentrations in the water of each muscle segment (C_{in}) over the concentration of the same labelled ion in the solution bathing the cut end of the muscle at the conclusion of the experiment (C_{ex}). Each point was the average of 4 (14·7 hr), 10 (38·7 hr) and 4 (63·0 hrs) experiments, respectively, the distance between the two longitudinal bars being twice the standard error.

RESULTS

The simultaneous influx of labelled K^+ and labelled Na^+ into cut sartorius muscles

The cut ends of frog sartorius muscles in EMOC tubes were exposed to normal Ringer-GIB solution doubly labelled with ^{42}K and ^{22}Na for 14·7, 38·7, and 63·0 hr respectively. The results are shown in Fig. 3, in which the average labelled K^+ and Na^+ concentrations in the different cut segments in micromoles per gram of tissue water (C_{in}) were represented as the ratio of C_{in} to the equilibrium concentration of the same labelled ions in the solutions bathing the cut end (C_{ex}), and these were plotted against the distance between the midpoint of the segment and the cut edge of the muscle.

Fig. 3 shows the differences between the pattern of accumulation of labelled K^+ and Na^+, which can be summarized as follows. (1) C_{in}/C_{ex} for labelled K^+ in regions away from the injured cut end rose to values considerably higher than unity; on the other hand C_{in}/C_{cx} for labelled Na^+ in the same region remained at a level far below unity. This difference in the K^+ and Na^+ accumulation resembles

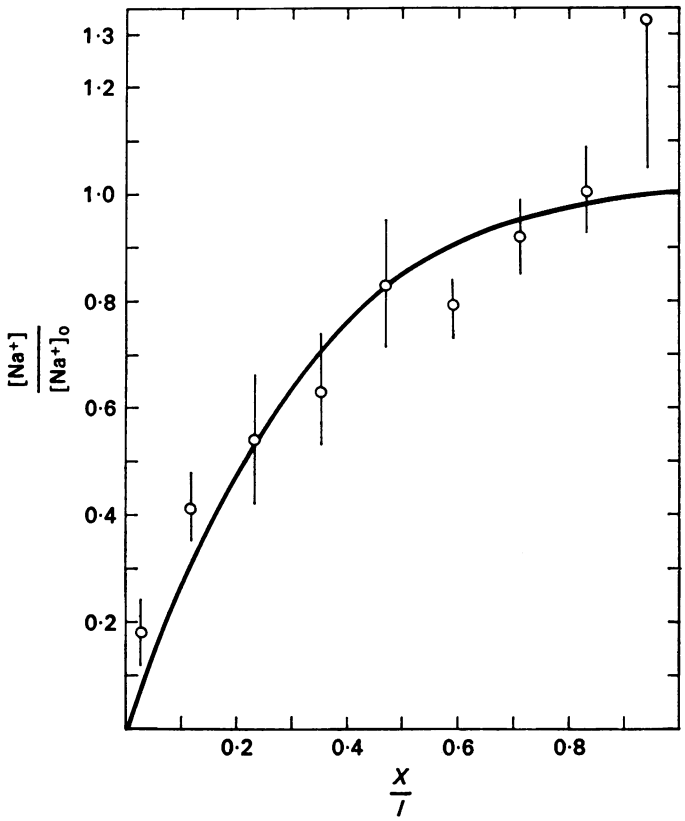

Fig. 4 Outward diffusion profile of labelled Na^+ at 25 °C from intact sartorius muscles equilibrated with ^{22}Na and freed of extracellular fluid by centrifugation. Data represent the average of six experiments. Lengths of vertical bars represent twice the standard error. Diffusion time was 19 hr. The abscissa is x/l where x is the distance from the outer edge of the silicone rubber gasket to the intact pelvic end of the muscle. The ordinate represents $[Na^+]/[Na^+]_o$ where $[Na^+]$ is the concentration of labelled Na at locus x and $[Na^+]_o$ is the initial concentration of labelled Na^+ in the muscle and was determined in a paired muscle similarly equilibrated with ^{22}Na and centrifuged. The large standard error of the point furthest to the right came from the high amount of labelled Na^+ contents of connective tissues and bits of cut fibres belonging to another adjacent muscle. The continuous line is a theoretical curve calculated with a diffusion coefficient of $1{\cdot}88 \times 10^6$ cm²/sec.

the familiar pattern of selective accumulation of K^+ and the exclusion of Na^+ seen in intact frog muscles after a relatively short exposure to a similar doubly labelled Ringer solution. (2) In regions farthest from the cut end, there was a steady increase in C_{in}/C_{ex} for labelled K^+, but a virtual standstill or very slow increase for labelled

Na^+. In regions nearest to the cut end, C_{in}/C_{ex} for labelled K^+ first increased and then decreased; on the other hand, C_{in}/C_{ex} for labelled Na^+ in these regions tended to increase only and showed no pronounced tendency to decrease with time.

