The Influence of Sodium-Free Solutions on the Membrane Potential of Frog Muscle Fibers

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ABSTRACT The membrane potential of frog sartorius muscle fibers in a Cl- and Na-free Ringer's solution when sucrose replaces NaCl is about the same as that in normal Ringer's solution. The K^+ efflux is also about the same in the two solutions but muscles lose K and PO_4 in sucrose Ringer's solutions. The membrane potential in sucrose Ringer's solution is equal to that given by the Nernst equation for a K^+ electrode, when corrections are made for the activity coefficients for K^+ inside and outside the fiber. For a muscle in normal Ringer's solution, the measured membrane potential is within a few millivolts of E_K. This finding is incompatible with a 1:1 coupled Na-K pump. It is consistent with either no coupling of Na efflux to K influx, or a coupling ratio of 3 or greater.

The resting potential of frog sartorius muscle fibers, equilibrated with normal Ringer's solution (2.5 mM K^+) is about 10 mv below the value deduced from the ratio of K^+ concentrations inside and outside the membrane (Nastuk and Hodgkin, 1950; Nastuk, 1953). It has been suggested that the membrane potential fails to reach E_K, the potassium equilibrium potential, because the fiber is not in a steady-state with respect to Na^+ and K^+. This tendency of fibers to gain Na^+ and lose K^+ has been treated by Hodgkin and Katz (1949) by applying the constant field theory of Goldman (1943) and leads to an expression for membrane potential given as Equation 1. For muscle, Cl^- has

$$E_m = \frac{RT}{F} \ln \frac{P_K[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i}{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o} \quad (1)$$

been shown to redistribute itself rapidly and passively in response to a change in membrane potential, hence Equation 1 without the Cl^- terms (shown as Equation 2) will represent E_m either in Cl^--free solutions or in Ringer's when sufficient time has been allowed for the redistribution of Cl^-.

$$E_m = \frac{RT}{F} \ln \frac{P_K[K]_o + P_{Na}[Na]_o}{P_K[K]_i + P_{Na}[Na]_i} \quad (2)$$

Experimentally, Equation 2 gives a good fit to the data for E_m *vs.* $[K]_o$ when P_K/P_{Na} is set as 100 (Hodgkin and Horowicz, 1959). An inspection of Equation 2 shows that with $[Na]_o = 110$ and $[K]_o = 2.5$ the term $(P_{Na}[Na]_o)$ contributes to the numerator about one-half as much as the term $(P_K[K]_o)$, while the sodium term in the denominator contributes negligibly to the total in the denominator. The experiments to be reported involve changing the composition of Ringer's solution such as to require that Equation 2 yield a potential that is substantially that given by $RT(F)^{-1} \ln[K]_o/[K]_i$.

In order to make Equation 2 approach a value for E_K, the potassium electrode potential, it is not sufficient to replace $[Na]_o$ by an equivalent $[choline]_o$ because, as Renkin (1961) has shown, the muscle fiber is about as permeable to choline as to Na^+. For this reason it appeared essential to replace Na^+ in Ringer's with osmotically equivalent concentrations of sucrose. Such a change greatly affects the specific conductance and ionic strength of Ringer's solution and thus leads to changes in the cable-like properties of the membrane. It does not, however, lead to any difficulty with respect to the measurements of membrane potential because the extra resistance introduced into the potential measuring circuit is negligible when compared with microelectrode resistance.

Alternate explanations for the discrepancy between E_m and E_K are: (*a*) that the activity coefficients of K^+ inside and outside the fiber are different, (*b*) that there is a current leak around the site of introduction of the microelectrode, (*c*) that $[K]_o$ of Ringer's solution is not equal to the [K] immediately outside the membrane or that metabolic pumps generate currents that contribute to the flux balance across the membrane and thus affect the membrane potential. These possibilities form the basis for the experiments to be reported.

METHODS

Membrane Potentials Measurements were made with glass micropipettes connected, *via* a chlorided Ag wire to a cathode follower which in turn was connected to the input of an oscilloscope. The reference electrode was a salt bridge connected to an Ag-AgCl electrode containing 3 M KCl. Tip potentials of the micropipettes used were no greater than −5 mv as measured in normal Ringer's solution and such potentials varied with the electrolyte composition of the Ringer solution. The micropipettes used were drawn from 1.5 mm kimax tubing and had DC resistances of from 5 to 7 megohm when filled with 3 M KCl. These pipettes were filled by the injection of a drop of KCl into the shank end of the pipette with a 31 gauge hypodermic needle. The KCl droplet was then advanced toward the micropipette tip with a glass rod of 1 mm OD drawn to a fine tip on one end. The time taken to fill a micropipette was *ca.* 30 sec. and such electrodes had, in many cases, negligible tip potentials. In order

to check on the response of the tip potential to salt solutions of low ionic strength, the micropipette was balanced to zero potential in Ringer's solution, the solution was then changed to sulfate-sucrose Ringer's and the change in potential measured. For electrodes with tip potentials between −3 and −5 mv, the potential increased in sucrose Ringer's from −5 to −7 mv. For tip potentials less than −3 mv, the change in potential in sucrose Ringer's was less than 1 mv. A number of micropipettes were broken in sucrose Ringer's in order to be sure that the change in potential was a tip—rather than a junction potential. In measuring membrane potentials, the tip potential change between Ringer's and sucrose Ringer's was checked before beginning and at the end of a series of measurements.

