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D. Geduldig
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ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
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D. GEDULDIG*
D. R. LIVENGOOD

* Department of Biophysics, University of Maryland, School of Medicine, Baltimore, Maryland

D. O. CARPENTER
Chairman
Neurobiology Department

LaWAYNE R. STROMBERG, MD
Colonel, USA
Director

ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
Defense Nuclear Agency
Bethesda, Maryland

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ABSTRACT

Surface fibers of Na-enriched muscles, bathed in K-free (Na-containing) saline, depolarize when the external sodium is replaced isosmotically with Tris. Subsequent addition of strophanthidin to the Na- and K-free bathing solution results in a hyperpolarization. If the main effect of strophanthidin in these experiments is to inhibit the electrogenic pump, then it may be concluded that before addition of the drug the net pump current was inward-going. This contrasts with the usual observation that cell membranes generally have hyperpolarizing electrogenic pumps which are caused by Na-K pump ratios greater than unity. These direct electrical measurements suggest that the enzyme machinery responsible for the active transport of sodium and potassium is not always obligated to extrude sodium out of the cell faster than potassium is actively accumulated. Since the muscles retain virtually all of their internal potassium when bathed in Na- and K-free saline, the passively lost potassium would appear to be largely regained by an efficient back-pump process, which may only modestly stimulate active Na efflux.

I. INTRODUCTION

When Na-enriched frog muscles are bathed in Na- and K-free saline, the small amount of potassium which could accumulate outside of the membrane after leaking from the fibers may be sufficient to stimulate the Na-K pump. Under these conditions, it had been noticed that the rate constant for K loss was unusually small, and it was suggested that this could be a consequence of a potassium influx pump which back-pumps much of the leakage K.[6,18] Thus, only a small fraction of the tracer K which actually leaked out of the muscle would be recoverable from the bathing solution. The rate constant for K loss in these situations was about an order of magnitude smaller than observed in control conditions, in which pump inhibitors (strophanthidin or ouabain) had been added to the same bathing solutions. If inhibition of active transport by the presence of these drugs does not affect the K permeability, then an interesting feature of the K pump (for these muscles bathed in the Na- and K-free saline) would be that possibly more than 90 percent of the potassium leaving the fibers is actually recovered by the muscle and does not diffuse through the external unstirred layer into the bulk phase of the bathing solution. At the same time that strophanthidin (or ouabain) permits the increased K loss from these muscles, the usual inhibition of Na loss is observed.

From the observed rate constants for the Na and K loss from these muscles into Na- and K-free solutions[6,18] (also see Appendix A), it is clear that the $[K]_i$ remains relatively constant for extended periods of time, while the $[Na]_i$ decreases exponentially with time. When the rate constants for Na and K loss in the presence of strophanthidin are also considered, the inward-going K pump current and the outward-going Na pump current can be calculated. These calculations show that the rates of active transport

of Na and possibly also K decrease with time, but also that the Na pump rate may attenuate considerably faster than the K pump rate.

If the outward-going Na pump current continually declines more rapidly than the inward-going K pump current, even if the Na-K pump ratio were to start out at values of 3:1 or 3:2, at some later time the pumped currents should be equal (giving a pump ratio of 1:1), and at even later times Na-K pump ratios smaller than unity could be expected. If the stoichiometry of the pump has particular significance for the behavior of active transport at a molecular level, this will be important.

If the Na-K pump ratio is greater than unity, the net actively transported cation efflux exerts a hyperpolarizing influence on the membrane potential. Two reviews on electrogenic pumps[14, 20] make it clear that for both nerve and muscle cells in the usual test solutions, the active transport system generally contributes a hyperpolarizing component to the membrane potential. Thus inhibition of electrogenic pumps commonly results in a measurable depolarization of the membrane, and this would be expected if the Na-K pump ratio had been greater than unity. One exception to this comes from a study by Villegas et al.[21] in which it was reported that a cardiac glycoside can hyperpolarize the membranes of the glial cells which surround the squid giant axon. The conclusion was made that the pump ratio was less than unity; that is, more K was actively transported into the cell than Na actively transported out of the cell.

Calculations made from flux data also indicate that at times the Na-K pump ratio may become less than unity (squid axons with reduced $[Na]_i$ when bathed in seawater;[16] and also frog sciatic nerves when bathed in low Na solutions[10]). Mullins and Brinley[13]

have also demonstrated the occurrence of an ATP_i-dependent K influx in the absence of any Na in the internal dialysate for squid axons.

However, a Na-K pump ratio which is demonstrably less than unity would not necessarily exert a depolarizing influence on the membrane potential. If Na on the outward-going carrier is merely replaced by another cation, then no change in electrogenicity could be expected, even though the Na-K pump ratio were altered. Only if the carrier-mediated Na efflux is reduced without an equivalent replacement of cations on the outward-going carrier, would a depolarization be expected. The electrogenicity of a carrier-mediated transport system is unambiguously tested by electrical measurements.

If Na-enriched frog muscle fibers (when bathed in Na- and K-free saline) do in fact actively take up external K (which had leaked out of the fibers) at a faster rate than the active extrusion of Na, the Na-K pump may then exert a depolarizing influence. Inhibition of the pump in this case may then result in a hyperpolarization. Data on the net fluxes and details of the analysis which at first led to the premise of Na-K pump ratios less than unity are provided in Appendix A to this report. The Appendix also provides the data which show that the strophanthidin-sensitive Na efflux can become smaller than the component of K movement associated with active K uptake. The main electrical evidence which is consistent with a depolarizing electrogenic pump is given in the body of the report itself. The electrical data suggest that when the carrier-mediated K influx remains constant, and the carrier-mediated Na efflux is reduced, other cations do not obligatorily replace Na on the carrier.

II. METHODS

Pairs of sartorii, attached to their halves of the split pelvic bone, were dissected from Rana pipiens (in the normal saline, below), suspended on separate Chromel "A" hangers, and immersed in about 10 ml of a K-free saline for Na-enrichment (5°C), usually for 2 days. A separate test tube was used for each muscle, and the K-free saline was changed two or three times each day. This procedure was used to exchange the internal K for external Na, thus elevating $[Na]_i$ to about 70 meq/kg fiber water and reducing the $[K]_i$ to about 55 meq/kg fiber water after 2 days. If muscles which are treated in this way are then exposed to a saline containing K (at room temperature), active extrusion of Na is stimulated.

Solutions. A phosphate-buffered K-free saline was used for Na-enrichment of the muscles at 5°C (see above). The main test solution was Na- and K-free (Tris-substituted) saline used at room temperature (ca. 22°C). The Na-free, K-containing saline was made by addition of KCl (from 0.5 M stock) to the Na- and K-free saline. Each day as required, strophanthidin (from a 10^{-2} M stock in ethanol) was added to the appropriate salines for a final concentration of 5×10^{-5} M. At the temperatures used, all solutions were at pH 7.2. Each of the above solutions was isosmolal (230 mosm ± 1 percent), as determined by a freezing-point depression osmometer. The external solutions are given in Table I.

Electrical measurements. Standard electrophysiological techniques were used to measure the membrane potentials of the muscle fibers. The microelectrodes (filled with 3 M KCl) generally had tip potentials less than 5 mV and resistances between 10 and 20 MΩ. Ag/AgCl/agar-3 M KCl bridge electrodes were used to connect electrolytic

Table I. External Solutions

Name	NaCl (mM)	KCl (mM)	$CaCl_2$ (mM)	Tris base* (mM)	Na_2HPO_4 (mM)	NaH_2PO_4 (mM)
Normal saline	116	2.5	1.8	3.3		
Na-enrichment	116		1.8		2.5	1.5
Na- and K-free			1.8	124.5		

* Tris base, neutralized with HCl to final solution pH 7.2 (21°C)

circuit elements with the preamplifiers, and all liquid junctions contained a 3 M KCl element. The membrane potential was detected differentially by using two preamplifiers whose outputs were connected to the inputs of both a pen recorder (Hewlett-Packard model 7100BM) and also an oscilloscope. The membrane potential data were collected and analyzed from the pen records. All microelectrode penetrations were made on surface fibers only.

At the time of the potential measurements, the chamber contained about 2 ml of solution, and this was perfused at about 6 ml/min. To test how fast the solutions could be changed at the fiber surface, the membrane potential of a fiber was monitored while the $[K]_o$ was switched (at the delivery tube to the chamber) from 2.5 mM to 10 mM and back to 2.5 mM, using constant [K] [Cl] product solutions.[8] For these solutions, no net KCl or water movements across the membrane are expected, and therefore the response of the membrane potential should be exactly in step with the actual concentration change at the surface of the fiber being measured. The 2 min required for the membrane potential to reach a new steady potential level (on changing solutions) can be taken as the actual time required to change solutions at the fiber surface in this chamber.

