The Efflux of Substances from Frog Ventricles to Sucrose and to Ringer's Solutions

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ABSTRACT The frog ventricle in sucrose solution contracts for several hours at 25°C, and for as long as 24 hours at 5°C. The possibility that a fraction of the extracellular fluid remains outside of the excitable membrane was examined by measuring the efflux of tracers. The half-time for the efflux to sucrose solution at 25°C of C^{14} sucrose is about 1 minute, for Na^{24} is 6.5 minutes, and for Cl^{36} is 4 minutes. There is no evidence for the retention of an extracellular Na fraction. The Q_{10} for Na and Cl efflux is about 1.3. The half-time for K^{42} efflux is about 180 minutes; the Q_{10} is 1.7. The efflux rates of Na^{24}, Cl^{36}, and K^{42} to sucrose and to Ringer's solutions are quite similar. Ca^{45} efflux is only one-fifth as fast to sucrose solution as to Ringer's; the retention of Ca^{++} may be important for maintaining excitability in sucrose solution. P^{32} efflux is five times faster to sucrose solution than to Ringer's solution, and there is a similar increase in the rate of inosine loss to sucrose solution. The Q_{10} for efflux to sucrose solution is 2.2 for $P^{32}O_4$ and 2.4 for inosine. We suggest that energy metabolism is abnormal in ventricles in sucrose solution and that low temperature prolongs excitability by slowing the metabolic change.

A frog heart immersed in isotonic sucrose solution continues to conduct action potentials and to contract for several hours (Singh, Sehra, and Singh, 1945; Singh, 1962; Van der Kloot and Rubin, 1962). This observation raises obvious difficulties for ideas about the inflow of Na^+ generating the action potential, and about the inflow of Ca^{++} triggering contraction. The simplest explanation would be that as long as the muscle remains active the sucrose solution does not completely replace the extracellular fluid. This possibility is now examined by measuring the ion contents of sucrose-soaked ventricles and the kinetics of the efflux of radioactive ions and certain other substances from cardiac muscles into sucrose and Ringer's solutions.

A second observation was also kept in mind when designing the experiments.

Ventricles in sucrose solution at 25°C continue to beat for 2 to 3 hours, while ventricles in sucrose solution at 5°C remain active for as long as 24 hours (Van der Kloot and Rubin, 1962). This means that the process which leads to the loss of electrical and mechanical activity by a ventricle soaking in sucrose solution has a high Q_{10}, roughly 2 to 3 (between 5 and 25°C). Therefore, if ventricles eventually stop because a substance is lost to the sucrose solution, the efflux of this substance would also have a Q_{10} between 2 and 3. For this reason, efflux measurements were made at both 5 and 25°C.

The next question is what substances should be studied. When ventricles which have lost excitability after immersion in sucrose solution are returned to Ringer's solution, normal action potentials can be recorded almost immediately, and about 50 per cent of the initial contractility is recovered in 30 minutes. This might mean that one or more of the ions present in Ringer's solution had to be reacquired by the ventricles; therefore Na, K, Ca, and Cl^- were studied first. It also seemed possible that the return to Ringer's solution might restore excitability because the metabolism of the ventricle was favored by Ringer's solution. This line of thought lead to measurements of the efflux of P^{32} and of inosine, cytidine, and lactate, which were assayed by chemical methods.

MATERIALS AND METHODS

Animals Frogs (*Rana pipiens*) were obtained from a dealer and were kept in running tap water until use. Only ventricles were used in the experiments reported here because preliminary measurements showed that the apparent extracellular space of auricles varied widely from one determination to the next, undoubtedly because of the difficulty in uniformly blotting the delicate tissue.

Solutions The following solutions were used. Concentrations are given in mM. Ringer's solution, 121.0 Na^+, 3.0 K^+, 2.7 Ca^{++}, 4.0 HCO_3^-, and 125.4 Cl^-. Sucrose solution, 240.0 sucrose.

Determination of Cellular Contents Sodium and potassium were determined on ventricles blotted on filter paper, weighed in tared crucibles, and dried to a constant weight at 105°C. The muscles were then ashed in a furnace at 450–500°C, the ash was dissolved in a drop of HNO_3, and diluted with water. The resulting solution was treated with $SnCl_4$ to remove interfering anions (Margoshes and Vallee, 1956) and the sodium and potassium concentrations measured with a Beckman flame photometer.

Chloride was determined on ventricles blotted on filter paper, weighed in tared glass tubes, and dried to a constant weight at 105°C. The dried muscles were soaked for at least 2 days in 4 ml of a solution containing 0.1 N HNO_3 and 1.7 N acetic acid (Cotlove, 1962). The chloride in the solution was measured with a Cotlove chloridometer.

The iodinated serum albumin space was determined on ventricles blotted on filter

paper, weighed in a tared container, and then incubated in a solution (either Ringer's or isotonic sucrose) containing I^{131} serum albumin (Volk Radiochemicals Company). After 90 minutes the ventricles were removed from the radioactive solution, weighed, and placed in a tube containing 1 ml of distilled water. The radioactivity in the tube was detected by a scintillation crystal, a gamma ray spectrometer with the window set for I^{131}, and the pulses were tallied by a scaler.

