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Effects on Sodium Efflux of Treating Frog Sartorius Muscles with Hypertonic Glycerol Solutions

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Summary. Efflux of sodium from frog sartorius muscles was measured during and after exposure to Ringer's fluid made hypertonic by addition of 400 mM glycerol. Effects of strophanthidin, removal of external Na, and variation of external K were determined. During exposure to glycerol-containing solutions, Na efflux increased. Upon return to Ringer's fluid, Na efflux at first increased further. After the initial increase, Na efflux gradually declined; for the first two hours the efflux of Na from treated muscles was higher than that from untreated muscles. In the second hour, the strophanthidin-sensitive fractions of Na efflux were slightly increased while the strophanthidin-insensitive fractions were slightly decreased when compared with untreated muscles. The responses of Na efflux to removal of external sodium and to varying external K were comparable in both treated and untreated muscles. This shows that, at first, the membranes which remained after glycerol treatment exhibited the normal characteristics of Na extrusion. For at least eight hours after glycerol withdrawal the Na efflux from treated muscles declined relative to that of untreated muscles. The decline was largely due to reduction in strophanthidin-sensitive fractions of efflux. Six to eight hours after glycerol withdrawal the Na efflux in treated muscles was less responsive to alterations in external K and Na than it was in untreated muscles. This indicates that aged glycerol-treated sartorii lost a substantial part of their capacity to actively transport sodium.

The mechanisms responsible for extrusion of sodium ions from muscle fibers of frogs and other cells have been the focus of much recent research. In one widely used approach, investigators have studied the responses of sodium fluxes to cardiac glycosides and aglycones. With these agents it has now been established that, in freshly isolated muscles, sodium efflux is about equally divided between glycoside-sensitive and glycoside-insensitive fractions (Edwards & Harris, 1957; Horowicz, 1965; Keynes, 1966; Keynes & Steinhardt, 1968; Horowicz, Taylor & Waggoner, 1970). Furthermore, it has been shown that external potassium activates the glycoside-sensitive fractions but has no effect on the glycoside-insensitive fractions (Keynes & Steinhardt, 1968). On the other hand, the effects of external sodium on the two fractions of sodium efflux defined by glycoside sensitivity are complex and depend on the history and treatment of the isolated muscles. The

response of the glycoside-insensitive fraction to external sodium is the simplest. About 75% of this fraction is activated by external sodium (Horowicz, 1965; Keynes, 1966); the other 25% remains after external sodium is replaced by lithium, choline or other cations. The glycoside-sensitive fractions, however, are either inhibited or activated by external sodium depending on the circumstances (Beaugé & Sjodin, 1968; Keynes & Steinhardt, 1968; Horowicz *et al.*, 1970; Erlij & LeBlanc, 1971; Sjodin, 1971). The factors involved are not fully resolved and clarification awaits the results of further studies.

Another method for studying membrane properties in muscle fibers has been developed recently. After muscles are exposed for an hour or longer to sufficiently hypertonic glycerol solutions, returning them to normal Ringer's fluid produces an uncoupling of contraction from excitation (Fujino, Yamaguchi & Suzuki, 1961; Howell, 1969). It has been generally thought that this is due to the disruption and disconnection of the transverse tubular system from the surface membrane which also occurs (Howell & Jenden, 1967; Krolenko, Adamjan & Shvinka, 1967; Eisenberg & Eisenberg, 1968). Recent evidence, on the other hand, suggests that although uncoupling of contraction from excitation and transverse tubular disconnection do occur, these events are separate (Fujino, Yamaguchi & Fujino, 1972; Dulhunty & Gage, 1973). Nevertheless, muscles treated with glycerol so as to 'detubulate' them have been useful in determining the distribution between transverse tubular membranes and surface membranes of such properties as total membrane capacitance, resting potassium conductance, and chloride conductance (Eisenberg & Gage, 1969; Gage & Eisenberg, 1969*a*).

The aim of the research described in this report was to determine how the various fractions of sodium efflux are altered by glycerol treatment. As will be seen, glycerol treatment produced relatively little change in sodium efflux early after glycerol withdrawal. Thus, 1 to 2 hr after glycerol withdrawal the normal characteristics of the sodium extrusion mechanisms remained largely intact; if there was any loss of efflux capacity it was more than compensated by the stimulation produced. As treated muscles age, there is a progressive loss in their capacity to extrude sodium.

An early account of this work has appeared elsewhere (Venosa & Horowicz, 1971).

Materials and Methods

All experiments were performed on paired sartorius muscles freshly isolated from *Rana pipiens*. Only those muscle pairs were used which were free of damage and parasites. The average muscle wet weight was about 60 mg. One muscle of each pair was left untreated and used as a control while the other was treated with glycerol-containing Ringer's fluid. The treatment consisted of a 60- to 75-min exposure to hypertonic glycerol-Ringer's

fluid followed by return either to normal Ringer's fluid or to one with increased calcium and magnesium concentrations.

The composition of normal Ringer's fluid was as follows: (in millimoles/liter): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.8; Na_2HPO_4, 2.15; NaH_2PO_4, 0.85. The solution with high concentrations of calcium and magnesium had the following concentrations of salts (mmoles/liter): NaCl, 115; KCl, 2.5; $CaCl_2$, 5; $MgCl_2$, 5; Na_2HPO_4, 1.07; NaH_2PO_4, 0.43. Hypertonic glycerol-containing Ringer's fluid used for the experiments was made by addition of 400 mmoles/liter glycerol to the ionic constituents listed above for normal Ringer's fluid. In some experiments the concentration of KCl in the external medium was altered without changing the other constituents. Lithium-containing Ringer's fluid was made by substituting LiCl for NaCl. Whenever strophanthidin was used it was added in a concentrated ethanol solution and its final concentration in the external medium was 3×10^{-5} M. The final ethanol concentration never exceeded 0.05% (v/v).

Sodium efflux was measured by following the loss of $^{22}Na^+$ from muscles previously loaded with this isotope. The loading solution was made by adding carrier-free $^{22}Na^+$ to Ringer's fluid in an amount to produce a specific activity of about 150 μC/ml. Muscles were first exposed to this solution for a period of 2 to 2.5 hr and then were fastened to stainless steel frames which had not been exposed to loading solution. $^{22}Na^+$ leaving the muscles was collected for periods of 10 min in tubes containing 4 ml of inactive solution. At the end of some long experiments the collecting periods were increased to 20 min. Stirring was achieved by rotating the tubes with a motor around the frames which were kept fixed. At the end of an experiment the muscles were removed from the frames and were put in 4 ml of distilled water in tubes similar to those used for collecting the radioisotope during the experiment. Both the collected samples and the muscles were counted in a crystal-well gamma spectrometer.

Efflux is expressed as the fraction of total $^{22}Na^+$ lost per minute during each collection interval. The total amount of isotope present in a muscle for any collection period was calculated by adding together the activities of all subsequent collection samples, the activity remaining in the muscle at the end of the experiment, and one-half of the activity leaving the muscle in the given collection period.

The sodium and potassium contents of muscles were measured by flame photometry following essentially the methods of Adrian (1956). ^{14}C-inulin spaces were determined and used as a measure of extracellular space.

Membrane potentials were measured with glass microelectrodes using a high input impedance amplifier. The microelectrodes were filled with 3 M KCl and had resistances ranging from 6 to 20 MΩ.

Isometric twitch tensions and action currents were measured in some muscles. In these experiments muscles were secured in a small Lucite chamber containing three compartments separated by two partitions. Each partition had a slit just large enough to permit a sartorius muscle to be threaded through. The partitions were spaced 21 mm apart and were 1.5 mm thick. Muscles were stimulated across one partition and the action currents were recorded across the other. Supramaximal stimuli were used. One end of each muscle was mechanically grounded and the other was attached to a tension transducer. Tension and action currents were displayed on an oscilloscope and photographed.

All experiments were performed at room temperatures (20 to 23 °C).