The longitudinal diffusion coefficients of Na^+ in frog muscle cytoplasm

Fig. 4 shows the averaged results of six experiments from the studies of the outward diffusion of ^{22}Na from radioactively labelled sartorius muscles following 19 hr of washing of the exposed (intact) tibial end of the sartorius muscles at

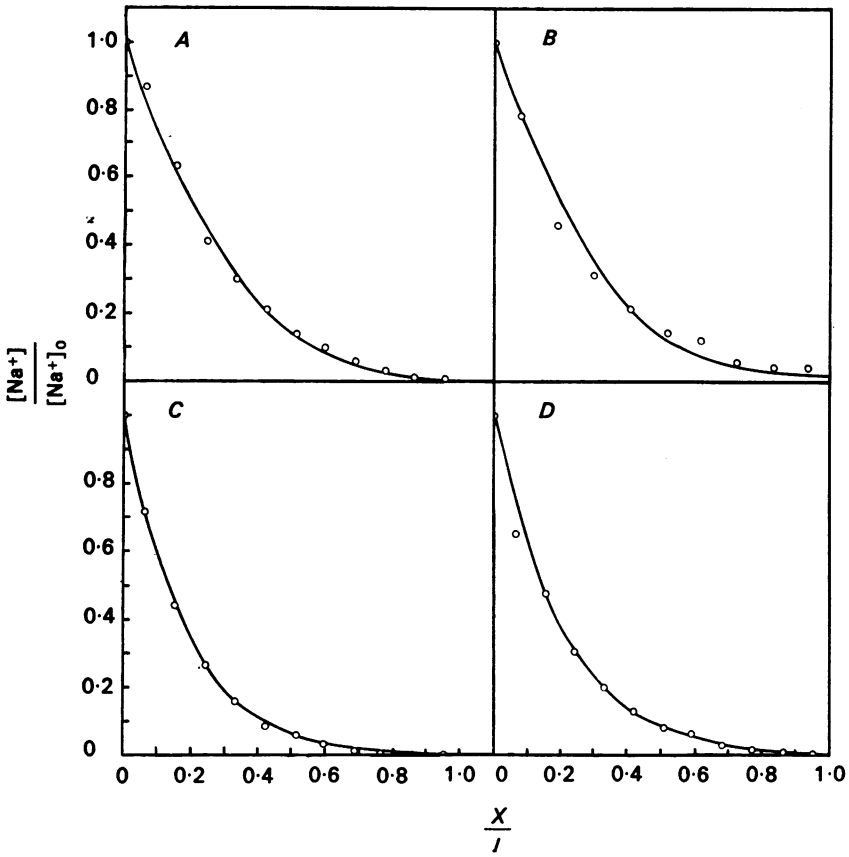

Fig. 5. Inward diffusion profile of labelled Na^+ at 25 °C into intact sartorius muscles. The abscissa, x/l represents the distance from the midpoint of the segment to the outer edge of the silicone rubber gasket. $[Na^+]$ is the concentration of labelled Na^+ in each segment while $[Na^+]_o$ is the surface concentration of labelled Na^+ which is taken as the labelled Na^+ concentration in the 1 mm segment immediately outside the outer edge of the silicone rubber gasket. Continuous lines are theoretical curves. The duration of all experiments was 19 hr. Further details of these illustrative experiments are given in Table 1, in which Experiment *A* here is designated as 6B02G; *B*, as 4DO2P; *C*, as 6BO2A; and *D*, as 6BO2F.

25 °C. The continuous line represents a theoretical profile of a diffusant in a similar situation with a diffusion coefficient equal to $1{\cdot}88 \times 10^{-6}$ cm^2/sec (see Crank, 1956).

Fig. 5 shows four experiments on the inward diffusion of labelled Na^+ into intact

sartorius muscles. The duration of these experiments was also 19 hr. A more complete list of all nine experiments, including those shown in Fig. 5, is given in Table 1. The average diffusion coefficient for Na^+ in muscle cytoplasm from these

TABLE 1. Diffusion coefficient of labelled Na^+ as measured by the influx method.

Data include the four sets of experiments shown in Fig. 5

Experiment no.	Length of muscle (cm)	D (10^{-6} cm^2/sec)*
4DO2M	1·95	1·67
4DO2N	1·95	1·95
4DO20	1·55	1·99
4DO2P	1·65	1·93
6BO2A	2·25	1·92
6BO2B	2·35	2·10
6BO2F	2·35	2·50
6BO2G	2·25	2·74
6BO2H	2·45	3·51
mean ± S.E.		2·25 ± 0·19

* Diffusion coefficient of Na^+ in frog muscle cytoplasm at 25 °C.