Analytical All the muscles used were ashed in platinum and the ash taken up in dilute HCl solution for flame analysis for Na and K. Some muscles were used for K efflux measurements. These were labeled with K^{42} for from 4 to 12 hrs. and then washed for 50 min. in inactive Ringer's before beginning the collection of samples for a count of the radioactivity emerging from the fibers. Although generally only changes in efflux were of interest, absolute values of efflux were computed using 415 cm^2/gm muscle as a conversion factor for surface area. The water content of each muscle was measured by weighing before and after drying at 105°C. Values for extracellular space were taken as 20 per cent of muscle weight (*cf.* Steinbach, 1961; Mullins and Frumento, 1963). Phosphate efflux into Ringer's solutions was measured by the methods of Martin and Doty (see Ernster, Zetterström, and Lindberg, 1950). Muscles were soaked in various Ringer's solutions for from 2 to 24 hrs. and the phosphate determination was made on the Ringer solution. All concentrations are expressed as mmoles/liter fiber water (mM) or as μmole/gm (initial weight) whole muscle.

Solutions The Ringer solutions used were prepared as shown in the tabulation below. Osmotic coefficients (G) are from Robinson and Stokes (1959) and these have been taken at a concentration of 100 mM. For Na_2SO_4 and K_2SO_4 the values are extrapolated to 55 mM. All solutions have equal osmotic pressures corresponding to a concentration of 210 milliosmoles.

		Solution concentrations, m_M		
Substances	G 25°C	Ringer's	Sulfate Ringer's	Sucrose-sulfate Ringer's
NaCl	0.93	110	—	—
KCl	0.93	2.5	—	—
$CaCl_2$	0.85	1.8	—	—
Na_2SO_4	0.84	—	55	—
K_2SO_4	0.84	—	1.25	1.25
$CaSO_4$	—	—	Saturated	4.8
Sucrose	1.02	—	65	202

Activity Coefficients Because of uncertainty regarding the activity coefficients of K^+ on either side of the membrane, and because the measurements to be described involve comparisons of ion activities in solutions of very different ionic strengths,

measurements were made of the activity coefficients γ_K and γ_{Na} in a variety of solutions. Because there is evidence (Kortüm and Bockris, 1951) that the single ion activity coefficients for NaCl do not vary in a way that makes it possible to obtain γ_{Na} from the mean activity coefficient for NaCl, $\gamma_\pm$, it seemed most suitable to measure γ_+, the cation activity coefficient, at a concentration where the Debye-Hückel limiting law applies and to refer all measurements to such a concentration. Accordingly, a cation-selective glass electrode (Beckman) was used together with an Ag/AgCl reference electrode. A saturated KCl salt bridge connected the reference electrode to the solution under measurement. The glass electrode, with a resistance of *ca.* 10^9 ohms, was connected to the input of an electrometer (Keithley) with a 10^{14} ohm input resistance. Potentials could be read to 0.1 mv and were reproducible to 0.3 mv; they were measured at 20°C. The cation-selective electrode was not specific for any particular cation but comparisons to be made involved only either Na or K and hence ion specificity of the electrode was not involved. At a concentration of 1 mM cation and a pH of 7, however, H_3O^+ contributes appreciably to the potential and hence such measurements were made at a pH of 11. Measurements of phosphate ester salts at a K concentration of 140 mM were made at pH 7.4; that there was no error from H_3O^+ at this pH was demonstrated by comparing 100 mM solutions of KCl adjusted to the two different pH values. All values for single ion activity coefficient given below depend, of course, on the assumption that a saturated KCl bridge abolishes any junction potential between the solution under measurement and the Ag/AgCl reference electrode. The single ion activity coefficient given by the Debye-Hückel limiting law is $\log \gamma_+ = -0.505\ z^2\ (\mu)^{1/2}$ where z is ion valence and μ is ionic strength. Experimental values of $\gamma_\pm$ follow the limiting law to within 1 per cent up to a concentration of about 5 mM; hence we have chosen 1 mM Na or K as a reference concentration for the measurements reported below. Because the creatine phosphate and ATP are labile substances and the amount of hydration of the salts is