Results

Excitation and Twitches During and After Exposure to Glycerol

Several experiments were performed to verify that the methods used both uncoupled contraction from excitation and 'detubulated' adequately the

muscles used for flux studies. The verification of 'detubulation' by electron-microscopy is the subject of a following paper [1]. Here, we present the effects of glycerol treatment on externally recorded action currents and isometric twitch tensions. For the first 5 min after muscles were placed in solutions made hypertonic with glycerol the peak twitch tension declined to low values. During the remainder of the period in glycerol-containing Ringer's fluid, the twitch tension slowly recovered and approached its value in normal Ringer's fluid. This recovery of twitch tension in hypertonic glycerol solutions in both whole muscles and single fibers has been reported on previously by several investigators (Fujino *et al.*, 1961; Yamaguchi, Matsushima, Fujino & Nagai, 1962; April, Brandt, Reuben & Grundfest, 1968; Caputo, 1968; Krolenko & Fedorov, 1972; Zachar, Zacharova & Adrian, 1972). After being in hypertonic glycerol solutions for 60 to 75 min, the muscles were returned to normal Ringer's fluid. The twitch declined and became undetectable 40 to 50 min after withdrawal of glycerol. This also confirms the results of previous investigators (Fujino *et al.*, 1961; Howell, 1969). During this same period, the action current amplitudes stabilized at 75% of their value in Ringer's fluid prior to the exposure to glycerol. In a number of instances muscles were tested for several hours after glycerol withdrawal and no sign of tension recovery was observed. Thus, for the protocol and muscles used, contraction was completely uncoupled from excitation within 1 hr of glycerol withdrawal.

In other experiments where recovery during the first hour after glycerol withdrawal was in Ringer's fluid with 5 mM Ca and 5 mM Mg (Eisenberg, Howell & Vaughan, 1971), tension fell as rapidly and completely as in the experiments mentioned above. The externally recorded action currents, however, stabilized out at values more nearly those found in Ringer's fluid prior to glycerol treatment.

Response of Sodium Efflux to Hypertonic Glycerol-Ringer's

Sodium efflux always increased whenever sartorius muscles were placed in hypertonic glycerol-containing solutions. A second increase in efflux occurred when muscles were returned to normal Ringer's fluid after they had been exposed to hypertonic glycerol-Ringer's fluid for a period of 1 hr. Experiments illustrating these increases in sodium efflux are shown by Curve *A* in Fig. 1 and Fig. 2. That these increases in sodium efflux were not much

1 Franzini-Armstrong, C., Venosa, R. A., Horowicz, P.: Morphology and Accessibility of the Transverse Tubular System in Frog Sartorius Muscle after Glycerol Treatment. *J. Membrane Biol. (To be published).*

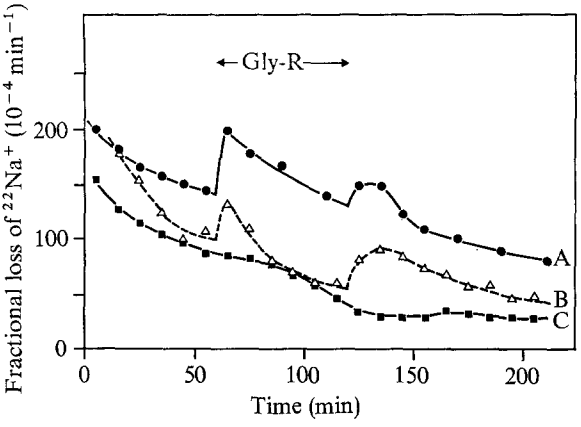

Fig. 1. Response of sodium efflux from sartorius muscles to application and withdrawal of 400 mM glycerol-Ringer's fluid. *A:* Expt. of 3/21/70, muscle in sodium solution. *B:* Expt. of 2/17/70, muscle in lithium solution throughout. *C:* Expt. of 2/17/70, muscle in sodium solution plus 3×10^{-5} M strophanthidin. The time taken as zero was 2 hr after the efflux of $^{22}Na^+$ was started

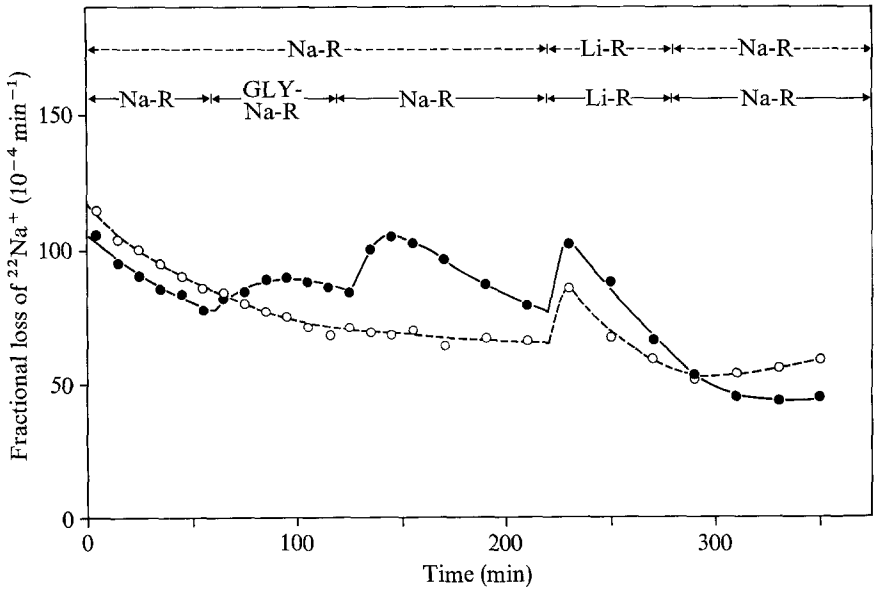

Fig. 2. Sodium efflux response to replacement of external sodium by lithium for a glycerol-treated muscle (solid curve, filled circles). Behavior of untreated, control muscle given by interrupted curve and open circles. Symbols used: Na-R, normal Ringer's fluid; Li-R, Ringer's fluid with LiCl replaced for NaCl; Gly-Na-R, 400 mM glycerol-containing Ringer's fluid. Expt. of 6/27/70

affected when sodium chloride was replaced completely by lithium chloride in the external fluid is illustrated by the experiment shown in Curve *B*, Fig. 1; in the experiment given, lithium was present for the entire time plot-

ted. When sodium efflux was inhibited by a maximally effective concentration of strophanthidin, the increments in sodium efflux were either reduced or absent. This result is illustrated by Curve *C*, Fig. 1. With strophanthidin present, the sodium efflux increased slightly on going into hypertonic glycerol-Ringer's fluid. However, the increment in efflux usually present on returning to Ringer's fluid was now absent. From these results it is clear that much of the first and all of the second increment in sodium efflux was due to stimulation of a strophanthidin-sensitive sodium efflux mechanism.

More quantitative estimates of the magnitude and time course of the stimulated efflux were made after allowing for the slow decline in rate coefficient for sodium loss normally present. This was done by taking the ratio of the rate coefficients for sodium loss in the treated muscle to those from the control muscle. The results from 12 pairs of muscles were analyzed, and the data show that withdrawal of glycerol produced an increment in efflux which at its peak value was 30% to 70% greater than the efflux from untreated muscles. After 4 hr in Ringer's fluid the rate coefficients in the treated muscles had fallen to about 90% of the untreated muscles.

A simple explanation for the initial increase in the fractional loss of ^{22}Na when muscles were first exposed to glycerol-Ringer's fluid is that increased internal concentrations produced by shrinkage of the fibers in the hypertonic solution caused the increase in sodium efflux. To examine whether shrinkage occurred, changes in muscle weight produced by exposure to hypertonic glycerol-Ringer's fluid were measured. At first the weight fell by 15%. Then, it increased slowly as the exposure to glycerol-Ringer's fluid continued; presumably, owing to entry of glycerol into the fibers (Zadunaisky, Parisi & Montoreano, 1963; Parisi, Montoreano & Lew, 1965; Caputo, 1968; Dulhunty & Gage, 1973). Upon returning to Ringer's fluid the muscles swelled to a volume 30% greater than they were when initially in Ringer's fluid. Control muscles not exposed to glycerol-Ringer's fluid did not show a significant change in volume during a comparable period. The initial decrease in volume produced by exposure to hypertonic glycerol-Ringer's fluid was comparable in magnitude and time course to the increase in sodium efflux produced by these solutions. Similar changes in muscle weight in response to hypertonic glycerol solutions have been reported by Miyamoto and Hubbard (1972). Hence, the initial increase in efflux produced by these solutions is that expected for a system in which sodium efflux is operating in a region where it is approximately a linear function of internal sodium concentration.