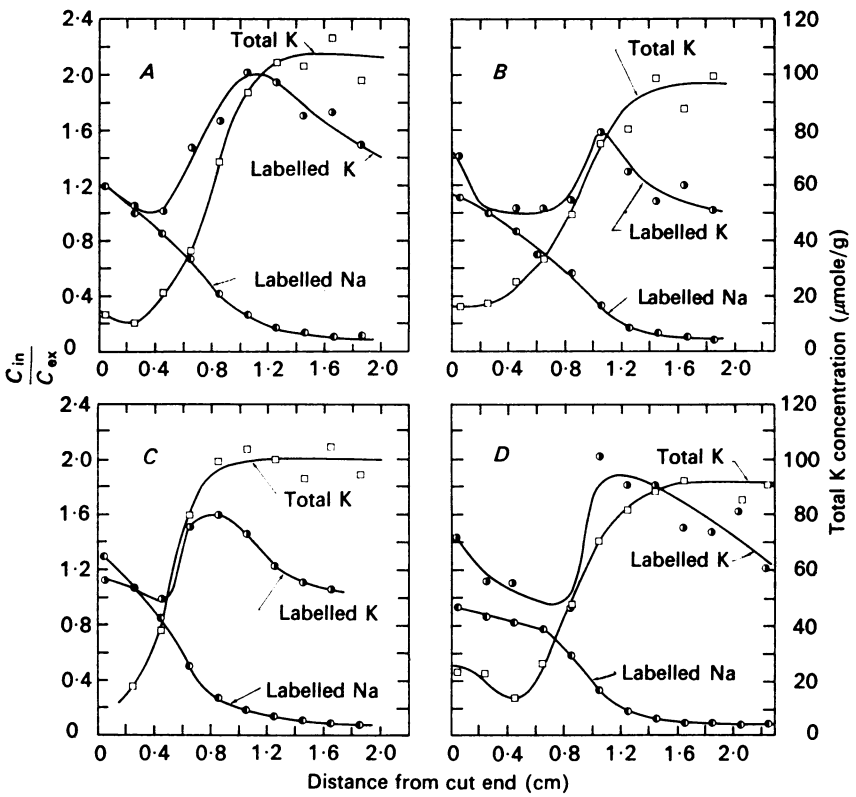

Fig. 6. The distribution of total K^+, labelled K^+, and labelled Na^+ in cut frog sartorius muscles. The duration of the experiment was 40 hr at 25 °C. Total K^+ of muscle segments was analysed after a second counting of the HNO_3 extract. Otherwise similar to data given in Fig. 3.

TABLE 2. Determination of the size of the extracellular space of sartorius muscles in the snug-fitting silicone rubber gasket. Intact sartorius muscles were maintained in EMOC tubes with about 0·5–1·0 cm of the intact tibial end directly bathed in the source Ringer solution labelled with ^{14}C-labelled inulin (total inulin concentration 0·128%) and ^{35}S-labelled sulphate (total sulphate concentration was 0·8 mM). After 66 or 72 hr of incubation at 25 ± 1·0 °C the average total cross-sectional areas of muscles were calculated with the aid of eqn. (1). Total amount of labelled inulin or labelled sulphate collected in the muscle beyond the inner edge of the silicone rubber gasket is denoted s. The final concentration of labelled inulin or sulphate in the Ringer solution was taken as C_1. The value s divided by the volume of extracellular space in muscles beyond the inner edge of the gasket (i.e. 9% of the fresh weight) gives an estimate of the final concentration of extracellular labelled inulin or sulphate. This concentration, divided by two, yields an average of C_2. A_{total} was obtained from the width of the muscle in the gasket and the slit width of the gasket measured with a micrometer. Two sets of mean ± S.E. are given. The larger value includes expt. 4H9H (which, owing to the extremely shallow silicone rubber gasket, apparently leaked); the smaller figure does not include the set of data from expt. 4H9H.

Experiment no.	Probe	Δt (hr)	s (counts)	C_1 (10^7 counts)	C_2 (10^7 counts)	A/l (10^{-4} cm)	l (cm)	A_{ecs} (10^{-2} cm^2)	A_{total} (10^{-2} cm^2)	$\frac{A_{ecs}}{A_{total}}$ (%)
3D18B	1	66	6,489	2·68	0·11	3·47	—	—	—	—
3D18D	1	66	7,977	2·57	0·10	4·43	—	—	—	—
3D18H	1	66	2,744	2·53	0·48	1·53	—	—	—	—
3D18J	1	66	1,900	2·36	0·39	1·12	—	—	—	—
3D18L	1	66	2,477	2·56	0·34	1·35	—	—	—	—
3D18M	2	66	197,000	19.10	3.42	5·62	—	—	—	—
3D18N	2	66	97,020	19·30	1·56	2·45	—	—	—	—
3D18O	2	66	29,445	18·00	0·37	7·48	—	—	—	—
3D18P	2	66	42,229	16·00	0·65	1·23	—	—	—	—
4G5A	1	72	1,450	1·00	0·015	1·85	0·45	0·0083	—	—
4G5B	1	72	3,494	1·05	0·025	4·30	0·37	0·0160	—	—
4G5C	1	72	2,739	1·07	0·016	3·27	0·35	0·0110	—	—
4G5D	1	72	3,159	1·20	0·018	3·36	0·45	0·0150	—	—
4G5E	1	72	1,871	1·08	0·016	2·21	0·40	0·0088	—	—
4G5F	1	72	3,159	1·07	0·025	3·83	0·30	0·0120	—	—
4G5G	1	72	574	1·04	0·004	0·70	0·85	0·0059	—	—
4G5H	1	72	2,655	1·03	0·020	3·35	0·40	0·0130	—	—
4H9A	1	72	2,472	0·44	0·030	7·56	0·20	0·0150	1·70	0·88
4H9B	1	72	750	0·42	0·010	2·30	0·40	0·0092	1·52	0·60
4H9C	1	72	2,492	0·44	0·030	9·12	0·15	0·014	3·65	0·38
4H9E	1	72	115	0·42	0·001	0·35	0·90	0·003	1·36	0·22
4H9F	1	72	1,880	0·41	0·020	6·00	0·20	0·012	3·24	0·37
4H9G	1	72	308	0·43	0·003	0·91	0·60	0·005	1·40	0·36
4H9H*	1	72	7,046	0·41	0·080	26·90*	0·15	0·040	1·14	3·51*
4H9I	1	72	600	0·41	0·007	1·86	0·50	0·009	1·06	0·85
4H9J	1	72	783	0·43	0·007	2·33	0·55	0·013	1·53	0·85
mean ± S.E.						4·19 ± 1·01		0·012 ± 0·002		0·89 ± 0·34
						3·28 ± 0·47*		—		0·56 ± 0·09*