Solution	Cation concentration	ΔE	γ_+ 20°C	$\gamma_\pm$ 25°C
(pH 11)	*moles/liter*	*mv*		
NaOH	0.001	0	0.966	0.966
NaCl	0.011	58.5	0.88	0.90
	0.101	112.0	0.82	0.78
	1.001	166.5	0.76	0.66
KCl	0.101	110.5	0.76	0.77
$K_3Fe(CN)_6$	0.101	110.5	0.76	—
K_2SO_4	0.101	108.5	0.70	0.52
(pH 7.4)				
KCl	0.140	0	0.75	—
K_3 creatine PO_4	0.142	−5.8	0.60	—
K_4ATP	0.141	−6.2	0.57	—

likely to be variable, trial solutions were made in water 0°C and brought to pH 7.4 with KOH. These solutions were then analyzed for K by flame photometry and the results of such analysis used to obtain a corrected weight for the phosphate ester

solutions. Such solutions were made up, measured with the cation glass electrode, and then analyzed for K. Values for $\gamma_{\pm}$ are from Latimer, 1952. A typical 1–3 electrolyte, $K_3Fe(CN)_6$, showed only a normal γ_K while both K salts of phosphocreatine and ATP showed large potential differences when compared with KCl. Such a finding makes it clear that no simple rule for obtaining γ_+ from $\gamma_{\pm}$ is likely to be valid. The result with ATP has been corrected for ionization on the basis that $pK_4 = 6.5$.

Muscles Sartorius muscles from *Rana pipiens* were used exclusively in this study. The muscles were carefully dissected and tied with thread at each tendon in cases in which the muscle was to be used for flux measurements. The muscles, so prepared, were mounted on plastic frames under sufficient tension to keep them at body length, and the frame and muscle were moved as a unit through a number of test tubes containing inactive Ringer's in order to follow efflux. For potential measurements, the muscles were dissected free only at the knee joint, with the pelvic tendon intact, and the pelvic bone cut so that the muscle, attached to the bone could be placed in various solutions and the potentials of its fibers measured. All measurements were at 20°C. At the end of an experiment the muscles were examined under a microscope for the presence of damaged fibers; more than five damaged fibers was a basis for the rejection of the data from the muscle. A number of muscles were equilibrated for 24 hrs. in sulfate-sucrose Ringer's, returned to normal Ringer's for 30 min., and then tested for excitability. Such tests showed that muscles do not apparently suffer irreversible loss of excitability in the experimental solution.

RESULTS

The activity coefficient measurements reported in the Methods section yield the following values for γ_K for the various solutions used: Ringer's, 0.75; sulfate Ringer's, 0.70; sucrose-sulfate Ringer's, 0.90 (calculated from the limiting slope for the ionic strength of S-S Ringer's); and inside K, 0.60 (taken as that of K creatine phosphate on the basis that while ATP will lower γ_K, other monovalent anions such as HCO_3^- will raise γ_K).[1] The equation for the potassium equilibrium potential at 20°C can be written as $E_K = 58 \log [a_K]_o/[a_K]_i$ where a_K is potassium ion activity. This can also be written as $E_K = 58 (\log [\gamma_K]_o/[\gamma_K]_i) + 58 \log [K]_o/[K]_i$ where [K] is analytical K^+ concentration. In Ringer's solution $E_K = 5.6 + 58 \log [K]_o/[K]_i$, using values for γ_K given above, or E_K is 5.6 mv lower than that deduced from the K^+ concentration ratio. The effect of this correction is to put E_K within 4 mv of E_m when $[K]_o$ is 2.5 mM. The objection may be raised that E_m has been shown to follow closely the equation $E_m = 58 \log [K]_o/140$ at high $[K]_o$ (*cf.* Hodgkin and Horowicz, 1959). There is, however, in isotonic K^+ solutions a large

[1] Another method for obtaining an estimate of γ_K is to apply the Debye-Hückel relationship to Ringer's ($\mu = 0.115$) and to 46.6 mM K_3CrPO_4 ([K] = 140 mM, $\mu = 0.280$). This calculation yields γ_K (Ringer's) = 0.68 and γ_K (creatine phosphate solution) = 0.54. The ratio is 1.26, compared with 0.75/0.60 = 1.25 (from potential measurements). While the concentrations used are clearly out of the Debye-Hückel range, it does seem reasonable to suppose that a solution with an ionic strength 2.5 times that of Ringer's should have a reduced γ_K.

Na^+ efflux which must be expected to exert a hyperpolarizing effect on the membrane potential. This point will be treated in the Discussion.

To examine the predictions of Equation 2, muscles were equilibrated in Na-containing or Na-free solutions as described below. In order to avoid the complications of contributions from the movement of Cl^- to the membrane potential, muscles were equilibrated in sulfate Ringer's solutions for 30 minutes prior to their treatment with sulfate-sucrose Ringer's solution. After periods of time in this solution varying from 0.5 to 24 hrs. from fifteen to thirty microelectrode penetrations were made on different fibers of each muscle and the muscle was then weighed, dried, ashed, and an electrolyte analysis made. The results obtained are shown in Table I; from these data it is clear that the membrane potential in sucrose-sulfate Ringer's is equal to E_K. The table summarizes measurements made in Ringer's solution at short and long equilibration times, as well as measurements in sulfate Ringer's. The analytical

TABLE I
MEMBRANE POTENTIALS OF MUSCLE FIBERS

Solution bathing muscle	Time of equilibration	Potential E_m	$[K]_i$	E_K
	hrs.	*mv*	*mM*	*mv*
Ringer's	0.5–1.5	−90±1 (14)*	132±2	−94
Ringer's	18–24	−90±1 (10)	120±4	−92
Sulfate Ringer's	0.5–1.5	−89±2 (12)	125±4	−94
Sucrose-sulfate Ringer's	0.5–1.5	−89±2 (5)	128±2	−88
Sucrose-sulfate Ringer's	18–24	−84±2 (5)	109±4	−84