The second increment in efflux which occurs upon returning to Ringer's fluid requires some comment. An increase in passive permeability of sufficient magnitude is an unlikely explanation since the increment was strophan-

thidin-sensitive. A decrease in sodium efflux should have been produced by the swelling of fibers upon glycerol withdrawal if efflux was a monotonic increasing function of internal sodium concentration. In addition, during the return to Ringer's fluid a decrease in efflux would also have been expected if a disconnection of transverse tubular membranes occurred (Eisenberg & Gage, 1967, 1969; Eisenberg & Eisenberg, 1968; Gage & Eisenberg, 1969*a*, *b*) and these membranes were responsible, at least in part, for sodium extrusion. Both these effects may very well have been present, but they were more than canceled by the increase in sodium efflux.

One likely explanation for the increase which occurred upon return to Ringer's fluid is that a sufficiently large depolarization was produced to stimulate active sodium extrusion (Horowicz & Gerber, 1965*a*, *b*). In several experiments transmembrane potentials in surface fibers were measured and 1.5 hr after glycerol withdrawal the resting membrane potential in 103 fibers was -71.4 ± 1.2 mV (mean $\pm$ SEM). Although the mean was near threshold for stimulation of the sodium pump in frog muscle fibers, more than 40% of the fibers had internal potentials more positive than -70 mV; these values are well into the range of potentials which stimulate active sodium extrusion. These findings suggest that depolarization may have accounted for the second increase in sodium efflux. However, it will be shown below that depolarization may not be the only factor causing an increase in efflux.

The second increment in the rate coefficient for sodium loss which occurred upon return to Ringer's fluid was not sustained but declined with time. In view of this decline the properties of sodium efflux were examined at times early (1.5 to 2.0 hr) and late (6 to 8 hr) after glycerol withdrawal.

Sodium Efflux 1.5 Hours After Glycerol Withdrawal

In frog muscles, sodium efflux can be fractionated by various means. To take one example, Keynes and Swan (1959) were the first to report reduction in sodium efflux from sartorius muscles when lithium replaces sodium in the external solution. This finding was interpreted in terms of an exchange diffusion mechanism (Ussing, 1947). Since that time numerous investigations have been made on the withdrawal of external sodium and the effects produced are more complex than those expected on the basis of the presence of a simple exchange diffusion mechanism (Beaugé & Sjodin, 1968; Keynes & Steinhardt, 1968; Horowicz *et al.*, 1970; Erlji & LeBlanc, 1971; Beaugé & Ortiz, 1972). For sartorius muscles freshly isolated from *Rana pipiens*, replacement of sodium by lithium in Ringer's fluid produces, in most instances, an initial increase of the sodium efflux rate coefficient which is soon followed

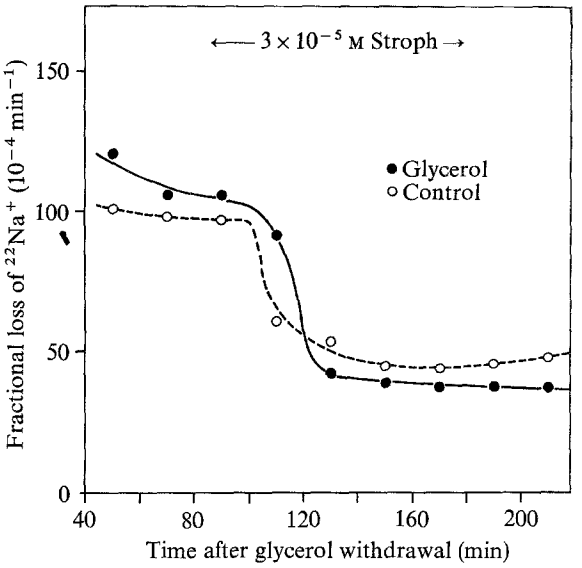

Fig. 3. Strophanthidin sensitivity of sodium efflux 90 min after glycerol withdrawal. Average of three pairs of muscles: filled circles — glycerol treated; open circles — untreated controls. Strophanthidin-to-control efflux ratios in glycerol-treated muscles were 0.60, 0.36, 0.22; and in untreated control muscles were 0.49, 0.44, 0.43

by a decline. The effect produced when sodium was replaced by lithium in a pair of muscles is illustrated in Fig. 2. One muscle was exposed to glycerol while the other was left in Ringer's fluid for use as a control. It is apparent that both muscles gave the usual transient increase in sodium efflux upon external sodium withdrawal; the increment was similar in magnitude and time course.

Another method which has been used to fractionate sodium efflux is to apply a specific inhibitor of active transport, such as strophanthidin. At a concentration of 3×10^{-5} M this aglycone maximally inhibits sodium loss in frog muscle (Horowicz *et al.*, 1970). The behavior of the fractional loss of sodium averaged from three pairs of muscles upon exposure to 3×10^{-5} M strophanthidin is illustrated in Fig. 3. Strophanthidin was added 95 min after transferring the experimental muscle from glycerol-containing Ringer's fluid to normal Ringer's fluid. It is clear that strophanthidin produced similar inhibitions in both the glycerol-treated and control muscles. The percentage inhibition was slightly larger for the treated muscles (61 %) than for the controls (55 %). This is a reflection in part of the fact that the sodium efflux increment early after glycerol withdrawal is strophanthidin-sensitive and in part of the fact that the strophanthidin-insensitive components are reduced.

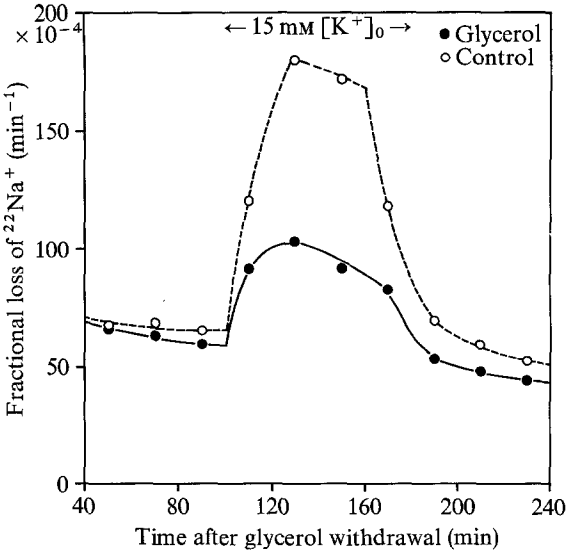

Fig. 4. Response of sodium efflux to increasing external potassium from 2.5 to 12.5 mM. Filled circles from glycerol-treated muscle; open circles from untreated control. Expt. of 7/7/70; Temp.=21 °C

Sodium efflux from frog muscles can be stimulated by increasing external potassium. The effect on sodium efflux of raising potassium from 2.5 mM to 15 mM 1.67 hr after glycerol withdrawal is illustrated by the experiment shown in Fig. 4. Although the rate coefficient for sodium loss from both the glycerol-treated and control muscles was increased, the response of the treated muscle was significantly smaller than that of the control.

One possible explanation is that the diminished responsiveness of sodium efflux to depolarization by potassium in glycerol-treated muscles was due to disruption and disconnection of the transverse tubules. Before accepting this suggestion, another factor has to be considered. As noted above, glycerol treatment also depolarizes fibers. With this in mind two types of experiment were performed to test whether depolarization rather than tubular disconnection was the factor determining the diminished responsiveness of sodium efflux to an increase in external potassium.