Analysis for muscle sodium and potassium. The pelvic bone was removed and the remaining muscle lightly blotted on low-ash filter paper, immediately before measuring its wet weight. Dry weights were taken after drying overnight at 105°C. The dried muscle was then ashed for 8 hours at 530°C in a Pt crucible, after which the ash was dissolved in deionized water. The solution for the ash was brought up to about 50 ml with deionized water and the amount of total ash solution was measured by weighing to the nearest 10 mg. The concentrations of Na and K in the ash solutions were measured by flame photometry (usually 10 to 100 μeq/l). Desmedt[3] noted that for his blotting procedure the extra-fiber water weight = 0.125 x (wet weight), and then also that the dry weight = 0.215 x (wet weight). This leaves the fiber water = 0.66 x (wet weight). From these figures one can calculate the ratio of fiber water to dry weight = 3.07, and also extra-fiber water weight = (wet weight - 4.07 x dry weight). The last two relationships should be insensitive to any errors which would arise from a variability of the blotting procedure. The ionic content of the muscle fibers (free of blotting errors and also corrected for extracellular ions) can be obtained from:

$$C_i = \frac{C_f W_a - C_o [WW - 4.07\ DW]}{DW}$$

where C_i is the ionic content of the fibers (in meq/kg dry weight of muscle), C_f is the ionic concentration of the ash solution (in meq/l), C_o is the ionic concentration of the last solution bathing the muscle before blotting (in meq/l), WW and DW are (respectively) the wet weight and dry weight of the muscle (in kg), W_a is the weight of the total ash solution (in kg). The weight of the ions in dilute solutions has been ignored. For all of the muscles reported in the present work, this method of analysis has been used,

but, the ionic contents are expressed as concentrations (meq/kg fiber water) by multiplying C_i by 3.07. A measure of the reproducibility afforded by this method is illustrated in the column comparing the $[K]_i$ of paired muscles in Table III (see Results).

Although errors due to blotting variations can be removed by this kind of analysis, if net movements of water occur across the fiber membrane a new error is introduced, for which it is difficult to correct regardless of the method of analysis. The extra-fiber water may no longer be a constant fraction of the wet weight of the muscle, and the ratio of fiber water to dry weight of the muscle may no longer equal 3.07. The flux data shown in Figure A-1 suggest that a net efflux of (Na + K) occurs when the muscle is bathed in Na- and K-free saline. Electroneutrality requires that this is largely balanced by an influx of other cations (Tris or Ca) and/or an efflux of anions by either active or passive means. If only an exchange of cations occurs to balance the system, no net water movement is expected. To the extent that anions also leave the fibers a loss of water and a fiber shrinkage may be expected.

III. RESULTS

Behavior of Na-enriched muscles bathed in Na- and K-free saline. When Na-enriched muscles are bathed with a Na- and K-free (Tris-substituted) saline which is continually perfusing through the chamber, the surface fibers depolarize and may reach fairly steady levels of membrane potential after about 1 hour. (Although the $[Cl]_o$ is slightly smaller in the Tris solution, this could account for no more than 1 mV of depolarization, and this should be a transient effect only.) The time constant for this depolarization is about 15 minutes, and for some of the muscle fibers an initial and transitory hyperpolarization can be observed before the main depolarizing effect of

these solutions occurs. In Figure 1 these effects are shown for a paired set of muscles, one of which had been Na-enriched for about 2 days, the other had been similarly treated for 1 day (see Methods for Na-enrichment of muscles). In about 1-1/2 hours, the fibers depolarized by more than 25 mV. (These results differ from those reported

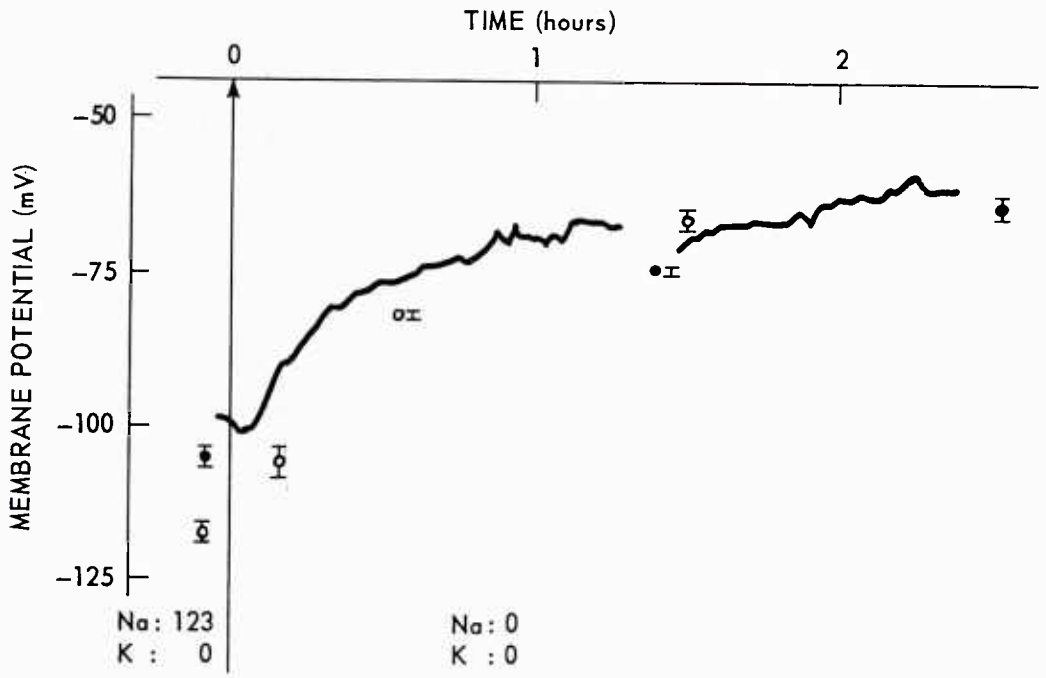

Figure 1. Depolarization of membrane during bathing period in Na- and K-free saline for paired muscles. Bathing solution changed from Na-enrichment to Na- and K-free at zero time. Filled circles and trace: muscle Na-enriched for 55 hours at 5°C. Each filled circle represents mean V_m for 7 to 15 fibers ± S.E. as indicated. The two line segments represent V_m for two different surface fibers in the same muscle. The lines were traced from points taken from a pen recording of V_m (obtained during a continuous impalement by microelectrodes) in each case. Open circles: paired muscle from same frog, Na-enrichment for 33 hours. Each open circle represents the mean V_m for 7 to 11 fibers. Final $[Na]_i$ and $[K]_i$ for muscles (in meq/kg fiber water): filled circles, 29, 59; open circles 25, 94. All electrical measurements shown in this and succeeding figures were taken at room temperature (about 22°C).

by Henderson,[6] which show about 20 mV of hyperpolarization for the membrane potential of Na-enriched muscles when the bathing solution is changed in a similar manner. Henderson also reported that subsequent addition of strophanthidin produced a depolarization which also contrasts with our measurements. We do not know why our results and Henderson's results differ.)

The continuous lines (and filled circles) show the variation in the membrane potential (V_m) for the muscle enriched for about 2 days while the open circles show mean values of V_m for fibers in the paired muscle which had been enriched for the shorter period. The depolarizing trace of the two fibers cannot be attributed to electrode damage of the membranes since the traces reasonably track the depolarization shown by other fibers of the same muscle (means represented by filled circles) penetrated only briefly by microelectrodes. In fact, the observed depolarizations can be simply interpreted as a shift in V_m resulting from bathing these muscles with Na- and K-free (Tris-substituted) saline.

It will be suggested in later sections of this report that much of the 25 to 30 mV of depolarization which is displayed in Figure 1 is due to a depolarizing electrogenic pump. Alternatively, one might consider that if Tris is more permeant than Na, then the depolarization might be due to ion selectivity rather than the appearance of a depolarizing pump.

Behavior of Na-enriched muscles on addition of potassium to Na-free solutions. If a Na-enriched muscle has been bathed in Na- and K-free solution and then K is added to the bathing solution, the rate constant for net Na efflux increases and the muscles also accumulate net K.[15,18] These ionic flux changes are sensitive to strophanthidin,

and produce membrane potential changes. In Figure 2, a muscle fiber is shown which depolarized about 22 mV during a 20-minute exposure to Na- and K-free saline. At 20 min, 2.5 mM K was added to the bathing solution and the fiber then hyperpolarized about 22 mV. This polarization was reversed when K was later removed from the bathing solution. A somewhat smaller hyperpolarization was observed for fibers of the same muscle when 2.5 mM K was reintroduced to the bathing solution about 3 hours later. This second K-induced polarization was reversed by the addition of strophanthidin.