* Data from expt. 4H9H deleted in calculation of S.E.

influx experiments is 2·25 ($\pm$ 0·19) $\times 10^{-6}$ cm²/sec. The average value for both influx and efflux data is 2·07 $\times 10^{-6}$ cm²/sec which is roughly 1/6 that of the diffusion of ^{22}Na in a 0·1 N-NaCl solution at 25 °C (Mills, 1961). The data leave no doubt that Na^+ diffuses 'freely' in the muscle cell cytoplasm, even though at a considerably lower rate than in a 0·1 N-NaCl solution, and that there are no impermeable barriers to Na^+ within the cell.

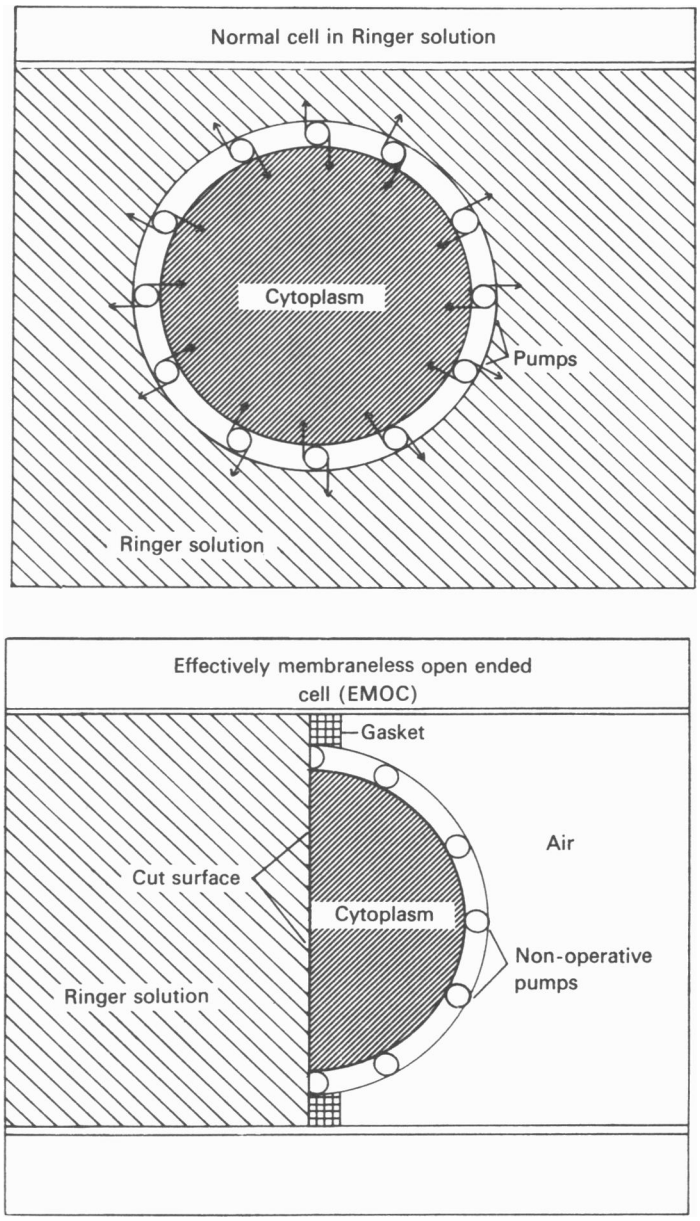

Fig. 7. Diagrammatic illustration of the effectively membraneless open-ended cell preparation. Diagram shows how amputation of part of the cell membrane and exposure of the remaining intact membrane to air incapacitates the plasma membrane pumps.