The first test consisted of depolarizing normal, untreated muscles with 6 mM potassium so that the resting membrane potential was at −71 mV; this was the average value found in fibers after glycerol treatment. After muscles had been depolarized to this level for 1.5 hr, the effect on sodium efflux produced by a further increase of external potassium was compared with that produced in control muscles which were not depolarized. Experiments of this type on two pairs of muscle are illustrated in Fig. 5. The experi-

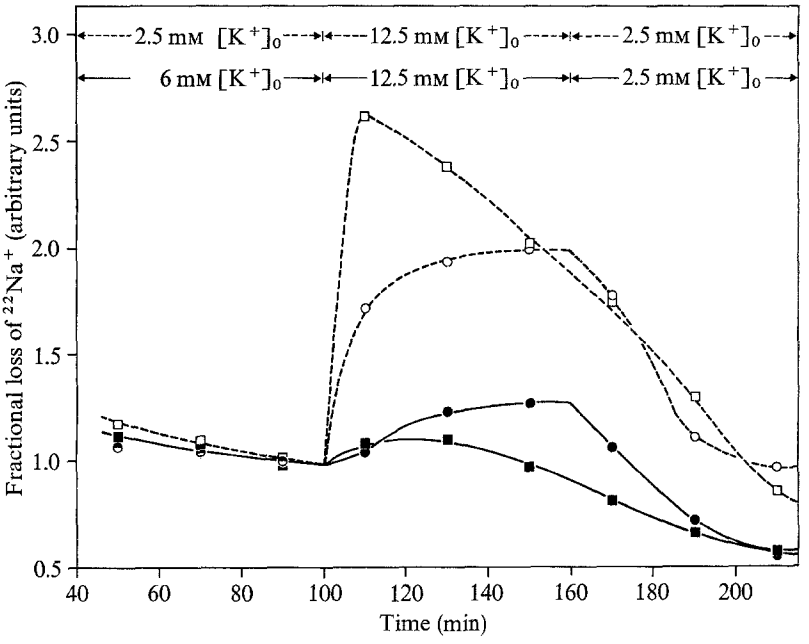

Fig. 5. Response of sodium efflux to 12.5 mM external potassium for muscles with different initial membrane potentials. Open symbols and interrupted curves for muscles starting from normal Ringer's fluid. Filled symbols and continuous curves for muscles initially depolarized by 6 mM potassium for 100 min. Ordinate, fractional sodium loss normalized to last value before application of 12.5 mM potassium. *See* text for additional comments. Zero time chosen at the time when the experimental muscles were placed in 6 mM external potassium. Expt. of 6/2/70

ments are normalized with respect to the last rate coefficient before external potassium was increased to 12.5 mM.[2] For the muscles which were first depolarized with 6 mM potassium, the increment in sodium efflux on raising the potassium to 12.5 mM was substantially smaller than the increment produced in control muscles kept in 2.5 mM potassium throughout. Both the increment in sodium efflux and the absolute value of the efflux produced by high potassium were reduced by the initial depolarization. Precise quantitative comparisons between experiments of this type and those on glycerol-treated muscles are difficult to make owing to the fact that not only the average membrane potential but also the distribution of membrane potentials in the fiber population are important. For any mean membrane potential the distribution is more widely spread out for the glycerol-treated muscles

2 This normalization procedure, although convenient for comparisons, does not show that increasing external potassium from 2.5 to 6 mM in one muscle of each pair produced a 25% increase in sodium efflux.

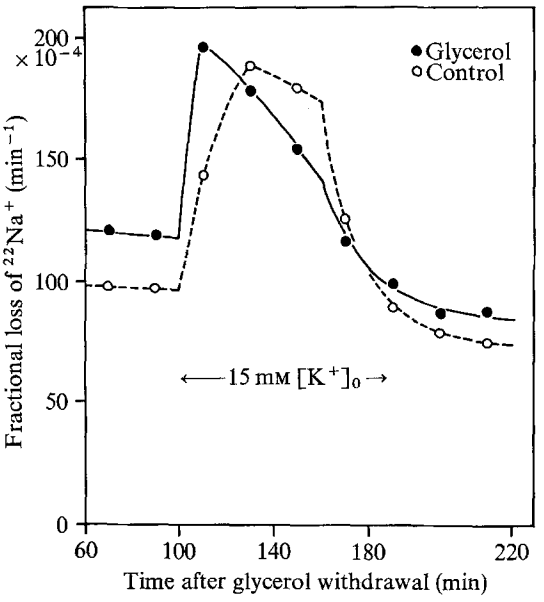

Fig. 6. Response of sodium efflux to increased external potassium in a glycerol-treated and control muscle. The glycerol-treated muscle was exposed to Ringer's fluid with 5 mM Ca^{2+} and 5 mM Mg^{2+} for the first hour after glycerol withdrawal. Expt. of 12/6/70

than is the distribution produced by raised external potassium. It is clear, nevertheless, that preliminary depolarization does reduce the responsiveness of sodium efflux to further depolarization by potassium and that the magnitude of the reduced responsiveness is comparable to that produced by withdrawing glycerol.

A second test was devised based on the findings of Eisenberg *et al.* (1971) that the depolarization which follows glycerol withdrawal can be reduced by first returning, for a brief period, to Ringer's fluid containing 5 mM calcium and 5 mM magnesium. Fig. 6 illustrates the response of the sodium efflux rate coefficients when external potassium was raised to 15 mM in a pair of muscles, one of which was exposed to 5 mM calcium plus 5 mM magnesium during the first hour of return in Ringer's fluid; the potassium was increased 100 min after withdrawal of glycerol. It is clear that the magnitude of the potassium-stimulated sodium efflux in the glycerol-treated muscle was comparable to that produced in the control, untreated muscle. Although the increments in efflux in the two muscles were not quite equal, there was much better correspondence in this situation than in the experiment illustrated by Fig. 4 where the experimental muscle was not exposed to high calcium-magnesium solutions. The result of this second test also suggests that de-

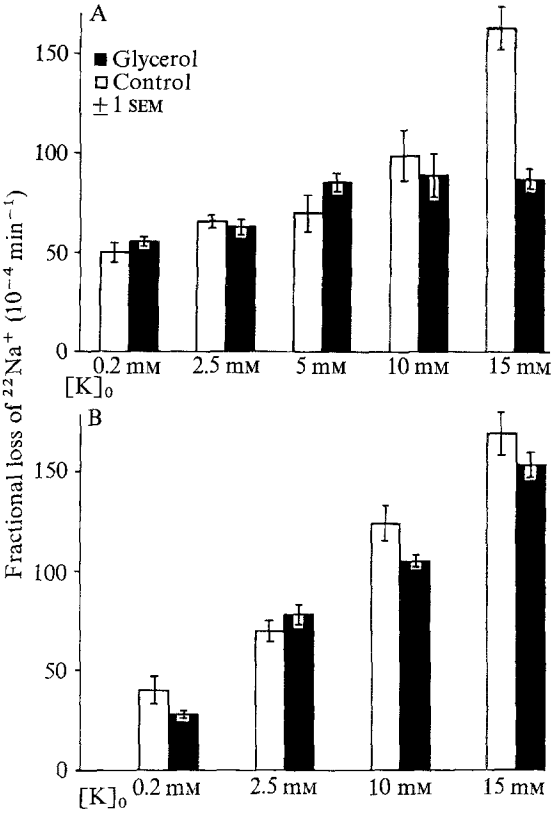

Fig. 7. Fractional sodium loss 1.5 to 2 hr after glycerol withdrawal as a function of external potassium concentration. Group *A* are from muscles returned to normal Ringer's fluid; Group *B* are from muscles returned to Ringer's fluid containing 5 mM Ca^{2+} and 5 mM Mg^{2+}. Heights of columns give average values and bars depict ± one standard error of the mean

polarization in the glycerol-treated muscles is the major factor which diminishes the responsiveness of the sodium efflux mechanisms to increased external potassium.

A summary comparison of the sodium efflux in solutions with different external concentrations from glycerol-treated muscles without and with the calcium-magnesium treatment is shown in Fig. 7. It is clear that the most significant difference between the calcium-magnesium treatment and that of direct return to normal Ringer's fluid is at the higher (15 mM) external potassium concentrations.