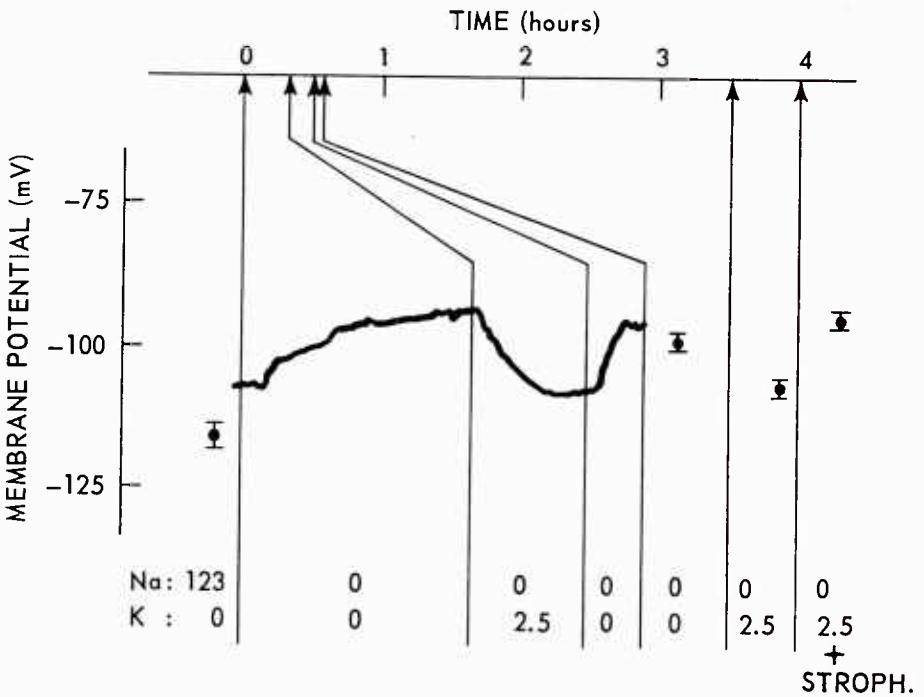

Figure 2. Effect of 2.5 mM K (and 5×10^{-5} M strophanthidin) on V_m. The muscle was Na-enriched for 55 hours at 5°C before electrical measurements shown. Each filled circle represents mean V_m for about seven fibers ± S.E. as indicated. The continuous trace (at expanded time scale) represents V_m for a single fiber of this muscle bathed sequentially in 0, 2.5, and 0 mM K, all Na-free salines (traced directly from the pen record).

Similar effects are shown for another muscle in Figure 3. In Na- and K-free saline, the muscle fibers depolarized by about 25 mV. About 6 hours later, when 2.5 mM K was introduced to the bathing solution, the membrane potential hyperpolarized by about 22 mV. Addition of strophanthidin again depolarized the membrane. It is well-established that this type of hyperpolarization is due to an activation of the Na-K pump by external K, whereas in the absence of pump effects the addition of external K would

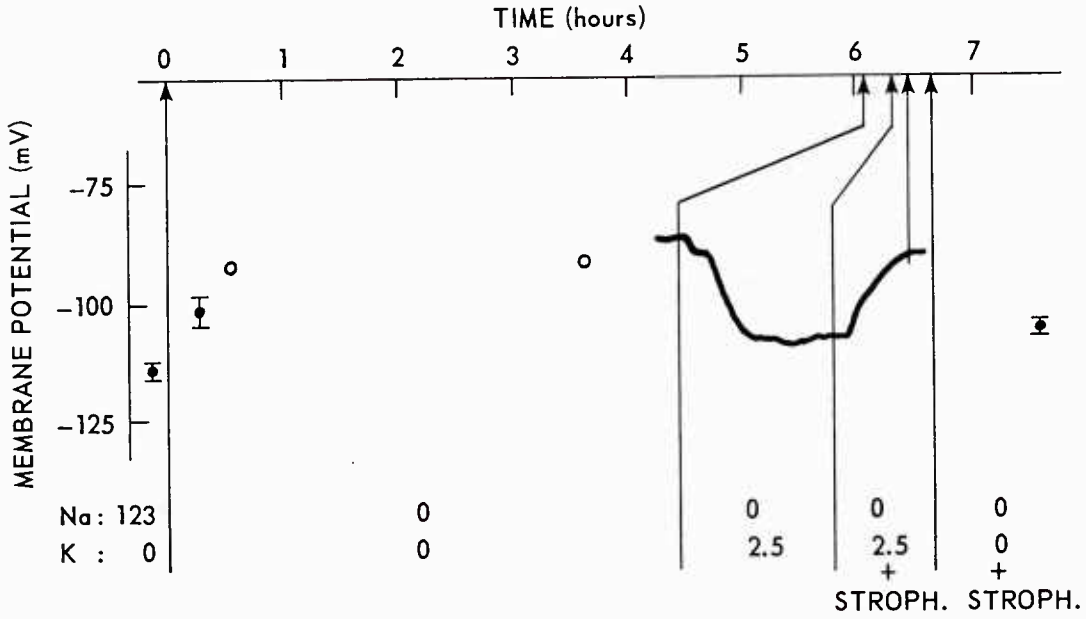

Figure 3. Effect of 2.5 mM K (and 5×10^{-5} M strophanthidin) on V_m. The muscle was Na-enriched for 53 hours at 5°C before electrical measurements shown. Each filled circle represents mean V_m for about seven fibers ± S.E. as indicated. The two open circles represent V_m for a different surface fiber at each time. The continuous trace (at expanded time scale) represents V_m for a single fiber of this muscle bathed sequentially in 0, 2.5 mM K and 2.5 mM K containing strophanthidin, all Na-free salines (traced directly from pen record). Final $[Na]_i$ and $[K]_i$ respectively (in meq/kg fiber water) for this muscle: 20 and 62; for the paired control muscle immediately after similar Na-enrichment: 61 and 63.

tend to depolarize rather than hyperpolarize the membrane. The Na-K pump ratio for this membrane bathed in Na-free, 2.5 mM K saline should be greater than unity. Both Figures 2 and 3 show the expected responses of the membranes to the presence of external K and also to the addition of strophanthidin. To explain the potential changes shown in Figure 2, it would be sufficient to require only that in both the Na-enrichment solution and in the Na-free, 2.5 mM K solution an ordinary hyperpolarizing electrogenic pump is operating (which extrudes Na at a rate faster than K is taken up). Thus, removal of Na from the K-free Na-enrichment solution might inhibit active Na and K transport, and removal of K from the Na-free, 2.5 mM K solution might also inhibit the pump. Although the overall effects of the removal of the Na in one case and the removal of K in the other case result in similar potential changes, the actual process or step by which this occurs could be different.

Alternatively, these potential changes can also be explained by a reversal of the net pump current. In the Na-free, 2.5 mM K saline, there is a net efflux of actively transported cations; in the Na- and K-free saline there may be a net influx of actively transported cations. In the Appendix the Na and K flux data are given for muscles bathed in the latter solution, from which it is apparent that more K can be actively transported into, than Na actively extruded from, these muscles. If the direction of the net pump current differs in the two solutions, then the tracing for V_m shown in Figure 2 demonstrates this reversal of the net pump current in the same fiber. Thus, for a change in pump ratio only a change in the $[K]_o$ would be required. Similar behavior can also be inferred from Figure 3. An interesting consequence of this explanation would be that the removal of external K decreases the active Na efflux to a greater

extent than the possible decrease in active K influx. However, the data (presented in Figures 2 and 3) cannot discriminate between the two explanations. Also in Figure 3, in the presence of strophanthidin, the removal of external K resulted in a large hyperpolarization, as expected for a reasonably good K electrode. For freshly dissected muscle fibers, where the electrogenic pump may be limited to only a few mV, Hodgkin and Horowicz[8] also noted that V_m hyperpolarized by about 20 mV when $[K]_o$ was reduced from 2.5 mM to 0.5 mM.

Effect of strophanthidin on the membrane potential of muscles bathed in Na- and K-free saline. Figure 3 suggests that it may be possible to hyperpolarize the membrane by simply adding strophanthidin to the Na- and K-free saline bathing Na-enriched muscles. However, the evidence for a strophanthidin-induced hyperpolarization (in Na- and K-free saline) from Figure 3 is weak as no comparable data (mean V_m in strophanthidin-free saline for this muscle) are available. Since strophanthidin inhibits the Na-K pump system, a strophanthidin-induced hyperpolarization could mean that when the muscles are bathed in Na- and K-free saline more cations are being actively transported into the fibers than are actively leaving. If this is the case, then the progressive depolarization of the Na-loaded muscles (shown in Figures 1 and 2) bathed in the Na- and K-free saline results from the appearance of a depolarizing pump current.