The relation between the concentration distribution of labelled K^+ and Na^+ and that of total K^+ in the cut sartorius EMOC preparation

In Fig. 6, four individual sets of illustrative data on the concentration distribution of labelled K^+ and Na^+ in the sartorius EMOC preparations are shown. The labelled K^+ profile in each case conforms to the distribution profile of the total K^+; the labelled K^+ concentration remained low in regions near the cut end where the total K^+ concentration had fallen to low values; the labelled K^+ concentration rose

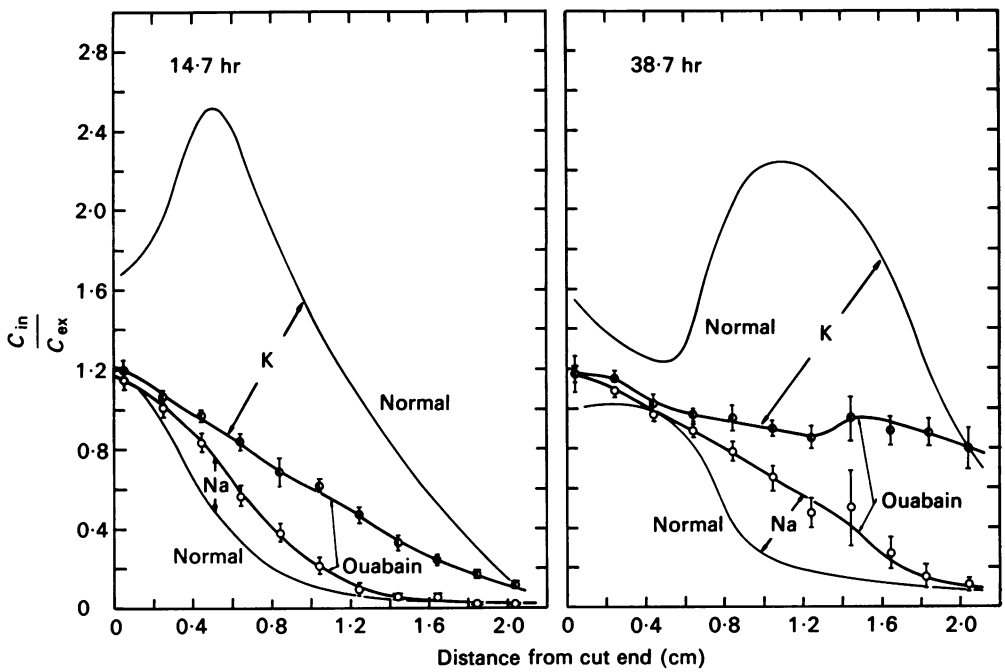

Fig. 8. The effect of ouabain on the accumulation of labelled K^+ and Na^+ in sartorius muscles through their cut ends. Experimental procedures used were the same as those described in the legend of Fig. 3 except that the Ringer solution bathing the cut end of the muscles contained initially 10^{-4} M-ouabain. Continuous lines without experimental points were reproduced from the data of Fig. 3 to provide a basis for visualizing the effect of ouabain.

to high values in regions where the total K^+ concentration remained high. The labelled Na^+ profile when converted into micromolecular concentrations was a fairly accurate mirror-image of the total K^+ distribution profile. The sum of the total K^+ and labelled Na^+ concentrations at any locus along the lengths of the muscles was roughly constant.

The effect of ouabain on the accumulation of labelled K^+ and Na^+ in the cut muscle

In a previous publication, it was shown that the effect of ouabain in increasing intracellular Na^+ concentration persisted in muscle cells whose postulated membrane-pump (if it exists) was made non-operative by the deprivation of a 'sink' in the EMOC preparation. Fig. 8 shows that the effect of ouabain in reducing cellular

K^+ concentration was also maintained in the effectively membraneless open-ended muscle cells. Inclusion of ouabain at an initial concentration of 10^{-4} M has all but eliminated the excessive uptake of labelled K^+ seen in the control muscles.

The size of compressed sartorius extracellular space in the silicone rubber gasket

Table 2 shows the cross-sectional area of the extracellular space in the gasket slit (A_{ecs}), $1{\cdot}2 \times 10^{-4}$ cm^2. This value is less than 1% of the average total (intra- and extracellular) cross-sectional area of the normal sartorius muscles used. Since the extracellular space of a normal sartorius muscle is 9% of the total muscle weight (Ling & Kromash, 1967; Ling & Walton, 1975), the tight-fitting gasket has reduced its volume by approximately 90%.

DISCUSSION

If we expose only the cut end of the cell to a labelled Ringer solution and suspend the remaining part of the cell in air we deprive the postulated pumps of their needed 'sinks' and 'sources' thereby producing an effectively membrane-pump-less open-ended cell (EMOC) preparation. From the viewpoint of the membrane-pump theory, the diffusion of labelled solutes from the Ringer solution into these open-ended cells will follow the laws of diffusion into open capillaries. That is, t sec after diffusion begins, the spatial distribution of a labelled solute along the length of the tubular cells would be uniquely defined by Dt/l^2, where D is the diffusion coefficient of the labelled solute and l is the total length of the long cell cylinders. In other words, if two labelled solutes K^+, and Na^+ diffuse into the same open capillary, then their spatial distribution profiles would be identical if $D_{K^+}t_{K^+} = D_{Na^+}t_{Na^+}$.