Thus, the total sodium efflux was only slightly altered 1.5 to 2.0 hr after withdrawing glycerol. The results of measurements on various components of sodium efflux from a number of muscles are summarized in Table 1, Part

Table 1. Effect of glycerol treatment on the various fractions of sodium efflux; initial recovery in 5 mM Ca plus 5 mM Mg-containing solutions[a]

Sodium efflux parameter	Control		Glycerol-treated		Ratio of glycerol-treated to control values
	10^{-4} min^{-1}	% of Total	10^{-4} min^{-1}	% of Total	
A. One and one-half hours after glycerol withdrawal. (Data from 4 to 17 muscle pairs)					
Total rate coefficient	75±6	—	85±6		1.16±0.06
Strophanthidin-sensitive fraction	—	40±2	—	56±7	1.36±0.22
Sodium-activated, strophanthidin-insensitive fraction	—	42±3	—	24±4	0.54±0.08
Residual fraction	—	17±2	—	20±3	1.09±0.13
B. Six to eight hours after glycerol withdrawal. (Data from twelve muscle pairs)					
Total rate coefficient	103±6		83±5	—	0.82±0.05
Strophanthidin-sensitive fraction	—	51±2	—	53±2	0.89±0.08
Sodium-activated, strophanthidin-insensitive fraction	—	37±2	—	27±1	0.61±0.04
Residual fraction	—	12±2	—	18±4	1.17±0.10

[a] Values listed are means ± one standard error of the mean.

A. A protocol of a typical experiment used for these measurements is given in Fig. 12; the times after glycerol withdrawal were in the 1.5- to 2.5-hr range.

Sodium Efflux 6 to 8 Hours After Glycerol

The situation for sodium efflux found early after glycerol withdrawal, however, was not stable. For all long-term experiments muscles were exposed to solutions containing added calcium and magnesium during the first hour after withdrawing glycerol. Several hours later the rate coefficient for sodium loss from treated muscles was consistently smaller than that from the untreated muscles. The behavior in time of the rate coefficient for sodium loss is given in Fig. 8. In this figure the ratio of the rate coefficient for sodium loss of glycerol-treated muscles to their paired control muscles is plotted as the ordinate. In view of the decline illustrated, it was of interest to determine which of the various sodium efflux fractions were altered.

Fig. 9 illustrates an experiment, 6 hr after withdrawing glycerol, in which Ringer's fluid was replaced by lithium-Ringer's fluid. The efflux from the

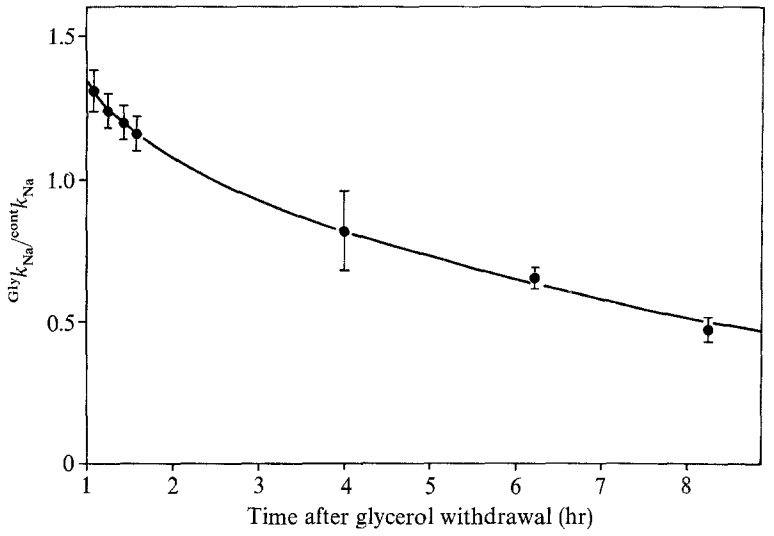

Fig. 8. Ratio of fractional sodium loss in glycerol-treated muscles to control muscles as a function of time after glycerol withdrawal. During the first hour after glycerol withdrawal muscles were exposed to Ringer's fluid containing 5 mM Ca plus 5 mM Mg. Bars indicate ± one standard error. Number of experiments included in average in points from left to right are 17, 17, 17, 17, 4, 35 and 14

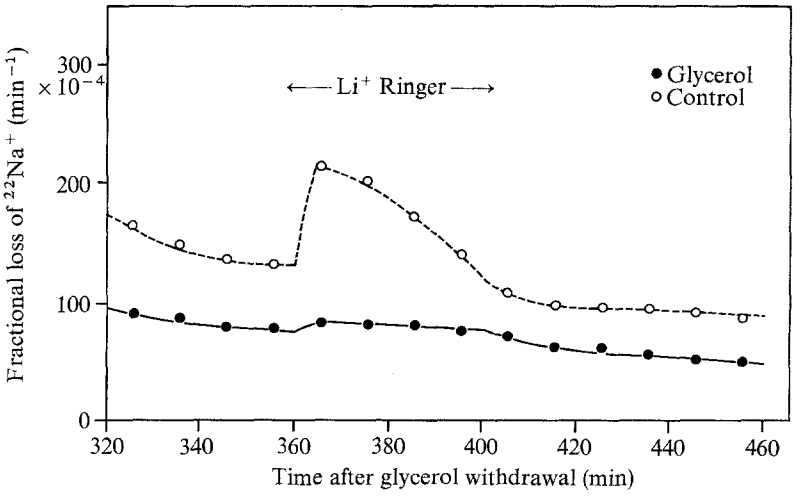

Fig. 9. Response of sodium efflux to replacement of external sodium by lithium 6 hr after glycerol withdrawal. Filled circles and full curve from glycerol-treated muscle. Open circles and interrupted curve from untreated control. Expt. of 5/12/71

control muscle showed the usual transient increase in the fractional loss of sodium while the glycerol-treated muscle showed little change. This difference between glycerol-treated and control muscles contrasts with the nearly identical

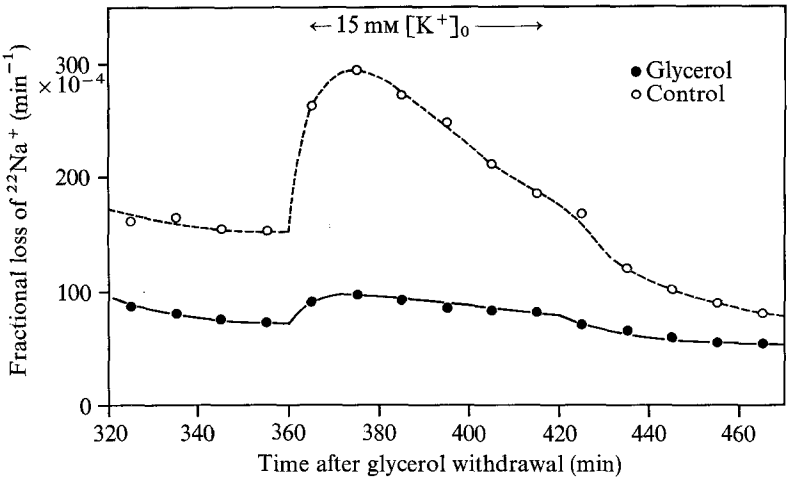

Fig. 10. Response of sodium efflux to 15 mM external potassium 6 hr after glycerol withdrawal. Filled circles and full curve from glycerol-treated muscle. Open circles and interrupted curve from untreated control. Expt. of 5/10/71

response of sodium efflux to external sodium removal early after withdrawal of glycerol.

Sodium efflux was also stimulated by increasing external potassium. Fig. 10 shows an experiment in which both glycerol-treated and control muscles were exposed to 15 mM external potassium for 1 hr. Once again the increment in fractional sodium loss produced by high external potassium was much smaller in the glycerol-treated muscle than in the untreated muscle. This is the case whether one expresses the increment in relative or dimensional units. At the peak of the response the rate coefficient for the control muscle was increased by more than 100% while the rate coefficient for the treated muscle was increased by only 42%.

The relation between the fractional sodium loss and external potassium concentration at these times for both treated and untreated muscles is illustrated by Fig. 11. The fractional sodium loss in treated muscles was smaller than in control muscles for all values of external potassium concentration in the range from 0.2 to 15 mM. It is noteworthy that sodium loss from treated muscles did not increase when potassium was increased from 10 to 15 mM while sodium loss from control muscles continued to increase between these two values of external potassium. In frog muscles, the effects on sodium efflux for this range of potassium concentrations are largely due to membrane depolarization rather than a specific effect of external potassium.