In order to show unambiguously the effects of strophanthidin, a number of Na-enriched muscles were bathed in the Na- and K-free saline, and the membrane potentials were recorded before and after the addition of the drug. The membrane consistently hyperpolarized when strophanthidin was added to the Na- and K-free bathing

solution, and this is clearly shown in the collected data of Table II and also in the single muscle fiber record in Figure 4. When Na-enriched muscles were transferred from the Na-enrichment solution to a solution free of Na and K, the mean V_m always depolarized, and when strophanthidin was later added to the bathing solution, the V_m began

Table II. Hyperpolarizing Effect of Strophanthidin When Added to Na- and K-Free Bathing Solution

Muscle number	Na-enrichment $\bar{V}m \pm$ S. E. (mV)	(N)	Wash period (hour)	Na- and K-free $\bar{V}m \pm$ S. E. (mV)	(N)	Na- and K-free + strophanthidin $\bar{V}m \pm$ S. E. (mV)	(N)	Strophanthidin effect $\Delta(\bar{V}m)$ (mV)	Final concentration (meq/kg-fiber water)	
									$[Na+]_i$	$[K+]_i$
267B	-104.6 ± 2.75	(7)	2:15	-70.0 ± 0.93	(6)	-80.7 ± 1.67	(14)	-10.7	25	68
268B	-105.3 ± 1.18	(15)	2:45	-63.6 ± 1.35	(13)	-78.9 ± 1.71	(7)	-15.3	29	59
269B	-116.8 ± 1.81	(8)	1:10	-78.6 ± 1.18	(8)	-87.1 ± 2.50	(20)	-8.5	33	71
270B	-113.1 ± 1.20	(11)	2:17	-75.6 ± 2.22	(9)	-85.1 ± 2.08	(9)	-9.5	41	68
273A	-104.4 ± 1.12	(14)	2:44	-74.7 ± 1.71	(10)	-85.2 ± 2.41	(12)	-10.5	32	67
277A	(not observed)		3:11	-87.0 ± 0.76	(21)	-107.7 ± 1.53	(13)	-20.7	27	55
278A	(not observed)		3:00	-74.2 ± 2.27	(5)	-82.0 ± 4.15	(4)	-7.8	24	54
283A	-116.2 ± 1.40	(12)	0:40			-117.1 ± 0.66	(12)		49	45
285A	-111.4 ± 1.41	(10)	0:20	-92.1 ± 2.22	(7)	-113.4 ± 1.22	(24)	-21.3	47	59
286A	-103.3 ± 1.14	(7)	0:20	-86.5 ± 3.03	(6)	-111.4 ± 1.23	(16)	-24.9	61	43
Mean values	-109.4 mV			-78.0 mV		-94.9 mV		-14.4 mV		

to hyperpolarize after a short delay. Hyperpolarization was usually complete in about 1/2 hour. When we started taking these membrane potential measurements, we believed that $[Na]_i$ would have to be considerably reduced to produce the net inward-going pump current. Our initial protocols therefore included a preliminary 2- to 4-hour bathing period in the Na- and K-free saline, to lower $[Na]_i$ before addition of strophanthidin. Later on, we decreased the initial bathing period, and expected to find that the strophanthidin-induced hyperpolarization would also be reduced. However, this was not the case. In fact, of the three muscles showing more than 20 mV

of hyperpolarization on addition of strophanthidin, two were bathed in the Na- and K-free saline for only 20 minutes before addition of the drug (see muscles 285A and 286A of Table II). For three of the muscles (277A, 278A, and 283A), a single muscle fiber was impaled just before addition of strophanthidin and the microelectrode remained in the fiber for more than 15 minutes while the fiber hyperpolarized. Even though for two of these fibers the microelectrode left the fiber early, before a steady V_m could be observed, the membrane potential had hyperpolarized by 13 and 14 mV respectively.

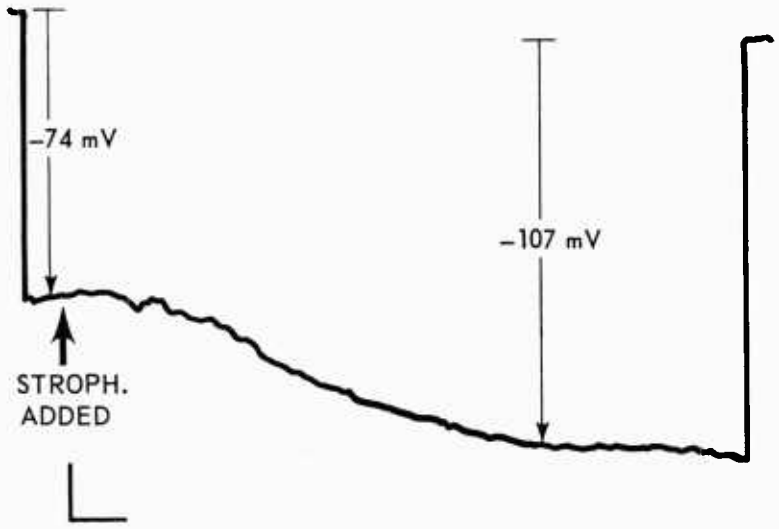

Figure 4. Hyperpolarizing effect of strophanthidin. 10^{-5} M strophanthidin added to Na- and K-free saline bathing muscle at time indicated. The trace (taken directly from a pen record) shows the hyperpolarizing effect of strophanthidin on a single muscle fiber continuously impaled by a microelectrode. Na-enrichment of muscle for 42 hours at 5°C. The vertical bar (lower left in figure) indicates 16 mV and the horizontal bar indicates 4 minutes, for the above record. The initial and final portions of the trace were made with the microelectrode in the external bathing solution before and after impalement of this fiber. Mean values of V_m for other fibers of this muscle (277A) in these solutions are given in Table II. Final $[Na]_i$ and $[K]_i$ for this muscle and its control pair are given in Table III.

For the third fiber, a complete hyperpolarization was observed and this is shown in Figure 4.

For a number of the muscles (apparently those associated with shorter bathing periods in the Na- and K-free saline) addition of strophanthidin to the Na- and K-free saline resulted in hyperpolarizations close to or beyond the values of membrane potentials observed in the initial Na-loading solution. For these muscles, if strophanthidin did not increase the membrane permeability for K[4,5] an explanation for the strophanthidin-induced hyperpolarization may be limited to an inhibition of a depolarizing electrogenic pump by strophanthidin. Alternatively, one might consider the possibility that the effect of strophanthidin was to simply decrease the Tris-permeability. Although it seemed possible that a portion of the depolarization, which appeared when the Na of the Na-enrichment solution was replaced by Tris, might have been due to an inhibition of a hyperpolarizing electrogenic pump, the substantial hyperpolarizations which appeared on a subsequent addition of strophanthidin to the Na- and K-free bathing solution cannot at the same time be explained by a further inhibition of hyperpolarizing electrogenic pumps. For the other muscles listed in Table II, which hyperpolarized in strophanthidin to levels less negative than -90 mV, it would not be surprising if extended bathing periods in the Na- and K-free saline (2 to 3 hours) could have produced a net loss of the $[K]_i$ in the surface fibers of the muscles without having appreciably affected the values of $[K]_i$ for the whole muscles. If this was the case, a somewhat depolarized level of V_m may be expected for the surface fibers bathed in Na- and K-free saline both before and after addition of strophanthidin. Nevertheless, the main

effect of the addition of strophanthidin to the bathing solution was to consistently produce large and significant hyperpolarizations of the membranes.

Effects of external Tris versus external sodium. When Na-enriched muscles are bathed in solutions where the Na and K have been replaced by Tris, the $[K]_i$ loss is negligible while the $[Na]_i$ decreases significantly during the bathing period (see Table III). A loss of 50 percent of the $[Na]_i$ in 3 hours is not uncommon. These effects were

Table III. Loss of Sodium and Retention of Potassium for Na-Enriched Muscles Bathed in Na- and K-Free (Tris) Saline*

Muscle number		Wash period (hour)	Retained Na and K (meq/kg-fiber water)		
			$[Na+]_i$	$[K+]_i$	$\Sigma[Na+]_i + [K+]_i$
270	A†		(64)	(65)	(129)
	B	2:17	41	68	109
272	A	4:33	29	61	90
	B†		(69)	(59)	(128)
273	A	2:44	32	67	99
	B†		(55)	(66)	(121)
275	A	3:40	23	68	91
	B†		(51)	(71)	(122)
276	A	3:28	22	70	92
	B†		(51)	(70)	(121)
277	A	3:11	27	55	82
	B†		(73)	(55)	(128)
278	A	3:00	24	54	78
	B†		(66)	(60)	(126)

* Each pair of muscles Na-enriched 45-56 hours at 5°C. Control muscles (†) analyzed immediately thereafter for internal ions. Experimental (paired) muscles additionally bathed for the time indicated in the second column in Na- and K-free (Tris) saline before analysis for retained Na and K.

noted also in Mg-substituted solutions.[18] Sodium leaves the muscle fibers and at the same time overall electroneutrality is preserved. Thus the muscle must lose anions or take up external cations, and these exchanges must be electrically equivalent with

the movements of Na. From the viewpoint of maintaining electroneutral exchange of ions when Na is lost, the required movement of other ions could be via either active or passive pathways. If the membrane is at all permeable to Tris, at least some of the Na loss must be balanced by a passive influx of Tris.