I The equalization of $D_{Na^+}t_{Na^+}$ and $D_{K^+}t_{K^+}$

Experiments described under results have yielded D_{Na^+} (25 °C) in normal frog muscle cytoplasm equal to $2{\cdot}07 \times 10^{-6}$ cm^2/sec; D_{K^+} is $2{\cdot}63 \times 10^{-6}$ cm^2/sec (Ling & Ochsenfeld, 1973*a*). Thus if the diffusion time for Na^+ (t_{Na}) is set longer than t_K by a factor equal to $2{\cdot}63 \times 10^{-6}/2{\cdot}07 \times 10^{-6}$ or 1·27, then $D_{Na^+}t_{Na^+} = D_{K^+}t_{K^+}$. The diffusion profiles of K^+ and Na^+ in the muscle cytoplasm should then be indistinguishable. If t_{Na^+}/t_{K^+} exceeds 1·27, the uptake of labelled Na^+ should exceed that of K^+. The same principle applies to diffusion in the extracellular space (e.c.s.) fluid, in which ionic mobility has been shown to be similar to that in a 0·1 N-NaCl solution (Ling, 1972). The D ratio for K^+ *vs.* Na^+ in this case is 1·57 (Mills, 1961).

Comparing the Na^+ profile in the 38·7 hr experiment of Fig. 3 with the K^+ profile of 14·7 hr experiment ($t_{Na^+}/t_{K^+} = 2{\cdot}6$), we find the same results as when we compare the 63·0 hr Na^+ profile with the K^+ profile of either the 38·7 hr experiment or the 14·7 hr experiment. In all cases, much greater uptake of K^+ than Na^+ persists even though we have more than compensated for the slower D_{Na^+} by a longer t_{Na^+}.

II A possible role of the injury potential

As a case of simple diffusion into open capillaries the observed accumulation of K^+ within the cells to levels several times higher than that in the external solution is unusual. However, in terms of the membrane theory, an uneven distribution of

the resting potential might have just such an effect. Thus, at the site of amputation, the cellular resting potential has all but disappeared; in regions away from the cut end, a resting potential has been shown to persist for quite a long time (Ling & Walton, 1976). Such a sustained potential away from the cut might concentrate cations in the intact end of the cells to a concentration above that of the source solution. However, if, as proposed, a residual resting potential has the effect of concentrating cations locally, this concentrating effect must operate on Na^+ as well as K^+. The data of Figs. 3 and 6, however, clearly show that this is not the case.

III The possible role of pumps

A. Regeneration of the amputated plasma membrane (pumps). In the older literature, there were reports of regenerations of destroyed cell membranes (Heilbrun, 1961). However, three different types of tests unanimously showed that under the conditions of the experiments similar to the present no membrane regeneration occurs (Ling, 1973; Ling & Ochsenfeld, 1973*a*; Ling & Walton, 1976).

B. Pumping activities of the subcellular particle membranes. The highest level of labelled K^+ accumulated in the muscle segments shown in Fig. 3, was 10 mM. Thus it appears possible that the extra amount of labelled K^+ beyond the level of Na^+ (normalized for D ratio) was localized in the subcellular particles. However, we shall see that this explanation is not tenable. To begin with, the aggregate volume of subcellular particles, including the mitochondria, the sarcoplasmic reticulum system, and the nuclei, is shown by electron micrography in the muscles studied to be below 25% of the total cell volume. However, for the safety of argument let us assume that they occupy 50% of the cell volume.

Fig. 6 shows that at the locus of maximum C_{in}/C_{ex} for labelled K^+, the *total* K^+ concentration was 100 mM. Although some evidence exists that living cells may have the power to discriminate between different K isotopes (Lasnitski & Brewer, 1941), this discrimination is negligible. Thus. if labelled ^{42}K is accumulated in the sub-cellular particles at a concentration of, say, n times higher than that in the surrounding cytoplasm, the same ratio would apply to the distribution of all K^+ ions. Again, for the safety and simplicity of argument, we shall assume that at the locus where C_{in}/C_{ex} for labelled K^+ is 2 (Fig. 3), that for Na^+ is not below 1 as observed but 1. Thus, if the excess of K^+ beyond that of equal distribution is in the subcellular particles here, then this excess of ^{42}K (and hence total K^+) concentration in the subcellular particles would be $1/0{\cdot}5 = 2$ or twice that of ^{42}K (and hence total K^+) concentration in the cytoplasm. The ratio of total subcellular particle K^+ concentration to cytoplasmic K^+ concentration is then 3:1. To partition 100 mM-total K^+ in the muscle cell between subcellular particles and cytoplasm at a ratio of 3 to 1 would require that $75/0{\cdot}5 = 150$ mM be within the particles and that $25/0{\cdot}5 = 50$ mM be outside of the particles. This asymmetrical K^+ distribution would create an osmotic imbalance because in this intact region of the muscle cells there were no other solutes at high enough concentration to make up the osmotic difference. As a result, water would move into the particles to equalize the osmotic pressure. Indeed, as long as the bulk of intracellular ions are assumed free, as in the membrane theory, there is no way for a large concentration gradient of K^+ to be maintained across the subcellular-particle membranes, since the bulk of intracellular cation is