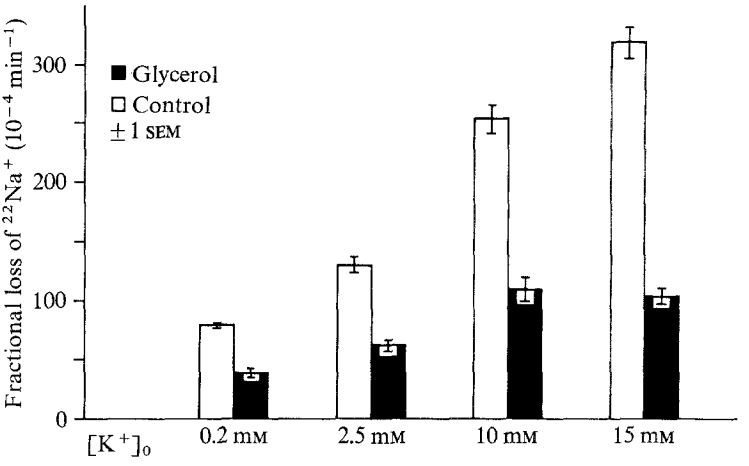

Fig. 11. Fractional sodium loss 6 to 8 hr after glycerol withdrawal as a function of external potassium concentration. Height of columns give average value and bars depict ± one standard error

These responses of sodium efflux in aged glycerol-treated muscles to removal of external sodium and increased external potassium concentrations suggest that the strophanthidin-sensitive efflux was smaller than in untreated muscles. Therefore, the strophanthidin sensitivity of the sodium efflux was measured in aged muscles. The results from one pair of muscles are given in Fig. 12 and they show, as expected, that the efflux from treated muscles was less sensitive to strophanthidin than it was from untreated muscles. In six pairs of muscles, before strophanthidin was added the average ratio of fractional loss of sodium from glycerol-treated muscles to untreated muscles was 0.65; after addition of strophanthidin this ratio increased to 0.80. These results indicate that the decline in fractional loss of sodium which occurs during aging of glycerol-treated muscles is largely, although not entirely, due to a reduction of the strophanthidin-sensitive components.

In the presence of strophanthidin complete replacement of sodium by lithium produces a marked reduction in sodium efflux. This reduction can be ascribed to an exchange diffusion component which is strophanthidin-insensitive. The component of efflux which remains in sodium-free, strophanthidin-containing salt solutions is called the residual sodium efflux. Fig. 12 also illustrates the behavior of these components in aged muscles. It is apparent that the external sodium-activated, strophanthidin-insensitive component of efflux was present in both treated and untreated muscles and

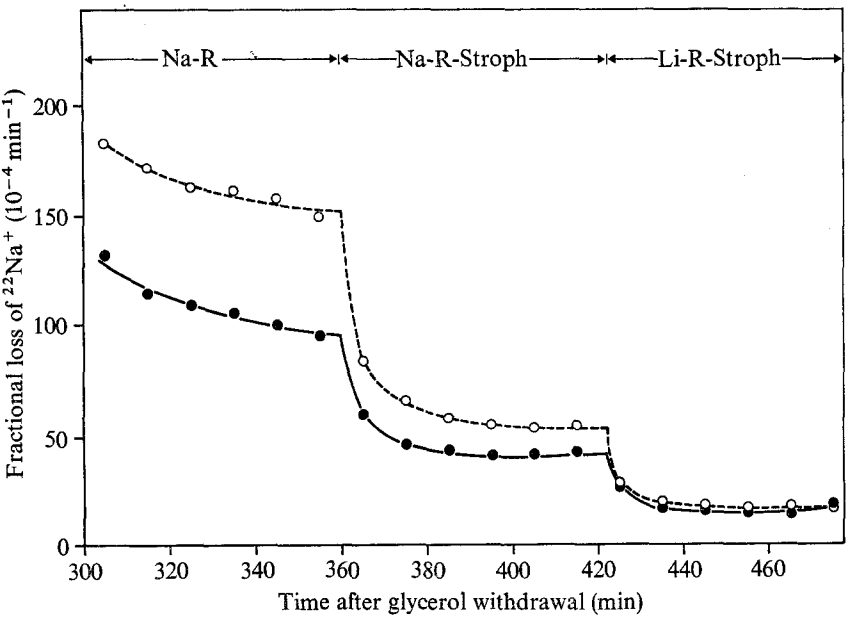

Fig. 12. Response of sodium efflux to strophanthidin and sodium removal 6 to 8 hr after glycerol withdrawal. Filled circles and full curve from glycerol-treated muscle. Open circles and interrupted curve from untreated control. Expt. of 9/22/71

that it was somewhat smaller in the treated muscle than it was in the untreated one. On the other hand, the residual efflux was essentially the same in both muscles. Table 1, Part B summarizes the data on the various components of sodium efflux in aged muscles.

Sodium and Potassium Contents of Glycerol-Treated Muscles

To compare sodium efflux from glycerol-treated muscles with that from untreated muscles both the fractional loss and the internal concentration of sodium are needed. Therefore, the sodium and potassium contents of aged glycerol-treated and control muscles were measured. Table 2 summarizes the results from 12 pairs of muscles 6 hr after one member of each pair was withdrawn from the hypertonic glycerol-Ringer's fluid. There was no statistically significant difference between the treated and untreated muscles in the intracellular contents of either water, sodium or potassium 6 hr after withdrawing glycerol. This means that the changes in the measured rate coefficients for sodium loss can, to a first approximation, be directly related to changes in sodium efflux.

Table 2. Intracellular water, potassium and sodium in glycerol-treated and control muscles six hours after glycerol-withdrawal with 5 mM calcium and magnesium treatment[a]

Muscle Parameter	Control	Glycerol	Ratio of glycerol to control values
Intracellular water in % of muscle weight	59.7 ± 3.0	59.3 ± 2.8	0.998 ± 0.024
$[K^+]_i$ (mmole/liter fiber water)	140.5 ± 7.3	140.1 ± 7.1	1.01 ± 0.04
$[Na^+]_i$ (mmole/liter fiber water)	15.3 ± 1.4	17.3 ± 1.3	1.26 ± 0.18

[a] Data are given as mean ± standard error of the mean and are based on the analysis of 12 pairs of muscles.

Discussion

The principal findings of this study can be summarized briefly. Early after glycerol withdrawal but at a time when contraction is uncoupled from excitation and the transverse tubules are disrupted and disconnected from the surface [1] both strophanthidin-sensitive and -insensitive fractions of sodium efflux are still present. The remaining strophanthidin-sensitive components are slightly stimulated while the strophanthidin-insensitive components are slightly depressed. The stimulation of the strophanthidin-sensitive components after glycerol withdrawal is somewhat reduced in muscles which are exposed for 1 hr to Ringer's fluid containing 5 mM calcium and 5 mM magnesium. The responsiveness of the efflux to either lowering or raising external potassium is almost normal if depolarization is reduced and delayed by the calcium-magnesium treatment. As time progresses the efflux becomes less responsive to alterations of both external sodium and potassium; this decline of responsiveness is primarily due to a reduction in strophanthidin sensitivity of the sodium efflux.

The initial stimulation of the efflux during the first 2 hr after glycerol withdrawal can be considered first. With the calcium-magnesium treatment the initial stimulation is about two-thirds of that found without the calcium magnesium treatment. As was noted above, depolarization occurs early after glycerol withdrawal. The transmembrane potential of surface fibers was measured in the second hour after glycerol withdrawal in muscles with the calcium-magnesium treatment and the average internal potential was −82 mV. This can be compared with the value of −71 mV found in muscles without the calcium-magnesium treatment. Furthermore, with the calcium-magnesium treatment the fraction of fibers with internal potentials equal to or more positive than −70 mV is smaller than it is for the muscles without this treatment; 12 out of a total of 78 impaled fibers fall into this category

in calcium-magnesium treated muscles as compared with 41 out of a total of 103 impaled fibers for the untreated muscles. For these values of internal potential it is known that the sodium pump is substantially stimulated (Horowicz & Gerber, 1965*a*, *b*). Since the calcium-magnesium treatment produces a one-third reduction only of the stimulation in sodium efflux upon glycerol withdrawal, it appears possible, therefore, that factors other than depolarization are responsible for a part of the initial stimulation of sodium efflux.