* Values in parentheses denote number of muscles used; variability of data is given as ±SD.

figures for $[K]_i$ together with values for γ_K make it possible to calculate E_K and it is clear that E_m is 2 to 5 mv below this value. It is somewhat difficult to decide whether the difference between E_m and E_K in Ringer's solution is real because the calculation of E_K depends upon measurements of K content, extracellular space, muscle water, and γ_K, all of which are subject to errors of the order of ±3 per cent. It is, thus, quite possible to have the estimate of $[K]_i$ in error by 10 per cent; this corresponds to 2.4 mv in E_K. A muscle in Ringer's solution has thus a value of E_m very close to E_K after 0.5 to 1.5 hrs. and a value equal to E_K (within experimental error) after 18 to 24 hrs. in Ringer's solution. A finding of some interest is that while $[K]_i$ for muscles in Ringer's solution after 18 to 24 hrs. is 120 mM, the corresponding figure for sulfate-sucrose Ringer's is only 109 mM. While the fall in $[K]_i$ in Ringer's may be expected to be accompanied by a gain in $[Na]_i$ (with the total osmotic pressure of the fiber remaining constant), no such possibility exists in sulfate-sucrose Ringer's, and indeed the result suggests that the fibers are losing both K and some anion.

A study by Fenn (1931) in which frog muscle was treated with isotonic solutions of sucrose alone, showed that under these conditions there was something like a 50-fold increase in the loss of inorganic phosphate into sucrose as compared with Ringer's solutions. It seemed important, therefore, to make measurements of the extent to which muscles in a sucrose solution containing normal concentrations of K^+ and Ca^{++} might also lose phosphate ions. The results of these measurements are shown in Table II together with the changes in wet weight of muscles treated with various solutions. It is clear the phosphate loss is roughly 4 times greater in sulfate-sucrose Ringer's than in normal Ringer's and the figure of 0.34 μmole is 0.68 μequivalent/gm hr. (with a valency of 2 for phosphate at pH 7.0). The loss of K over 24 hours is 17 μeq/gm or 0.70 μeq/gm hr.; this is just about the measured phosphate loss in sucrose-sulfate Ringer's. The loss of water (18 per cent) is consistent with the K loss (23 per cent) if the assumption is made that a compound K_2X is being lost.

TABLE II

PHOSPHATE LOSS, WATER CONTENT, AND $[K]_i$ OF MUSCLES

Solution bathing muscle	Time of Equilibration	$HPO_4^=$ loss	Wet weight as per cent initial weight	$[K]_i$
	hrs.	*μmole/gm hr.*		*μmole/gm*
Ringer's	2–4	0.11±0.03 (4)	99	72.8
Ringer's	24	0.09±0.03 (9)	96	69.4
Sucrose-sulfate Ringer's	2–4	0.44 (2)	98	73.4
Sucrose-sulfate Ringer's	24	0.34±0.099 (10)	80	56.0

A number of the Ringer solution samples which had been in contact with muscles for various periods of time were subjected to acid hydrolysis at 100°C before analysis for phosphate. Such treatment failed to affect the analytical result. In order to evaluate the contribution to the membrane potential made by the observed loss of K_2HPO_4, it was necessary to measure K fluxes in sulfate-sucrose Ringer's.

The results of K efflux measurements in the various experimental solutions are shown in Table III. It is clear that there is no great change in K efflux in going from Ringer's to sulfate-sucrose Ringer's solution and the value for efflux in this latter solution (8.1 pmole/cm^2 sec.) can be used together with the net loss of K previously found (0.70 μeq/gm hr. = 0.46 pmole/cm^2 sec.) to evaluate influx, and, therefore, the flux ratio. Influx is 7.6 pmole/cm^2 sec. and influx/efflux = 0.94. This ratio corresponds to a difference between E_m and E_K of about 1 mv which is, within experimental error, equal to the observed difference of zero. The net flux of K is so small that it is less than the error with which the K efflux can be measured.

When freshly isolated frog sartorius muscles are first placed in Ringer's

solution, they begin to gain Na and lose K. These changes in internal electrolyte are substantially complete in a few hours as shown by the data in Table IV. The increase in Na in muscle between 3 and 12 hours at 20°C is not statistically significant, and to a good approximation the muscle is in a steady state with respect to both Na and K. The K loss is in agreement with a Na gain and a $HPO_4^=$ loss each of comparable magnitude (see Table II). The most likely explanation for the initial increase in $[Na]_i$ is that the dissection and handling of the isolated muscle increase the P_{Na} of its fibers and that $[Na]_i$ then increases to a new level where the Na pump is able to balance passive influx. In some recent experiments (Conway *et al.*, 1963) it has been