If the membrane were significantly more permeable to Tris than to Na, then this permeability difference alone would produce a significant component of the depolarization which was observed when the external Na was replaced by Tris. But if the electrogenicity of the pump can also be altered by the replacement of external Na with Tris, then the change in membrane potential accompanying the change in external solution does not necessarily indicate the more permeant ion. In the presence of strophanthidin, the change in potential which accompanies the replacement of Na should tell whether Na or its replacement is more permeant. The results of Table IV and Figure 5 show the effect of replacement of the external Na by Tris in the presence of strophanthidin.

Table IV. Substitution of External Sodium with Tris (in the presence of strophanthidin)*

Muscle	Na-enrichment	Na-enrichment + strophanthidin $\overline{V}m \pm$ S.E.	Na- and K-free + strophanthidin mV
283B	-110.9 ± 0.82 (13)	-99.3 ± 1.21 (7)	-107.0 ± 1.52 (15)
285A	-111.4 ± 1.41 (10)	-106.6 ± 2.17 (9)	-113.4 ± 1.22 (24)
285B	(not observed)	-99.3 ± 0.63 (11)	-109.9 ± 0.87 (33)

* For muscle 285A, the Na- and K-free (+ strophanthidin) saline bathed the muscle before bathing in Na-enrichment + strophanthidin solution. Figure 5 is a record taken for a single fiber of muscle 285A.

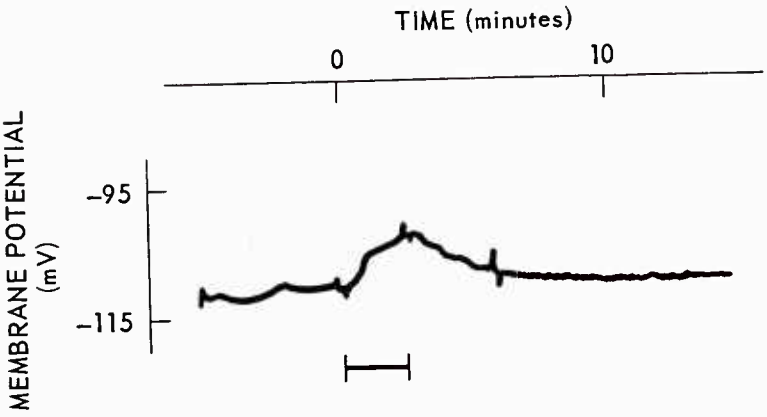

Figure 5. Effect on V_m (single fiber) of substitution of Tris by Na in the bathing solution. Both solutions contain 5 x 10^{-5} M strophanthidin. Na-enrichment of muscle by soaking 54 hours at 5°C in Na-loading solution. After bathing about 2 hours in Na- and K-free saline, and 1-1/2 hours in a solution containing strophanthidin, 123 mM Na (K-free) saline bathed the muscle for a little more than 2 minutes. (Duration in the Na-containing solution is indicated by the horizontal bar under the tracing.) The bathing solution was returned to Na- and K-free saline + strophanthidin when the bar ends.

The membrane potential hyperpolarized by about 7 mV when the Na of the Na-enrichment + strophanthidin solution bathing the Na-enriched muscles was replaced by Tris, and this was a reversible effect. This implies that in the presence of strophanthidin, the Tris-permeability is smaller than the Na-permeability. These data do not rule out the possibility that in the absence of strophanthidin, the Tris-permeability might be significantly greater than the Na-permeability. If in the absence of strophanthidin, Tris depolarizes the membrane simply because of a membrane permeability putatively greater for Tris than for Na, then it would also be required that the drug reduces the Tris-permeability to a value less than the Na-permeability. Whether or not Tris is actually more permeant than Na in the absence of strophanthidin is not easily resolved. If it were, then one could expect that after the chloride had

redistributed across the membrane (following the depolarization induced by the external Tris), the membrane potential should show some sign of a hyperpolarization as Tris is accumulated by the muscle fiber. No sign of such a delayed hyperpolarization was observed, even during a 6-hour period in the Tris saline.

Addition of strophanthidin to Na-enrichment solution. A second feature of the data shown in Table IV is that the membrane will depolarize (about 5 to 10 mV) when strophanthidin is added to the Na-enrichment solution bathing the muscles. This implies that even in the absence of external K, Na-enriched muscles pump electrogenically, and that in this solution the pump contributes a small hyperpolarizing potential to the membrane.

IV. DISCUSSION

The strophanthidin-induced hyperpolarization. The main observation reported here is that Na-enriched muscle fibers bathed in a Na- and K-free saline hyperpolarize when strophanthidin is introduced to the bathing solution. If the main effect of strophanthidin is to inhibit the active transport of Na and K, then it may be concluded that before the pump-inhibiting drug was added the net pump current was inward-going, and therefore the Na-K pump ratio was less than unity. On the other hand, if the effect of strophanthidin were to increase $[Na]_i$ or $[K]_i$, a hyperpolarization could be expected even if no electrogenic pump had been present. Although this does not seem likely, one may consider the possibility that strophanthidin might alter the P_{Na}-P_K ratio. A reduction of this ratio by any means would also produce a hyperpolarization. In fact, this is the very mechanism which has been proposed by Gorman and Marmor[5] for the hyperpolarization which appears about 4 hours after addition of strophanthidin to

solutions bathing the Aplysia neurons. For frog muscle we do not believe that a strophanthidin-induced reduction of the P_{Na}-P_K ratio causes the observed hyperpolarization for the following reasons. Figure 4 suggests that if an alteration of the P_{Na}-P_K ratio were responsible for the strophanthidin-induced hyperpolarization, these permeability changes may be slightly delayed, after introduction of the drug. It is difficult to resolve (at early times) possible changes in the rate constants for Na and K loss into Na- and K-free solutions containing strophanthidin from the wash-out data given in the Appendix (Figure A-1). However, good time resolution is available from the ^{42}K wash-out data reported by Sjodin and Beaugé,[18] and no sign of an increasing P_K appears during the first 3 hours. Possibly the best evidence that strophanthidin does not intrinsically increase P_K for muscle comes from Sjodin and Beaugé.[17] When strophanthidin was added to a 5 mM K Ringer solution bathing low $[Na]_i$ muscles, the K efflux rate constant showed only a slight increase (ca. 3 percent). (By using low sodium muscles, the possible influence of the back flux of K on the rate constant for K efflux was minimized.) Again, there is no indication that strophanthidin by itself will noticeably increase the P_K for frog muscle fibers.

The membrane depolarization resulting from removal of external Na. When Na-enriched muscles were transferred from a Na-containing K-free saline to the Na- and K-free saline, the membrane potential depolarized, usually by more than 20 mV. This was not a fast change in potential, nor could this have been fast for Cl-permeable muscle fibers bathed in Cl-containing solutions. (The change in $[Cl]_o$ could account for no more than 1 mV of depolarization.) In view of the size of hyperpolarization which followed addition of strophanthidin to this bathing solution, it is possible that the

earlier depolarization comes from a depolarizing electrogenic pump. However, other alternatives for the depolarization should also be considered. These include: (1) the membrane might be more permeable to the external Tris ions than to external sodium (2) the internal potassium ions might leave the fibers more quickly in the presence of external Tris; (3) the P_{Na}-P_K ratio might increase at the same time that $[Na]_i$ decreases; (4) a reduction of the pump ratio (via inhibition of both Na- and K-active transport) might result from substitution of external sodium with Tris; and (5) a reduction of the pump ratio might occur via stimulation of the K pump. For neither of the last two possibilities would a depolarizing electrogenic pump be required. These changes would simply reduce the normal hyperpolarizing electrogenicity.