Examination of the data summarized in Table 1, Part A allows an assessment of the status of the various efflux fractions during the latter part of the second hour after glycerol withdrawal. At this time the total efflux in the glycerol-treated muscles is only slightly above that of the untreated muscles. This situation is the resultant of a stimulation in the strophanthidin-sensitive fractions of efflux and an inhibition of the external sodium-activated, strophanthidin-insensitive efflux component. This last is, on present evidence, thought to be a sodium exchange diffusion component which is strophanthidin-insensitive. The residual efflux which occurs in sodium-free solutions in the presence of strophanthidin is apparently unaffected by the glycerol treatment.

The behavior of muscles 6 to 8 hr after glycerol withdrawal can be assessed by considering the data summarized in Table 1, Part B and Fig. 11. The total sodium efflux at these times is depressed. The situation, in this instance, is the resultant of a depression of both the strophanthidin-sensitive components of efflux and the external sodium-activated, strophanthidin-insensitive component. The depression of the latter component in the treated muscles relative to the controls does not change significantly between the second and eighth hour after glycerol withdrawal (0.54 ± 0.08 as compared with 0.61 ± 0.04). As was the case early after glycerol withdrawal, the residual fraction of efflux is unaltered. From a comparison of Fig. 11 with Fig. 7, it is clear that the protection conferred by the calcium-magnesium treatment on the efflux's responsiveness to external potassium was significantly decreased between the second and eighth hour. In particular, it is apparent that the efflux is about the same in both 10 and 15 mM external potassium. Both this lowered saturation level of sodium efflux with respect to external potassium and the overall depression of the sodium efflux at all levels of external potassium as the result of aging is probably due in large part to depolarization. In many fibers the transmembrane potential declines during prolonged aging after glycerol withdrawal even with the calcium-magnesium treatment. For example, the average transmembrane potential in 66 fibers, 7 to 8 hr after glycerol withdrawal, was -67 mV. Examination of the values obtained indicates that the distribution is bimodal; 43 fibers belong to a population of

internal potentials having a mean and standard deviation of -82 ± 7 mV while the other 23 fibers belong to a distribution having a mean standard deviation of -38 ± 14 mV. Thus, about one-third of the fibers are extensively depolarized. When all fibers in sartorius muscles are depolarized to these values by increasing external potassium, a rapid but transient increase in sodium efflux occurs with the final steady value of the efflux being lower than that found initially in normal Ringer's fluid (Erlij & Horowicz, *unpublished observations*). It seems, therefore, likely that prolonged depolarizations of large magnitudes drives fibers into a different metabolic state in which the sodium transport characteristics are altered.

The depolarization is probably due to an increased sodium-to-potassium permeability ratio and to the anomalous rectification characteristic of the potassium permeability system. Such an increased permeability ratio could be the result of either an increased sodium permeability or a decreased potassium permeability or both. Since there is no significant change in internal sodium content in muscles 6 hr after glycerol withdrawal (*see* Table 2), it appears unlikely that a large change in sodium permeabilty occurred. When one considers that the rate coefficient for sodium loss was of the order of 10^{-2} min^{-1}, this implies that the influx must have nearly balanced the efflux during the 6-hr aging period after glycerol withdrawal and that it must not have been drastically different from that of the control muscles. Nevertheless, it is possible that a small increase in sodium permeability occurred which in combination with a reduction in potassium permeability such as is known to happen in muscles treated with glycerol (Eisenberg & Gage, 1969; Henderson, 1970) could easily account for the large depolarizations encountered in many fibers. For this situation one would expect that the membrane potential becomes relatively insensitive to variations in external potassium. In addition, as was noted in the results section, prolonged pre-depolarization inactivates the response of the sodium pump to subsequent depolarization. An explanation for this inactivation of the sodium pump is not available at present.

It has been noted that the internal sodium, potassium and water contents of glycerol-treated muscles were no different from untreated muscles 6 hr after glycerol withdrawal. In fact, the measurements yield values which are comparable to those found in freshly isolated muscles. This contrasts with the recent report of Henderson (1972) which indicates that glycerol withdrawal produces a 5- to 10-fold increase in sodium influx and a greater than twofold increase in internal sodium concentration. A possible explanation for the difference between the findings in this report and those of Henderson is that for long-term experiments we have exposed all muscles to a Ringer's fluid

with added calcium and magnesium for the first hour after glycerol withdrawal.

The recent interest in the ionic and electrical properties of glycerol-treated muscles stems from the observation that a dramatic uncoupling of the action potential from the mechanical response occurs during the first minutes after glycerol withdrawal (Fujino *et al.*, 1961; Howell, 1969). Morphologically there appears to be an extensive disruption of the transverse tubules and/or a disconnection of these tubules from the surface membrane (Howell & Jenden, 1967; Krolenko *et al.*, 1967; Eisenberg & Eisenberg, 1968). The evidence upon which this view rests is a large reduction in the numbers of transverse tubules present in electron-micrographs and an inaccessibility of peroxidase molecules to internal compartments and the deeper remnants of transverse tubules in glycerol-treated muscle. Electrically there is a considerable reduction in the measured membrane capacitance after glycerol treatment which speaks in favor of at least some form of disconnection between the transverse tubules and the surface membranes (Gage & Eisenberg, 1969*a*).

The extent of transverse tubular disruption and the precise nature of the disconnection of the transverse tubules from the surface membrane depend upon the preparation under study and the procedures used in glycerol treatment. For example, only a transient or reversible uncoupling of contraction from excitation occurs when concentrations of glycerol lower than that employed in this study are used (Krolenko & Fedorov, 1972; Zachar *et al.*, 1972). With 400 mM glycerol, however, the uncoupling seems to be irreversible or, at the very least, long-lasting (Krolenko & Fedorov, 1972). Nakajima, Nakajima and Peachey (1973) have made observations which appear to indicate that after glycerol treatment enough of the transverse tubule system remains and is accessible to small ions such as K to retard the speed at which single fibers repolarize when external potassium is reduced after a long period of equilibration in high potassium solutions. The investigators interpret these findings to indicate that the transverse tubules are not totally disrupted or destroyed, that the uncoupling of concentration from excitation is of an electrical nature, presumably owing to an increased 'access' resistance (*see* Peachey & Adrian, 1973), and that small ions can move between the lumen of those transverse tubules which remain after glycerol treatment and the extracellular space. In a following paper, using lanthanum as an extracellular marker, it is shown that, for the muscles and glycerol treatment chosen in this study, there is, 1.5 hr after glycerol withdrawal, extensive disruption and damage of the transverse tubules and only 10% of the original 'transverse' tubules are accessible to extracellular lanthanum [1]. This is true for fibers both in the center and at the surface of the muscles.

The experimental results of this report make it clear that no component of the sodium efflux mechanism is completely destroyed by glycerol treatment. The significance of this result depends upon the extent of transverse tubular disruption. However, the effects on sodium efflux produced by glycerol treatment are not simple and cannot be used to make estimates about the distribution of the various components of sodium efflux between surface and transverse tubular membranes even in the present case where 90% of the transverse tubules are disconnected from the surface membrane. The major difficulty of making such an estimate results from the fact that the sodium efflux remaining after glycerol withdrawal is stimulated. Thus, any part of the efflux apparatus lost is masked by the stimulation produced in the remainder. Nevertheless, since glycerol treatment disconnects 90% of the tubules, then the fact that all major components of sodium efflux are present at about normal levels after glycerol treatment implies that no component is confined largely to the transverse tubules. This finding makes it unlikely, for example, that the tubular membranes are the only location either for the strophanthidin-insensitive exchange diffusion component (Keynes & Steinhardt, 1968) or for the sodium pump (Zierler, 1972). It can, however, be argued that a fraction of the exchange diffusion sites reside in the walls of the tubular membrane since the fraction of sodium-activated, strophanthidin-insensitive sodium efflux is reduced both early and late after glycerol treatment (*see* Table 1).

There is some evidence in the literature based on another type of experiment which bears on the question of whether or not the sodium pump may be located in the tranverse tubules. Constantin and Podolsky (1967) have shown that chloride-induced contractures in skinned fibers are sensitive to strophanthidin. This can be interpreted as indicating that the membrane polarization of the transverse tubules which remain after skinning is maintained by the sodium pump and that this permits depolarization when chloride is applied to the tubular remnants. However, the finding that the chloride conductance in frog muscle fibers is largely confined to the surface membranes (Hodgkin & Horowicz, 1960; Eisenberg & Gage, 1969; Luff & Atwood, 1970) somewhat complicates the interpretation advanced above of the results on skinned fibers and suggests that more than tubular remnants are left after skinning. The point to be emphasized is that neither the results from glycerol-treated fibers nor those from skinned fibers provide unambiguous evidence for or against assigning the sodium pump to the tubules.