TABLE III

POTASSIUM EFFLUX AND MUSCLE ELECTROLYTE CONCENTRATIONS

Solution bathing muscle	Equilibration time	K^{42} efflux	$[K]_i$	$[Na]_i$
	hrs.	*pmole/cm² sec.*	*mM*	*mM*
Ringer's	0.83	9.6±1.4 (36)	131	16
Ringer's	24	9.1±1.1 (5)	127	20
Sulfate Ringer's	0.83	10.4±2.2 (10)	126	18
Sucrose-sulfate Ringer's	0.83	8.1±1.3 (14)	127	6
Sucrose-sulfate Ringer's	24	7.7±1.0 (4)	109	3

TABLE IV

[Na] AND [K] OF MUSCLES IN RINGER'S SOLUTIONS AT 20°C

Time of immersion, *hrs.*	0	3	12
[Na], *μmole/gm*	26.6±0.7 (10)	29.9±1.0 (8)	31.2±0.9 (8)
[K], *μmole/gm*	75.8±0.9 (10)	72.4±0.8 (8)	69.7±1.1 (8)
Na flux, *μmole/gm hr.* (net)	—	1.1	0.14
K flux, *μmole/gm hr.* (net)	—	−1.0	−0.30

shown that muscles equilibrated in Ringer's at 0°C also gain Na very slowly over the period of from 4 to 24 hrs.; the calculated net flux for Na is 0.23 μmole/gm hr. There is, therefore, a period in which muscles are gaining Na, followed by a period of steady state. The membrane potential measured after short and long times in Ringer's solution is not different but the muscles equilibrated for 12 hours or more have membrane potentials closer to E_K. This finding suggests that the gain of Na at short times in Ringer's may result in a depression of the membrane potential in agreement with Equation 2. This point will be examined further in the Discussion.

It might be supposed that an influx of Ca^{++} would exert a depolarizing action on the membrane potential. Changes in $[Ca]_o$ from 1.8 to 10 or 25 mM, (prepared by adding $CaCl_2$ to sucrose-sulfate Ringer's) however, resulted either in no change or an increase in E_m by 1 to 2 mv. Such a finding suggests

that Ca^{++} influx is too small to contribute to the membrane current in a measureable way. As the fibers are essentially Na- and Cl-free (Na efflux is less than 0.1 pmole/cm^2 sec. and not measureable) the only ions that are able to contribute to the potential are K^+, and the organic anions and cations inside the fiber. The findings in 10 mM and 25 mM $CaCl_2$ added to sulfate-sucrose Ringer's have some relevance to the question of whether an ion leak around the site of insertion of the microelectrode may be holding the membrane potential at a value below that existing generally inside the fiber. The current flow into a membrane leak will vary inversely with the sum $r_i + r_o$ where these are the specific resistivities of the solutions inside and outside the muscle fiber. With a constant r_i and with r_o roughly twenty-five times that of normal Ringer's (sulfate-sucrose Ringer's) the microelectrode leak should be greatly diminished, while the addition of 25 mM $CaCl_2$ to sulfate-sucrose Ringer's would yield a solution with a specific resistivity of about twice that of normal Ringer's. The potential change is either nil or a small increase so that the observation suggests that a leak around the microelectrode is not a significant factor in potential measurements. The experiment is not conclusive, however, because Ca^{++} may have other actions besides changing the specific resistivity of the Ringer solution.

The muscle fiber has a system of tubules or channels that contribute to the complexity of its surface membrane. A model has been presented by Adrian and Freygang (1962); this consists of two membranes in series with a small space between them. The system is so set up that with current flow the [K] in the space between the membranes is changed. The permeability constants for the outer and inner membranes are given as 1800 A/sec. (P_K') and 100 A/sec. (P_K) at a resting potential of -90 mv. If muscles are placed in K-free Ringer's, a current of K^+ flows outward, and on the assumption that there is a negligible potential difference across the outer membrane, $\bar{m}_K = P_K'([K]_o' - [K]_o)$ where the primed concentration is that in the space between the inner and outer membranes. With a K efflux of 4 pmole/cm^2 sec. (this is the net flux across both membranes in K-free solution) $[K]_o'$ works out to be 0.22 mM. With the usual activity coefficient corrections, E_K is -146 mv in K-free Ringer's. Because a muscle in K-free Ringer's is clearly in a non-steady-state, it seemed useful to have measurements of membrane potential and K efflux under this condition and to compare these findings with those for a muscle in sulfate-sucrose Ringer's. Muscles were equilibrated in K-free solutions for from 1 to 2 hours at 20°C and membrane potentials were measured. Fluxes were obtained by changing muscles loaded with K^{42} from normal Ringer's to either K-free sucrose-sulfate or Ringer's solution and the change in K efflux rate constant evaluated after sufficient time to allow the fibers to become Cl-free (in the case of sucrose-sulfate Ringer's) or the extracellular space K-free, in the case of Ringer's solution. The results obtained are shown in Table V.