It has already been suggested that if Tris is more permeant than Na, the membrane would ordinarily depolarize on replacement of the external Na by Tris, and then a depolarizing electrogenic pump would no longer be required to explain the observed depolarizations. Since no reliable estimates for P_{Na} and P_{Tris} have been reported for muscles which have been stored for several days in K-free saline at reduced temperatures (and which are then bathed in a Tris saline), a good comparison of P_{Na} with P_{Tris} is not yet possible. Nevertheless, a crude value for P_{Tris} can be calculated from our data. Na-enriched muscles which are bathed in Na- and K-free (Tris) saline lose Na but retain virtually all of their K. If all of the Na loss is replaced by an equivalent uptake of Tris, and if this is an entirely passive uptake, then one can compute the upper limit for P_{Tris}. (If the muscle takes up Ca or loses anions during this period of net Na loss, our estimate for P_{Tris} would be correspondingly too great.) Na-enriched muscles which have been bathed for several hours in the Tris saline have membrane

potentials of about -72 mV and the $\lceil Na \rceil_i$ is about 32 meq/kg-fiber water (data from Table II). By combining these values with the concentration of external ionized Tris (112 mM) and the rate constant for Na loss (0.45 hour^{-1}, from Figure A-1), P_{Tris} could be as high as 30 x 10^{-9} cm/sec, for an assumed mean fiber diameter of 100 μm. This estimate is not necessarily greater than what one might find for P_{Na} for these Na-enriched muscles bathed in Tris. But even if this estimate for P_{Tris} were sufficiently large to account for the observed depolarizations, then one would be compelled to believe that strophanthidin can reduce P_{Tris}, and thereby produce the observed hyperpolarizations. Furthermore, if 2.5 mM K were also present in the bathing solution, then the combined effects of strophanthidin (on both P_{Tris} and the hyperpolarizing electrogenic pump) would now result in a substantial net depolarization. It is cautioned that although these ad hoc properties of the effects of Tris and strophanthidin on the membrane do not seem to make this a likely alternative for a depolarizing pump, these characteristics taken separately are not entirely implausible, and perhaps this should be considered as a qualification on the conclusions made in this report.

If, when the muscle is bathed in Na- and K-free saline, K were lost, then this might result in a depolarization without requiring the presence of a depolarizing pump. However, the wash-out data given in the Appendix show that there is no significant loss of internal K for whole muscle bathed in the Tris-substituted saline. In particular there is no sign of a loss of K which extends only for the 1st hour or so, after which it has been noted that the membrane potential remains fairly constant. The electrical measurements were made for the surface fibers only, and it is possible that the surface fibers could lose K without having this appear in the wash-out data taken on whole

muscles. For some of the muscles tabulated (Table II), a loss of K from the surface fibers might have occurred, considering the fact that their potentials after addition of strophanthidin remained well below 100 mV. Nevertheless, since some muscles, also bathed in strophanthidin, exhibited membrane potentials in excess of 100 mV, any substantial loss of K from the surface fibers may be incidental, and does not necessarily accompany the depolarizations observed in the Na- and K-free saline.

If the P_{Na}-P_K ratio were to increase as Na leaves the muscles in the Na- and K-free saline, this too could produce a depolarization. We have no direct measures for this possibility, but Mullins and Awad[12] have suggested that elevation of the $[Na]_i$ can increase the P_{Na}. However, the increased Na fluxes observed when $[Na]_i$ rises may indicate that the exchange diffusion of Na, rather than P_{Na}, was increasing.[18] Thus it seems unlikely that the strong depolarizations which were observed could have resulted from P_{Na} changes arising from a loss of internal Na.

The two remaining alternatives concern the possibility that the depolarizations (which were observed when the muscles were transferred to Na- and K-free saline) come from a change in the pump currents, but in such a way that when the depolarization occurs, the pump ratio need not become less than unity. A pump-dependent depolarization generally means only that the outward-going component of the pump current has decreased (or that the inward-going component has increased). It is not required that the pump ratio ever goes to values less than unity. However, removal of external Na increases the net Na extrusion.[15] Also, removal of external Na (when only leakage amounts of external K exist) may result in an increase of the active uptake of K. Thus when the external Na is replaced by Tris, there is no reason to believe that the active

transport of both Na and K decrease in a way which would require the pump ratio to be reduced but still remain greater than unity. For this solution change, although the K pump could increase, this still does not show that the pump ratio becomes less than unity. Nevertheless, it would be difficult to support the notion that the pump ratio actually remained greater than unity on removal of external Na when a subsequent addition of strophanthidin now results in a clear hyperpolarization.

The strophanthidin-insensitive fluxes. In the Appendix the values for the strophanthidin-insensitive rate constants for K loss and Na loss from Na-enriched muscles into Na- and K-free saline are given. If these values represent only the purely passive effluxes, then the ratio of these rate constants should equal the P_{Na}-P_K ratio. (In fact, use of the K efflux and Na influx data, in the absence of strophanthidin, from Hodgkin and Horowicz,[7] permits a calculation of a P_{Na}-P_K ratio of about 0.01 which is close to an independent estimate made from their electrical data.[8]) The ratio of our values of the strophanthidin-insensitive rate constants is considerably larger (0.675) than the expected P_{Na}-P_K ratio. This is not surprising, as analyses from other strophanthidin-insensitive flux data (from squid giant axons[1] and also from frog muscle[17]) also produce larger than expected permeability ratios. However, when Brinley and Mullins removed ATP_i from the squid giant axon by internal dialysis, then the ratio of effluxes of Na and K (each divided by their internal concentrations) comes more closely to the expected P_{Na}-P_K ratio. Moreover, the strophanthidin-insensitive Na efflux from freshly dissected frog muscles into Na-free (Li-substituted) saline[9] also seems to be at least an order of magnitude larger than what would be expected if all active extrusion of Na had been prevented by the drug. At the very least all of this is

a good indication that the strophanthidin-insensitive fluxes can include components of the Na pump and/or K pump.

The passive characteristics for the K efflux. When the muscles are bathed in Na- and K-free (Tris) saline, and strophanthidin is not present, at most only a negligible K efflux can be observed, and so we have assumed that under these conditions, the passive loss of K from the muscles will be approximately equal to the active uptake of K. Due to the expected active uptake of K, however, it is then impossible to directly measure the passive efflux of K at these times. Nevertheless, it should still be possible to estimate the magnitude of the passive K efflux. This is not a simple computation, and several assumptions are required. First, the K efflux may vary with V_m according to the constant field constructs: $\phi^K_{eff} = P_K[K]_i F(V_m)$, where $F(V_m)$ $= (V_m F/RT)/[1-\exp(V_m F/RT)]$. Thus V_m can influence the K efflux, and a depolarization will increase the calculated values of the efflux. Second, an estimate of P_K is required. However, as Hodgkin and Horowicz[8] have shown, for muscle fibers, P_K is strongly dependent on the driving force potential (V_m-E_K). Generally, as (V_m-E_K) becomes more positive, P_K will decrease.

Despite the inherent uncertainty for a value of P_K at any specific time, it may be of some interest to follow a model calculation to its conclusion, and to further see if these constructs contradict in any way the measurements which have been made. Before proceeding with the main calculation (that is, the estimation of the passive efflux in the absence of strophanthidin), P_K in the presence of strophanthidin can be estimated from our data. The figure for the rate constant for K loss in strophanthidin ($0.17\ hour^{-1}$) is taken from Figure A-1. Table IV gives -110 mV as a working estimate for the V_m

of these muscles when bathed in the Tris saline + strophanthidin. (The protocol for the muscles of Table IV bears a closer resemblance, than that of the muscles of Table II, to the protocol for the muscles of Figure A-1.) By using these values for the rate constant and V_m (and by assuming throughout these calculations that the mean fiber diameter is about 100 μm), one can calculate a value for P_K in the presence of strophanthidin of about 2×10^{-6} cm/sec. This is several times greater than what one usually figures for P_K, but it is within the estimates made by Hodgkin and Horowicz (1-2 $\times 10^{-6}$ cm/sec) when $(V_m - E_K)$ is close to zero. At these times the passive K efflux can also be calculated as about 7 pM/cm^2-sec.

When the Na-enriched muscles are bathed in the Tris saline, and in the absence of strophanthidin, one may suppose that $(V_m - E_K)$ can be about +25 mV. P_K may then be about 0.5×10^{-6} cm/sec (estimated from Table 8 of Hodgkin and Horowicz[8]). Then by using -70 mV for V_m and 60 mM for $[K]_i$, the passive K efflux can be evaluated, and is about 6 pM/cm^2-sec. If the electrogenic potential is taken as +25 mV, then by using the estimated K efflux, the K conductance (G_K) comes to 24 μmho/cm^2. This estimate is smaller than what one usually considers for G_K (100 μmho/cm^2). However, for striated muscle, the potassium conductance does decrease considerably with depolarization; in fact this estimate is close to those made by Hodgkin and Horowicz[8] for fibers having $(V_m - E_K)$ about +25 mV (their Table 8). Calculations of this sort are not entirely satisfying since the uncertainties and variations are unavoidable, and these resulting calculations can provide only crude values for the fluxes. Nonetheless, the approach may be of some interest in illustrating some of the complexities involved with the estimation of passive K fluxes for striated muscle. Further,

it is useful to know that the K fluxes are not necessarily inadequate to support pump-dependent depolarizations of the observed magnitudes.