K^+. Next, let us see if outward Na^+ pump of the subcellular particles may offer an explanation of the low level of Na^+ seen.

If one postulates that the Na^+ pumps are so efficient as to maintain a zero concentration of Na^+ in all the subcellular particles, the level of Na^+ in the total cell would equal the volume fraction of the cytoplasm namely, 75%, which is inadequate to account for the observed much lower steady level of labelled Na^+ seen in, for example, the 1·2 cm segment, i.e. 20–30%.

C. The inward K^+ pump. In the EMOC preparations pumping of labelled K^+ into the intact portions of the cells must be limited to the amount of labelled K^+ that leaked through the compressed ecs in the silicone rubber gasket. With the aid of eqn. (1) and the necessary data already given, the maximum average concentration of labelled K^+ that could have leaked through the e.c.s. in 14·7 hr was found to be $1{\cdot}85 \times 10^{-2}$ μmole/g which is trivial.

D. The outward Na^+ pump. The operation of an outward Na^+ pump is, of course, the heart of the Na pump theory in explaining the low Na^+ and high K^+ in living cells. However, since air cannot accommodate Na^+, the elimination of the necessary 'sink' in the EMOC preparation has already disarmed the pump and rendered it non-functional; nevertheless, it is important to examine the theory in the greatest detail, in order to make certain that no oversight is committed.

In the EMOC set-up, to create an observable lowering of the total Na^+ concentration in the muscle, the cell must eliminate from its intact end, the labelled Na^+ that the Na pump might have pumped into the e.c.s. However, the only repository for this load is the Ringer solution bathing the cut end. Since there is no longitudinal pump in the e.c.s., this transport of labelled Na^+ to the source Ringer solution could only be by diffusion. To do so the concentration of labelled Na^+ in the e.c.s. must be raised to and maintained at a high level.

We have already shown that the labelled Na^+ profile after 38·7 hr of diffusion is more than adequately 'normalized' in the differences in D when compared with the 14·7 hr labelled K^+ profile. If the lower Na^+ accumulation after normalization is attributed to pumping, then one can find out how much labelled Na^+ must have been dumped back into the Ringer solution in order to equalize the K^+ profile and the normalized Na^+ profile. By graphical integration one calculates that this amounts to 4·12 μmole.

Again with the aid of eqn. (1), one can calculate how high the concentration of labelled Na^+ must be maintained in the e.c.s. fluid to transport 4·12 μmole of labelled Na^+ back to the solution bathing the cut end in 38·7 hr as follows: D_{Na^+} in the e.c.s. fluid is $1{\cdot}28 \times 10^{-5}$ cm^2/sec, A/l is $4{\cdot}19 \times 10^{-4}$ cm^2/sec, and C_2 is 100 μmole/cc. Thus $4{\cdot}12 = 38{\cdot}7 \times 3{\cdot}6 \times 10^3 \times 1{\cdot}28 \times 10^{-5} \times 4{\cdot}19 \times 10^{-4} (C_1 - 100)$. $C_1 = 5{\cdot}75 \times 10^3$ μmole/cc, or 5·75 M.

A 5·75 M-NaCl is beyond the solubility of NaCl, and unquestionally hypertonic. Thus, even if the pump could succeed in raising the level of labelled Na^+ to 5·75 M, it would promptly cause water to move from the surrounding cells to dilute this solution and in so doing would defeat the purpose of building up a 'diffusion head'. The only effect achievable by the membrane pump is a steady increase of the ecs. Table 3 shows that *in fact* after 50–53 hr of incubation, the labelled Na^+ in the e.c.s. fluid of the intact end of the muscle was only $95{\cdot}5 \pm 0{\cdot}3$ mM and thus slightly

TABLE 3. The volume of the extracellular space and the concentration of labelled Na^+ in the extracellular space of the intact part of the muscle that had been suspended in air after 50 (to 53) or 144 hr of incubation at 25 °C

Volume of extracellular space (e.c.s.) is expressed as the percentage of the total muscle weight. Temperature of the last six experiments was 4 °C (for 96 hr) followed by 25 °C (for the remaining 48 hr). Initial and final wts. refer to muscle wts. before and after centrifugation to extract e.c.s. fluid