The membrane potential is clearly much higher in sulfate-sucrose Ringer's

than in Ringer's when both are K-free. While the value for E_K calculated on the basis that an external space retains K is -146 mv in K-free Ringer's, the potential calculated with Equation 2, using $P_K/P_{Na} = 100$ is -110 mv or a value identical with the observed potential. The experimental conditions are such that little can be concluded about the possibility of K-Na pump coupling because external K is so low. If $[K]_o'$ is assumed to be zero rather than 0.22 mM, the calculated potential from Equation 2 is -117 mv, a value considerably higher than that observed. It is concluded that the discrepancy between E_K and E_m in K-free Ringer's is accounted for by assuming that $[K]_o'$ is not zero and that Na gain by the fiber is important. We have not ruled out the possibility that anion loss (such as $HPO_4^{=}$) contributes appreciably to the potential at -110 mv and that the agreement given by Equation 2 is fortuitous.

TABLE V

MEMBRANE POTENTIALS AND K EFFLUXES IN K-FREE SOLUTIONS

	K-free Ringer's	K-free sucrose-sulfate Ringer's
Membrane potential, *mv.*	-110 ± 7 (3)	-130 ± 5 (3)
Rate constant for K efflux, $hr.^{-1}$	0.08	0.16*
$[K]_o'$, *mM* (calculated)	0.22	0.44
E_K, *mv* (calculated)	-146	-132

* We are indebted to Dr. R. A. Sjodin for this value.

In K-free sucrose-sulfate Ringer's a surprising finding is that the rate constant for K efflux is twice that in K-free Ringer's and about equal to that in normal Ringer's. The reason for this statement is that, on a constant field basis, K efflux should be expected to be given approximately by $(\text{flux})_2/(\text{flux})_1 = [\exp(E_2 - E_1)F/RT]E_2/E_1$. For the change from -90 to -130 mv this works out to a K flux reduced to 0.3 of that at -90 mv. The inference to be drawn from this finding is that P_K is increased 3.3-fold by the potential. The potential change may also be expected to increase phosphate loss, and a calculation such as that above (allowing for a doubly charged anion) shows that phosphate efflux should increase 17-fold. This would bring the loss observed at -90 mv of 0.68 μeq gm hr. (or 0.45 pequivalent/cm² sec.) to 7.7 peq/cm² sec., a value that agrees with the observed K efflux. The value calculated for E is very close (within 2 mv) to the measured membrane potential; the difference $(E_m - E_K)$ would seem to be too small to account for the large K efflux that is observed, even if a 3.3-fold increase in P_K has taken place. The constant (P_K') used to calculate $[K_o']$ is not, however, known very accurately. Further, the properties of the model may be expected to be altered rather markedly depending on whether or not sucrose penetrates into the tubular space. In view of the uncertainties involved no very precise estimation of E_K is possible. The important point demonstrated by the measurements in K-free

sucrose-sulfate Ringer's is that E_m can reach very high values and these are consistent with values necessary to bring the Donnan anion leak up to measured values for K efflux. The measurements also confirm that P_K increases with hyperpolarization.

DISCUSSION

In order to consider whether Equation 2 properly represents the conditions actually obtaining in a muscle fiber in Cl-free Ringer's solution, it is necessary to examine the theoretical basis for the equation as well as the basis for other equations that may be used to predict membrane potentials as a function of external and internal [Na] and [K].

In the absence of external sources of current, one may write (3) where C

$$-C\,dE/dt = I_1 + I_2 + \cdots I_n \tag{3}$$

is membrane capacitance in farads/cm², E is membrane potential in volts, t time in seconds, and I is membrane current density in amperes/cm² for a particular ion. When the potential E is constant with time, as is the case for the resting potential, $dE/dt = 0$ and we may write Equation 4, where z is ion charge and $\bar{m}$ is the net flux of an ion. The sign convention to be used is that $\bar{m}$ is positive for inward currents of positive ions. Similarly, influx of cations, m_i, is a positive quantity while efflux, m_o is a negative quantity so that $m_i + m_o = \bar{m}$. For simplicity in notation, [K], [Na], will be used to represent a_K, a_{Na}, in the section that follows. Potentials have a conventional (inside-outside) sign, and ion valence, z, is generally omitted from the equations as these consider mainly the movements of monovalent cations. The equation for

$$z_1\bar{m}_1 + z_2\bar{m}_2 \cdots z_n\bar{m}_n = 0 \tag{4}$$

ion flux as a function of concentration and potential can be integrated to yield the net flux as a function of ion permeability P, and C_o the outside and C_i

$$\bar{m} = P(C_o - C_i e^{EF/RT}) f(E) \tag{5}$$

the inside concentration (Equation 5); the factor $f(E)$ is a term describing the potential dependence of the flux. Equation 5 is thus not a constant field derivation. The factor $f(E)$ has a value that depends upon a particular model[2]

[2] For the constant field case, $f(E) = EF/[RT(1 - e^{EF/RT})]$; for a particular variable field case (Mullins, 1961), $f(E) = e^{-EF/2RT}$. The general differential equation for flux,

$$\bar{m} = \frac{uRT}{F}\left[\frac{dC}{dx} + \frac{zFC}{RT}\frac{dE}{dx}\right]$$

can only be integrated when some assumption has been made regarding dE/dx. Equation 5 has been obtained by integrating the first term in the brackets above between $x = o$ and $x = i$. The

for the distribution of the electric field in the membrane. It will appear from the treatment below that it is not necessary to specify a distribution of the field as the factor will cancel in the equation that is obtained.