The external K dependent pump stoichiometry. Perhaps what is most unusual about these results is that a depolarizing electrogenic pump was observed, and that this would require an active influx of K which exceeds the active efflux of Na. This cannot be a constant property of the electrogenic pump in frog muscle, since the presence of 2.5 mM K in the solution bathing similarly treated muscles makes the pump take on the opposite polarity. This at least implies a rather flexible stoichiometry for the actively transported Na and K. In the absence of external Na the pump stoichiometry depends very strongly on the external K concentration. It is recalled that the effect of strophanthidin is to depolarize the membrane potential when the bathing solution contains 2.5 mM K, but to hyperpolarize the membrane when the bathing solution contains neither Na nor K. It now remains to be seen how these results can fit with current ideas of the active transport of Na and K.

In Na-free (Tris-substituted) solutions, the $[K]_o$-sensitive portion of the Na efflux rate constant behaves with a Michaelis-Menten kinetic.[15] Thus, as the $[K]_o$ increases, the rate constant for Na efflux increases to a saturation level, with an apparent Michaelis constant of about 3 mM for external K. In addition to activating a strophanthidin-sensitive Na efflux, the K which leaks from the muscle will also be accumulated by the muscle fibers, and this influx also will be strophanthidin-sensitive. Although for squid giant axons the ATP_i-dependent K influx is linear with the $[K]_o$,[13] for muscle we still do not know how the $[K]_o$ affects the active uptake of K. That this is not a linear relationship in muscle can nevertheless be suggested from the present

work. If activation of Na-efflux saturates with increasing $[K]_o$, then it would be difficult to explain how removal of external K converts a hyperpolarizing electrogenic pump into a depolarizing one, unless the active uptake of K also not only saturates with increasing $[K]_o$, but does so with a smaller effective Michaelis constant.

In the presence of very small amounts of external K (and Na), it is therefore possible to suggest that external K can move onto the transport system for a rapid translocation into the fiber, and that a reduced steady-state density of K on some part of the transport system might also limit activation of the Na transport system. When the $[Na]_i$ is also not excessive, then under the conditions of a reduced activation of Na transport less Na would be pumped out than K pumped in, giving a pump ratio less than unity. A slight increase of $[K]_o$ should increase both the K and the Na pump currents. However, if the effective Michaelis constant for the $[K]_o$-dependent K transport process were somewhat smaller than the Michaelis constant for the $[K]_o$-dependent Na transport process, the stimulating effect of increasing the $[K]_o$ would be smaller for the K than the Na transport systems. In this way a small increase of $[K]_o$ should increase the pump ratio (and reduce the net inward-going pump current). As $[K]_o$ is continuously increased, this would lead to Na-K pump ratios greater than unity and the appearance of hyperpolarizing electrogenic pumping of the kind which was observed in Na-free 2.5 mM K-containing solution.

For muscle, external K apparently has two distinct functions for the Na-K active transport system. Potassium may combine with external sites in a saturating manner (possibly with a Michaelis-Menten kinetic) for inward translocation. External K also combines with sites in a saturating manner for activation of Na-efflux. That these may

be different sets of sites is suggested by the apparent necessity for different affinities of K for the sites responsible for the two transport processes.

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APPENDIX A

Flux Analysis for a Depolarizing Pump

Rate constants for Na loss and K loss from Na-enriched frog muscles into Na- and K-free solutions (in both the presence and absence of ouabain) have been reported by Henderson[6] (where Tris ions were employed as the substituting cation) and also by Sjodin and Beaugé[18] (where Mg ions were employed as the substituting cation). For each of these reports the rate constants were obtained by counting ^{22}Na and ^{42}K appearing in the bathing solutions coming from the whole muscles, and the appropriate corrections were made for tracer material coming from extracellular spaces. The values of the rate constants are several times greater in Tris than in Mg solutions. An alternative measure of these rate constants can come from an analysis of the total retained Na and K of the muscles themselves after different bathing periods in the Na- and K-free solutions.

Freshly dissected muscles were Na-enriched by soaking in K-free solution for periods of 40 to 50 hours at $5^{o}C$. Each muscle was then bathed for different periods of time in the Na- and K-free saline (at room temperature) before removal for analysis of total retained Na and K. Each muscle was individually suspended in its own test tube containing about 10 ml of wash solution. The solutions bathing the muscles were discarded and replaced with fresh solutions at least as often as every half hour. Muscle Na and K were analyzed by flame photometry (as detailed in Methods) on removal from the wash solution. For each bathing period (in a particular solution) four muscles were used. Only one member of any pair was used for a particular bathing period; the paired muscle was used for the bathing period either just preceding or following.

The mean values of Na and K retained by the muscles (in three different bathing solutions) are plotted as a function of the wash period (Figure A-1). It should be clear that the rate constant for the retained Na (for example, in Na- and K-free saline) would also have to be the rate constant for the net Na loss from the muscle into this solution.

The flux components, which can be calculated from the rate constants shown in Figure A-1, are net fluxes only. Thus the strophanthidin-sensitive Na efflux (into Na- and K-free saline) has a rate constant of 0.341 hour^{-1} (0.455 hour^{-1} less 0.114 hour^{-1}). This can result from the effects of strophanthidin on (1) active transport, and/or (2) passive transport, but is unrelated to any possible effect of the drug on the one-for-one Na-Na interchange component.

For the lowest set of data in Figure A-1, the bathing solution is Na-free but contains 2.5 mM K. For this solution, the rate constant for Na loss is 1.40 hour^{-1} for the first 2 hours (or for $[Na]_i$ greater than 5 mM). This compares with 1.38 hour^{-1}, the value obtained by Sjodin (Table II)[15] under similar circumstances, but by the tracer technique. When the internal Na becomes less than 5 mM, the rate constant for Na loss decreases sharply by about one order of magnitude, which also corresponds to Sjodin's findings. This may indicate an inhibition of the Na pump due to a reduced $[Na]_i$. (Compare the reduced Na efflux rate constant in this case with the Na efflux rate constant observed in the presence of strophanthidin, in the central portion of Figure A-1.) Surprisingly, not much K was accumulated by the muscles in the presence of 2.5 mM K in the bathing solution.

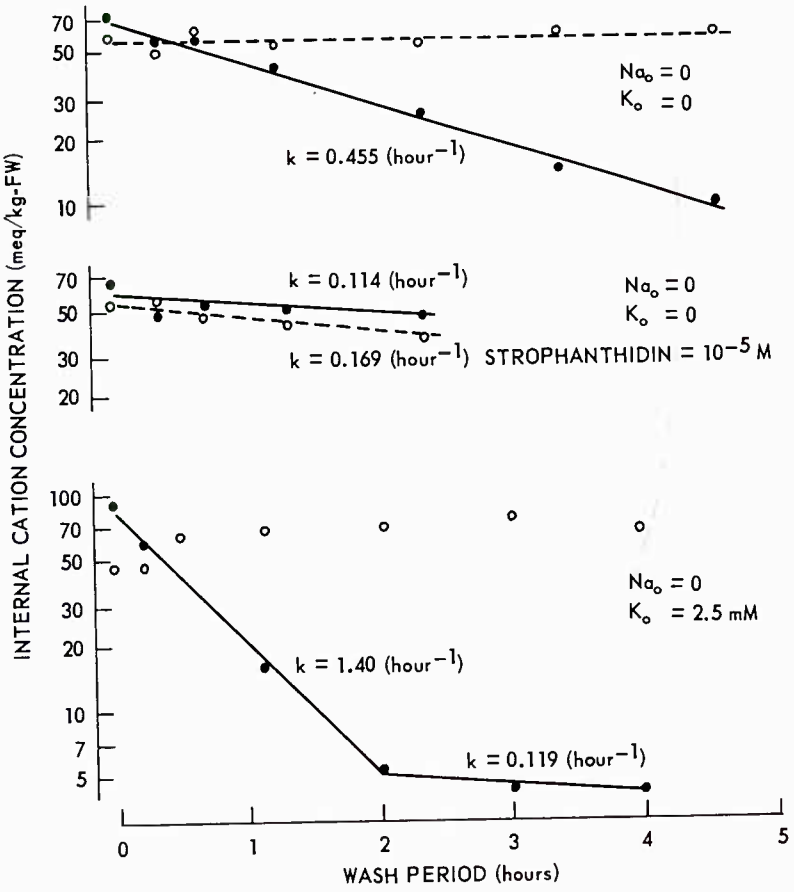

Figure A-1. Sodium and potassium retained by muscles after being bathed for different periods in the indicated solutions. (Before being bathed by these Na-free solutions, the muscles were Na-enriched for about 2 days at 5°C.) Internal sodium concentrations: filled circles (solid lines); internal potassium concentrations: open circles (interrupted lines). Bathing solutions: upper pair, sodium- and potassium-free; central pair, sodium- and potassium-free but with 10^{-5} M strophanthidin added; lower pair, sodium-free, 2.5 mM potassium. Where rate constants are indicated, the semilog regression was calculated from the data and an appropriate line was drawn. The line for potassium concentration in the upper pair was drawn to approximate the data; a zero slope was picked. Each point in the figure represents four muscles, with the pair of any given muscle bathed in the same solution but for a different period. Standard errors for each point are not indicated but for all points average to 10 percent of their means, with a range of 2 to 15 percent.