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References

Adrian, R. H. 1956. The effect of internal and external potassium concentration on the membrane potential of frog muscle. *J. Physiol.* **133**:631

April, E., Brandt, P. W., Reuben, J. P., Grundfest, H. 1968. Muscle contraction: The effect of ionic strength. *Nature* **220**:182

Beaugé, L. A., Ortiz, O. 1972. Further evidence for a potassium-like action of lithium ions on sodium efflux in frog skeletal muscle. *J. Physiol.* **226**:675

Beaugé, L. A., Sjodin, R. A. 1968. The dual effect of lithium ions on sodium efflux in skeletal muscle. J. Gen. Physiol. **52**:408

Caputo, C. 1968. Volume and twitch tension changes in single muscle fibers in hypertonic solutions. *J. Gen. Physiol.* **52**:793

Constantin, L. L., Podolsky, R. J. 1967. Depolarization of the internal membrane system in the activation of frog skeletal muscle. *J. Gen. Physiol.* **59**:1101

Dulhunty, A. F., Gage, P. W. 1973. Differential effects of glycerol fluxes on membrane capacity and excitation-contraction coupling in toad sartorius fibres. *J. Physiol.* (*In press*)

Edwards, C., Harris, E. J. 1957. Factors influencing the sodium movement in frog muscle with a discussion of the mechanism of sodium movement. *J. Physiol.* **135**:567

Eisenberg, B., Eisenberg, R. S. 1968. Selective disruption of the sarcotubular system in frog sartorius muscle. *J. Cell Biol.* **39**:451

Eisenberg, R. S., Gage, P. W. 1967. Frog skeletal muscle fibers: Changes in electrical properties after disruption of transverse tubular system. *Science* **158**:1700

Eisenberg, R. S., Gage, P. W. 1969. Ionic conductances of the surface and transverse tubular membranes of frog sartorius fibers. *J. Gen. Physiol.* **53**:279

Eisenberg, R. S., Howell, J. N., Vaughan, P. C. 1971. The maintenance of resting potentials in glycerol-treated muscle fibers. *J. Physiol.* **215**:95

Erlij, D., LeBlanc, G. 1971. The effects of ethacrynic acid and other sulphydryl reagents on sodiun fluxes in frog muscle. *J. Physiol.* **214**:327

Fujino, M., Yamaguchi, T., Fujino, S. 1972. Glycerol effect in various kind of muscle cell. *Jap. J. Physiol.* **22**:477

Fujino, M., Yamaguchi, T., Susuki, K. 1961. "Glycerol effect" and the mechanism linking excitation of the plasma membrane with concentration. *Nature* **192**:1159

Gage, P. W., Eisenberg, R. S. 1969*a*. Capacitance of the surface and transverse tubular membrane of frog sartorius muscle fibers. *J. Gen. Physiol.* **53**:265

Gage, P. W., Eisenberg, R. S. 1969*b*. Action potentials afterpotentials, and excitation-contraction coupling in frog sartorius fibers without transverse tubules. *J. Gen. Physiol.* **53**:298

Henderson, E. G. 1970. Potassium exchange and afterpotentials in frog sartorius treated with glycerol. *J. Gen. Physiol.* **56**:692

Henderson, E. G. 1972. The strophanthidin sensitivity of sodium efflux and potassium uptake in frog sartorius muscles treated with glycerol. *Biophys. Soc. Abstr., 16th Annu. Meeting*, p. 60a

Hodgkin, A. L., Horowicz, P. 1960. The effect of sudden changes in ionic concentrations on the membrane potentials of single muscle fibers. *J. Physiol.* **153**:370

Horowicz, P. 1965. Sodium movements in frog's sartorius muscle. *Acta Physiol. Acad. Sci. Hung.* (*Suppl.*) **26**:14

Horowicz, P., Gerber, C. J. 1965*a*. Effects of external potassium and strophanthidin on sodium fluxes in frog striated muscle. *J. Gen. Physiol.* **48**:489

Horowicz, P., Gerber, C. J. 1965*b*. Effects of sodium azide on sodium fluxes in frog striated muscle. *J. Gen. Physiol.* **48**:515

Horowicz, P., Taylor, J. W., Waggoner, D. M. 1970. Fractionation of sodium efflux in frog sartorius muscles by strophanthidin and removal of external sodium. *J. Gen. Physiol.* **55**:401

Howell, J. N. 1969. A lesion of the transverse tubules of skeletal muscle. *J. Physiol.* **201**:515

Howell, J. N., Jenden, D. J. 1967. T-tubules of skeletal muscle: Morphological alterations which interrupt excitation contraction coupling. *Fed. Proc.* **26**:553

Keynes, R. D. 1966. Exchange diffusion in frog muscle. *J. Physiol.* **184**:31

Keynes, R. D., Steinhardt, R. A. 1968. The components of the sodium efflux in frog muscle. *J. Physiol.* **198**:581

Keynes, R. D., Swan, R. C., 1959. The effect of external sodium concentration on the sodium fluxes in frog skeletal muscle *J. Physiol.* **147**:591

Krolenko, S. A., Adamjan, S. J., Shvinka, N. E. 1967. Vacuolization of skeletal muscle fibers. I. Vacuolization after efflux of low molecular weight non-electrolytes. *Tsitologiya* **9**:1346

Krolenko, S. A., Fedorov, V. V. 1972. Recovery of isometric twitches after glycerol removal. *Experientia* **28**:424

LeBlanc, G., Erlij, D. 1969. Effects of ethacrynic acid on sodium fluxes in frog sartorius muscle. *Biochim. Biophys. Acta* **173**:149

Luff, A. R., Atwood, H. L. 1970. The effect of nitrate ion on glycerol-treated frog skeletal muscle fibers. *Canad. J. Physiol. Pharmacol.* **48**:813

Miyamoto, M., Hubbard, J. I. 1972. On the inhibition of muscle contraction caused by exposure to hypertonic solutions. J. *Gen. Physiol.* **59**:689

Nakajima, S., Nakajima, Y., Peachey, L. D. 1973. Speed of repolarization and morphology of glycerol-treated frog muscle fibres. *J. Physiol.* (*In press*)

Parisi, M. N., Montoreano, R., Lew, V. 1965. Osmotical behavior of isolated muscle fibres. *Acta Physiol. Latinoamer.* **15**:38

Peachey, L. D. 1965. The sarcoplasmic reticulum and transverse tubules of frog's sartorius. *J. Cell Biol.* **25**:209

Peachey, L. D., Adrian, R. H. 1973. Electrical properties of the transverse tubular system. *In:* Muscle Structure and Function. G. H. Bourne, editor. 2nd Ed., Vol. 3, p. 1. Academic Press Inc., New York

Sjodin, R. A. 1971. The kinetics of sodium extrusion in striated muscle as functions of the external sodium and potassium ion concentrations. *J. Gen. Physiol.* **57**:164

Ussing, H. H. 1947. The interpretation of the exchange of radiosodium in isolated muscle. *Nature* **160**:262

Venosa, R. A., Horowicz, P. 1971. Sodium efflux from glycerol-treated muscle. *Proc. XXV Int. Congr. Physiol. Sci.* **9**:584

Yamaguchi, T., Matsushima, T., Fujino, M., Nagai, T. 1962. The excitation-contraction coupling of the skeletal muscle and the "glycerol effect". *Jap. J. Physiol.* **12**:219

Zachar, J., Zacharova, D., Adrian, R. H. 1972. Observations on "detubulated" muscle fibres. *Nature, New Biol.* **239**:153

Zadunaisky, J. A., Parisi, M. N., Montoreano, R. 1963. Effect of antidiuretic hormone on permeability of single muscle fibres. *Nature* **200**:365

Zierler, K. L. 1972. Sodium flux and distribution in skeletal muscle. *Scand. J. Clin. Lab. Invest.* **29**:343