If we consider a somewhat simplified case, namely that only the passive ion fluxes of Na and K contribute to the setting of the membrane potential

$$\bar{m}_{Na} + \bar{m}_K = 0 \tag{6}$$

we can rewrite Equation 4 as 6. Substituting for the terms in Equation 6 the relation given by Equation 5 yields Equation 7; rearrangement of these terms and dividing through by $f(E)$ gives Equation 8 and, after taking logarithms of both sides this is identical with Equation 2. It is clear, therefore, that Equation 2 is much more general than the constant field case and might appropriately be called the equation for membrane potential with net ion flow.

$$\begin{aligned} P_{Na}[Na]_o f(E) - P_{Na}([Na]_i e^{EF/RT}) f(E) \\ + P_K[K]_o f(E) - (P_K[K]_i e^{EF/RT}) f(E) = 0 \end{aligned} \tag{7}$$

$$\frac{P_{Na}[Na]_o + P_K[K]_o}{P_{Na}[Na]_i + P_K[K]_i} = e^{E_m F/RT} \tag{8}$$

The recent demonstration by Kernan (1962) that the membrane potential in frog muscle fibers can rise to values higher than E_K makes it clear that either the membrane potential depends on other considerations or we have not summed enough terms in Equation 6. The most obvious omission from Equation 6 is any representation of the active, metabolically linked ion fluxes. There is evidence (Keynes and Swan, 1959; Mullins and Frumento, 1963) that Na extrusion takes place by the outflux of 3 Na^+ per cycle of the pump and there is also evidence that K^+ may be pumped inward, with this pumping coupled to the Na extrusion. If we denote by r the coupling ratio or number of Na^+ pumped out per K^+ pumped inward, and if we assume that the Na fluxes in the muscle fiber are in balance (as is the case for the intact animal) we can write (9) where m_i and m_o are passive influx and efflux and p_o is the

$$m_i^{Na} + m_o^{Na} + p_o^{Na} = 0 \quad \text{or } \bar{m}_{Na} = -p_o^{Na} \tag{9}$$

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integral of the second term in the brackets is denoted $f(E)$. It will apply equally to all electrically charged particles of the same valency and sign whatever the assumptions regarding dE/dx may be. Because $f(E)$ may not be the same for cations and anions when both are flowing across the membrane, some further simplification is required for Equation 5 to be strictly valid. In the case of muscle, permeant anions such as Cl redistribute so rapidly that their net flux can be considered zero after only a few time constants of the extracellular space. Alternately, we can consider potential-flux relationships in sulfate Ringer's solution.

pumped efflux and is, following the sign convention, a negative quantity. Similarly for the K fluxes we can write Equation 10. Now, by definition

$$m_o^{\mathrm{K}} + m_i^{\mathrm{K}} + p_i^{\mathrm{K}} = 0 \quad \text{or} \quad -\bar{m}_{\mathrm{K}} = p_i^{\mathrm{K}} \tag{10}$$

$p_o^{\mathrm{Na}} = -rp_i^{\mathrm{K}}$ so that the passive fluxes must be related as shown in

$$\bar{m}_{\mathrm{Na}} = -r\,\bar{m}_{\mathrm{K}} \tag{11}$$

Equation 11. The substitution of this result for Equation 6 followed by the

$$E_m = \frac{RT}{F} \ln \frac{P_{\mathrm{Na}}[\mathrm{Na}]_o + rP_{\mathrm{K}}[\mathrm{K}]_o}{P_{\mathrm{Na}}[\mathrm{Na}]_i + rP_{\mathrm{K}}[\mathrm{K}]_i} \tag{12}$$

operations that lead to Equation 7 now yield Equation 12. Some points of interest in this result are the following: where there is no coupling between Na pumped efflux and K influx (corresponding to $r = \infty$) the expression for potential reduces to that for a potassium electrode. When $r = 1$ (corresponding to an electroneutral pump) the equation is equivalent to 2 or the current flow case. When $r = 3$ (a result consistent with some experimental data) the equation predicts a membrane potential much closer to E_{K} than Equation 2 (for the same values for the relative permeabilities of Na and K). The intrinsic limitation on the usefulness of Equation 12 is the assumption necessary in its derivation that Equations 9 and 10 are obeyed; *i.e.*, $[\mathrm{Na}]_i$ and $[\mathrm{K}]_i$ are in a steady-state. For an imbalance in the fluxes, recourse must be had to an analytical expression for pumped Na efflux as a function of $[\mathrm{Na}]_i$ and other parameters affecting pumping.