The remainder of the data shown in Figure A-1 (middle and upper sections) was obtained in Na- and K-free bathing solutions, and illustrates some of the effects of strophanthidin. Strophanthidin clearly decreases the rate constant for Na loss and increases the rate constant for K loss from Na-enriched muscles bathed in Na- and K-free solutions. There is no appreciable net loss of K in the absence of strophanthidin, but there is a significant loss of K when strophanthidin is present.

In the absence of strophanthidin, $[K]_i$ does not appreciably change, and after about 1 hour, V_m also remains fairly constant (see Figure 1). Under these conditions, the passive K efflux should remain constant. However, since $[K]_i$ does not change while the muscle is bathed in the Tris saline, then it should follow that K is continually pumped into the muscle at a constant rate.

For Na efflux into Na- and K-free (Tris) saline the situation is not the same. After a wash period of about 1/2 hour, the strophanthidin-sensitive Na efflux has a rate constant of about 0.34 $hour^{-1}$. If the main effect of strophanthidin is to inhibit active transport, then the Na pump current seems to decrease exponentially with time. Thus with the passage of time, although the outward-going Na pump current continues to decrease, the inward-going K pump current remains essentially constant, and a negative (or inward-going) net pump current should appear when the K pump current exceeds the Na pump current.

Analysis. If ϕ_{eff} is the net efflux, then:

$$\phi_{eff} = -\frac{V}{A}\frac{dC}{dt} \text{ (in pM/cm}^2\text{-sec)} \qquad (1)$$

where V/A is the volume-area ratio for the fiber (expressed in cm) and dC/dt is the rate of change of the internal concentration (expressed in pM/cm^3-sec). Since the upper and middle sections of Figure A-1 indicate that $[Na]_i$ and $[K]_i$ decrease reasonably exponentially with time, then regardless of the particular mechanism for Na or K loss, $dC(t)/dt = -kC(t)$, where k is the rate constant for this process. For a cylindrical fiber V/A can be replaced by d/4 where d is the fiber diameter, and then it follows that:

$$\phi_{eff}(t) = -\frac{d}{4} kC(t) \quad . \tag{2}$$

Now let ϕ_1^{Na} represent the Na efflux in the absence of strophanthidin, and ϕ_2^{Na} represent the Na efflux in the presence of strophanthidin. If ϕ_μ^{Na} represents the strophanthidin-sensitive portion of the efflux, then $\phi_\mu^{Na} = \phi_1^{Na} - \phi_2^{Na}$, and using the same superscript and subscript notation on the rate constants gives:

$$\phi_\mu^{Na}(t) = \frac{d}{4}[Na(t)]_i \{k_1^{Na} - k_2^{Na}\} \quad . \tag{3A}$$

For K, likewise:

$$\phi_\mu^{K}(t) = \frac{d}{4}[K(t)]_i \{k_1^{K} - k_2^{K}\} \quad . \tag{3B}$$

A single rate constant for the strophanthidin-sensitive Na efflux can now be written as:

$$k_\mu^{Na} = k_1^{Na} - k_2^{Na} \quad .$$

To the extent that the actively transported components of the fluxes can be identified with the strophanthidin-sensitive net fluxes, a neutral pump (or Na-K-pump ratio = 1) would require $\phi_\mu^{Na} = -\phi_\mu^{K}$. By setting these terms equal, one obtains a simple

relationship which is now independent of fiber diameter:

$$[Na]_i \{k_1^{Na} - k_2^{Na}\} = [K]_i \{k_2^K - k_1^K\} \ .$$

Since $[Na]_i$ and $[K]_i$ may be generally represented as exponential functions of time (see Figure A-1, in the absence of strophanthidin and when bathed in Na- and K-free solution), then at any time (t) after immersion of the muscle into the Na- and K-free saline:

$$[Na]_i = Na^o \exp(-k_1^{Na} t)$$

and

$$[K]_i = K^o \exp(-k_1^K t) \ ,$$

where Na^o and K^o represent the initial internal concentrations of these ions (at the time the Na-enriched muscle first enters the Na- and K-free saline).

Then, using the three previous relationships:

$$\frac{K^o}{Na^o} \exp(k_1^{Na} - k_1^K) t^* = \frac{k_1^{Na} - k_2^{Na}}{k_2^K - k_1^K} \ , \qquad (4)$$

where a solution for t* gives the time at which the strophanthidin-sensitive flux ratio should become unity. Tentatively, we may identify the strophanthidin-sensitive flux ratio with the pump ratio. Then at times earlier than t*, the electrogenic pump should be hyperpolarizing (or a Na-K pump ratio greater than unity); at later times the electrogenic pump should be depolarizing (a pump ratio of less than unity). So long as these rate constants for the net Na and K effluxes do remain constant, the pump

should become increasingly depolarizing. Even if the rate constants themselves become functions of time, this kind of analysis can still be used to compute t*, if the rate constants appropriate to the time of t* have been employed.

When the rate constants and the initial concentrations taken from Figure A-1 are applied to equation (4), the time of occurrence of a neutral pump t* is reckoned at about 2 hours. Thus, if strophanthidin is added after a 2-hour bathing period in Na- and K-free (Tris) saline, a hyperpolarization would be expected, while an earlier addition of strophanthidin should result in a depolarization. However, a number of factors can influence the calculated values of t*. For any particular muscle bathed in a strophanthidin-containing saline for a considerable time, K^o should be greater than the terminal value of $[K]_i$ obtained by flame photometry. The rate constants may be different for whole muscles immersed in standing solutions (i.e., data for Figure A-1), and for fibers bathed by solutions continually streaming over their surfaces (i.e., the electrical data showing membrane polarizations). The calculations of the internal concentrations (see Methods), and therefore also the estimation of the rate constants, will contain volume errors if any net water movements occurred during the wash period. Finally, if k_1^{Na} depends on the value of $[K]_i$, this too would change the estimate for t*.

In spite of the obvious differences in the ways fibers make contact with the external solutions, Sjodin and Henderson[19] have suggested that the rate constants for K fluxes in whole muscle may be close to the rate constants for single isolated fibers. Errors produced by fiber volume changes are probably small. During a 2-hour wash period in Na- and K-free saline, the total (Na+K) loss of the muscle (if also accompanied with an equivalent anion loss) could result in a substantial fiber volume decrease.

However, to correct equation (4) for the volume changes, the important factor is the relative volume change produced only by the presence of strophanthidin, and not the total change due to the Na- and K-free saline. Since the presence of strophanthidin might produce at most a 7 percent volume change in about 2 hours, an appropriate volume correction may reduce t* by less than 10 percent.

However, a 30 percent increase of $[K]_i$ (from 140 mM) results in a 50 percent decrease of strophanthidin-sensitive Na efflux for muscle;[2] and for red blood cells with reduced $[K]_i$ (about 60 mM), a 10 percent increase of $[K]_i$ results in a 10 percent decrease in Na efflux.[11] If these effects also occur in a Na-enriched muscle (where $[K]_i$ has been reduced to levels smaller than 50 percent of normal), then small increases in $[K]_i$ could have significant effects on k_1^{Na}, and this also would reduce the calculated value of t*. In fact, an increase in K^o from 55 meq/kg fiber water to 75 meq/kg fiber water would bring t* from about 2 hours down to about zero hours.

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13. ABSTRACT

Surface fibers of Na-enriched muscles, bathed in K-free (Na-containing) saline, depolarize when the external sodium is replaced isosmotically with Tris. Subsequent addition of strophanthidin to the Na- and K-free bathing solution results in a hyperpolarization. If the main effect of strophanthidin in these experiments is to inhibit the electrogenic pump, then it may be concluded that before addition of the drug the net pump current was inward-going. This contrasts with the usual observation that cell membranes generally have hyperpolarizing electrogenic pumps which are caused by Na-K pump ratios greater than unity. These direct electrical measurements suggest that the enzyme machinery responsible for the active transport of sodium and potassium is not always obligated to extrude sodium out of the cell faster than potassium is actively accumulated. Since the muscles retain virtually all of their internal potassium when bathed in Na- and K-free saline, the passively lost potassium would appear to be largely regained by an efficient back-pump process, which may only modestly stimulate active Na efflux.

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