Experiment no.	Incubation (hr)	Temp. (°C)	Initial wt. (mg)	Final wt. (mg)	ΔW (mg)	E.c.s. (%)	Labelled Na^+ in Spun-Off Fluid (mμmoles)	$[Na^*]_{ecs}$ (mM)
2128III	50	25	85·7	76·0	9·7	11·3	10·3	105
2128V	50	25	79·9	67·8	12·1	15·1	8·8	73
2K15E	50	25	77·0	65·4	11·6	15·1	9·4	84
2K15G	50	25	46·6	42·7	3·9	8·3	3·1	80
2J2A	53	25	69·3	62·2	7·1	10·2	7·4	104
2J2B	53	25	62·8	56·4	6·4	10·2	5·3	83
2J2E	53	25	103·5	91·8	11·7	11·3	12·8	109
2J2F	53	25	135·3	129·0	6·3	4·6	8·1	129
2J2G	53	25	96·3	88·0	8·3	8·6	8·5	102
2J2H	53	25	105·4	97·5	7·9	7·5	7·1	90
512OA	144	4 → 25	100·3	92·5	7·8	7·7	—	—
512OC	144	4 → 25	99·0	92·0	7·0	7·1	—	—
512OE	144	4 → 25	99·4	86·6	7·4	7·4	—	—
512OG	144	4 → 25	118·2	109·2	9·0	7·6	—	—
512OI	144	4 → 25	114·3	101·8	12·5	10·9	—	—
512OK	144	4 → 25	113·0	104·6	8·4	7·4	—	—
mean ± S.E.						9·4 ± 0·72		95·9 ± 5·3

lower than that in the Ringer solution (see also Ling, 1973). The expected 'diffusion head' was not found. Table 3 also shows that the average percentage of e.c.s. volume was $9{\cdot}4 \pm 0{\cdot}72\%$, which is roughly the same as in normal muscle: 8–10% (Ling & Kromash, 1967; Ling & Walton, 1976). Thus, not only was there no evidence that any amount of labelled Na^+ was actually 'pumped' back to the only sink available; but there was also no evidence that such an attempt was made. If it had been, the e.c.s. volume would have increased.

Taken together, the data shows that the high level of K^+ and the low level of Na^+ seen in the resting frog muscle cells could not have been maintained by postulated pumps in the cell membrane. Next, let us examine if the association-induction hypothesis can explain the new experimental findings; with special emphasis on the two key observations: (1) C_{in}/C_{ex} for K^+ is much higher than that for Na^+, and (2) the level of labelled K^+ first rises and then falls near the cut end while that of Na^+ steadily rises.

According to the association-induction hypothesis, the living cell is seen as maintained in a high-energy cooperative state, the living state. In maintaining this living state, certain substances called 'cardinal adsorbents' have controlling influence. Chief among the cardinal adsorbents is ATP, which by interacting with the protein molecules through its adsorption on certain specific binding sites, controls the electronic and steric conformations of the proteins. Thus, when ATP is adsorbed, the protein–water–ion system may exist in the resting (living) state. In this state the bulk of intracellular K^+ is adsorbed on anionic sites of certain protoplasmic proteins which have more favourable adsorption energies for K^+ than for Na^+. As a result only a small concentration of intracellular Na^+ is adsorbed. The rest remains in the cell water at a reduced level because the cell water, existing in the state of multilayers polarized by a matrix of extended polypeptide chains equipped with alternatingly positive (NH) and negative (CO) sites, has diminished solubility for Na^+ and other solutes. When ATP is removed during cell deterioration, the system goes into an alternative co-operative state, in which selectivity for K^+ adsorption is lost and the bulk of cell water is depolarized. As a result, the levels of Na^+ and K^+ approach those of the surrounding medium (for evidence, see Ling, 1962; Gulati, Ochsenfeld & Ling, 1971; Ling & Ochsenfeld, 1973*b*; Ling, 1974).

Thus in the association-induction hypothesis, the level in the cell of a particular non-metabolized solute such as K^+ and Na^+ depends on two factors: (*a*) solubility of the solute in the cell water and (*b*) adsorption on proteins and other macromolecules (see also Troschin, 1966). The low solubility of hydrated Na^+ as well as K^+ in cell water would have reduced levels of both ions to below that in their external solution. However, for K^+ but not for Na^+ the preferential adsorption on intracellular sites more than compensates for this effect, thus raising the value of K^+_{in}/K^+_{ex} to much higher values than Na^+_{in}/Na^+_{ex}.

At the very beginning of the diffusion experiment the cytoplasm near the cut end was not far from normal. Exchange of the labelled K^+ with the selectively adsorbed non-labelled K^+ originally in the cell caused the initial rise of the level of labelled K^+ near the cut edge as observed. The amputation of the cell soon took its toll reducing the local level of ATP. Deterioration then set in, at first only near the cut end but then gradually spreading toward the intact end of the muscle. With

this deterioration and ATP loss, the selectivity for K^+ adsorption declined, and a drop in the concentration of adsorbed K^+ (labelled as well as that not yet labelled) followed as a result while at the same loci, depolarization of water in the deteriorating cytoplasm raised the level of Na^+.

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