For the non-steady–state, if the assumption is made that m_i^{Na} is constant while p_i^{K} is zero, and if estimates for m_o^{K} and p_o^{Na} are available, then $\bar{m}_{\mathrm{K}}$ can be evaluated from the relationship $\bar{m}_{\mathrm{K}} + \bar{m}_{\mathrm{Na}} + p_o^{\mathrm{Na}} = 0$. The K flux ratio[3] $m_o^{\mathrm{K}}/m_i^{\mathrm{K}}$ can be obtained from $\bar{m}_{\mathrm{K}}$ and m_o^{K} and this set equal to $\exp(E_m - E_{\mathrm{K}})F/RT$ thus yielding Equation 13. The membrane potential can be either greater or less than E_{K} depending upon whether p_o^{Na} is greater

$$E_m = \frac{RT}{F} [\ln m_o^{\mathrm{K}}/m_i^{\mathrm{K}} + \ln [\mathrm{K}]_o/[\mathrm{K}]_i] \tag{13}$$

or less than $\bar{m}_{\mathrm{Na}}$.

In the non-steady–state with 1 : 1 coupling of p_o^{Na} and p_i^{K}, the simple relation $\bar{m}_{\mathrm{K}} + \bar{m}_{\mathrm{Na}} = 0$ must hold, so that Equation 2 will give the membrane po-

[3] A long pore effect may make for errors in estimating the flux ratio if $\bar{m}_K$ is large; we are concerned here, however, mainly with flux ratios differing from unity by a few per cent.

tential. For a muscle fiber in sulfate Ringer's solution, the situation with respect to membrane potential under varied conditions is tabulated below.

Line	Condition	Flux	Fiber gaining	Pump coupling Na^+ out/ K^+ in	(Membrane potential) Value	Equation
A	Non-steady-state	$\bar{m}_K + \bar{m}_{Na} + p_o^{Na} = 0$	Na	None	$<E_K$	13
B	Non-steady-state	$\bar{m}_K + \bar{m}_{Na} + p_o^{Na} = 0$	K	None	$>E_K$	13
C	Non-steady-state	$\bar{m}_K + \bar{m}_{Na} = 0$	Na or K	1:1	$<E_K$	2
D	Steady-state	$\bar{m}_K = 0 = \bar{m}_{Na} + p_o^{Na}$	0	None	E_K	—
E	Steady-state	$\bar{m}_K + \bar{m}_{Na} = 0$	0	1:1	$<E_K$	2
F	Steady-state	$r\bar{m}_K + \bar{m}_{Na} = 0$	0	r:1	$<E_K$	12

Fibers equilibrated in Ringer's solution for 0.5 to 1.5 hrs. are not in a steady state with respect to $[Na]_i$ and $[K]_i$ (see Table IV) so that their potential should within the stated assumptions be given by lines *A* or *C* in the table above. It is easy to show that line *C* gives a potential that is substantially below that measured (86 mv). Further, some data for K and Na fluxes (unpublished, from our laboratory) show Na efflux = 2.7, Na influx 3.5, K efflux 8, and K influx 7.2 pmole/cm² sec. If a 1:1 coupling demanded by line *C* existed, $m_i^K = 4.5$ and $m_i^K/m_o^K = 0.56$ or E_m is 14 mv from E_K. Even if 40 per cent of m_o^{Na} is considered as exchange diffusion (and there are reasons for supposing that exchange diffusion is an artifact, see Mullins and Frumento, 1963), the difference between E_m and E_K is 9 mv. This result does not rule out a coupling ratio greater than 1 but does preclude a 1:1 coupled Na-K pump. The experimental values found in this study are adequately represented by Equation 13. The finding of Kernan (1962) that when fibers are losing Na and gaining K, E_m is $>E_K$ also argues against a 1:1 coupling as from the table, E_m cannot exceed E_K when coupling is 1:1.

After some few hours in Ringer's solution fibers are, to a good approximation, in a steady state with respect to $[Na]_i$ and $[K]_i$. To a fair approximation, E_m is equal to E_K; a somewhat closer approximation to the measured E_m is given by Equation 12 with $r = 3$ and $P_K/P_{Na} = 100$ but errors of various sorts prevent this from being proof that coupling is 3:1.

For a muscle fiber in sulfate-sucrose Ringer's we expect the relationship $\bar{m}_K + z\bar{m}_{PO_4} = 0$ to hold. With $[K]_o = 2.5$ mM, it has been shown that net K flux is so small when compared with unidirectional fluxes that to a good approximation $E_m = E_K$. When $[K]_o$ is zero, the membrane potential is consistent with net K flux equalling net phosphate flux.

Two different methods of estimating γ_K (Ringer's)/γ_K (fiber water) agree and give a value of 1.25 for this ratio. The use of activity ratios requires that

a muscle fiber have a potential that is 5.6 mv less than that given by $E_m = 58 \log [K]_o/140$. The data of Hodgkin and Horowicz for single fibers in Ringer's are, in fact, better fit by using a correction of 5.6 mv but their data in sulfate Ringer's are not. A reason for this discrepancy may be that with $[K]_o$ high, Na efflux may be more than doubled from resting values. Potassium influx at 100 mM $[K]_o$ is about 70 pmole/cm^2 sec. and Na efflux might be about 7 pmole/cm^2 sec. This pump activity would contribute *ca.* 2.5 mv to the potential. Minor volume changes because of the difficulties in getting osmotic balance with a 1:2 electrolyte (K_2SO_4) and possible alterations in anion leaks from the fibers are other possible sources of error.

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