Isotope Effluxes Weighed ventricles were tied to a frame of stainless steel wire; the tying was identical with the method used in preparing ventricles for tension recordings (Van der Kloot and Rubin, 1962). The mounted muscles were placed in 10 ml of a Ringer's solution containing Na^{24}, Cl^{36}, K^{42}, $P^{32}O_4$, Ca^{45}, or C^{14} sucrose. The isotopes were usually used in concentrations of about 10 μc/ml. Muscles were kept in the radioactive Ringer's for 16 hours at 4–7°C unless specified otherwise. The ventricles were then passed at 5 or 10 minute intervals through a series of tubes, each containing 5 ml of solution at 25°C or at 5°C. At the end of the experiment, the muscles were placed in 5 ml of 0.1 N HNO_3. Na^{24} and K^{42} were measured by using a scintillation detector, a spectrometer, and a scaler. One ml aliquots of the solutions containing P^{32} or C^{14} sucrose were dried on flat planchets and counted with a thin-window, gas-flow counter. The counts were corrected for self-absorption. In later experiments, 2 ml aliquots of the radioactive solutions were added to 15 ml of *p*-dioxane (containing 120 gm/liter naphthalene, 7 gm/liter 2,5-diphenyloxazole, 0.5 gm/liter 2-*p*-phenylenebis (5-phenyloxazole), and counted in a Nuclear Chicago three channel liquid scintillation counter at room temperature.

Lactate An 0.5 ml aliquot was mixed with 2 ml of 0.5 M glycine–0.4 M hydrazine buffer at pH 9, and 0.2 ml of 0.027 M NAD. 30 μl of lactic dehydrogenase (2 mg/ml; California Biochemical Corp.) was added and the mixture was incubated for 60 minutes at 25°C. The absorbance at 340 mμ was then determined with a Beckman DU spectrophotometer.

Nucleosides Xanthine oxidase (20 mg/ml) was purchased from California Biochemical Corp., and a fraction of rat liver containing nucleoside phosphorylase was prepared by the method of Kalckar (1947). Most of the methods for the separation and analysis of nucleosides will be introduced along with the results.

Amino Acids The concentrations of amino acids and peptides were estimated by the Cu^{++} salt method of Spies (1957). Glycine was used as the standard.

Inorganic Phosphate P_i was measured by the method of Seraydarian *et al.* (1961) except that the phosphomolybdate complex was extracted into isobutanol:benzene (1:1).

RESULTS

The measurements of the total Na^+, K^+, Cl^-, and the I^{131} serum albumin space are given in Table I. The table compares the contents of ventricles soaked for 90 minutes in Ringer's solution with those soaked in isotonic sucrose

solution. Intracellular concentrations were calculated by assuming that the I^{131} albumin space is equal to the extracellular space.

Our results on the ventricles kept in Ringer's solution were quite similar to those of Krogh, Lindberg, and Schmidt-Nielsen (1944) on the entire heart; they used thiocyanate to estimate the extracellular space. Both series of measurements lead to the conclusion that the "cellular" compartment contains a significant amount of chloride. Krogh *et al.* calculated a higher intracellular Na^+ concentration (72 to 157 mM/kg cell H_2O) and a somewhat lower intracellular K^+ (82 mM). Both series of measurements show that the

TABLE I

ION AND WATER CONTENTS OF FROG VENTRICLES

Treatment	Total concentrations			H_2O	I^{131} serum albumin space	Calculated intracellular concentrations		
	Na	K	Cl			Na	K	Cl
	meq/kg H_2O			*gm/kg wet weight*	*per cent muscle H_2O*	*meq/kg H_2O*		
In Ringer's solution 90 min.	63.1±1.6 (20)	103.3±2.8 (13)	67.8±3.5 (15)	809±7 (35)	19.9±2.4 (10)	48.6	128	53.5
In isotonic sucrose solution 90 min.	17.1±3.1 (20)	66.3±6.5 (20)	4.1±0.65 (15)	761±5 (35)	16.4±1.4 (10)	20.4	79.3	4.9

Means ± SE and number of samples given in parentheses.

total cation inside the cell substantially exceeds the concentration of cations in the Ringer's solution. Our figures show an excess of about 50 meq/kg H_2O inside the cell, while Krogh *et al.* found an excess of 35 to 120 meq/kg H_2O. They concluded that the excess intracellular cation was accounted for by the binding of about half of the intracellular K^+ in an osmotically inactive form, but this conclusion was based on tracer evidence for the non-exchangeability of some of the intracellular K^+ (Hahn, Hevesy, and Rebbe, 1939) which has subsequently been challenged.

While our results agree substantially with those of Krogh *et al.*, we find a higher intracellular Na^+ concentration than was reported by Hajdu (1953) or by Johnson (1957). We also calculated a higher value for the intracellular K^+ than Hajdu (1953). The reason for these differences is not known; rather slight differences in the composition of the Ringer's solutions may be important.

When the ventricles were soaked for 90 minutes in large volumes of sucrose solution, the concentrations of all the ions in the tissue markedly decreased. The intracellular concentrations were calculated by assuming that the con-

centrations of the ions remaining in the extracellular fluid are low enough to be neglected. The cells appear to retain about 42 per cent of the initial Na^+, 62 per cent of the K^+, but only about 9 per cent of the Cl^-. In evaluating the physiological significance of these measurements, it is important to recall that after 90 minutes in sucrose solution, ventricles are usually still conducting action potentials, and contracting.

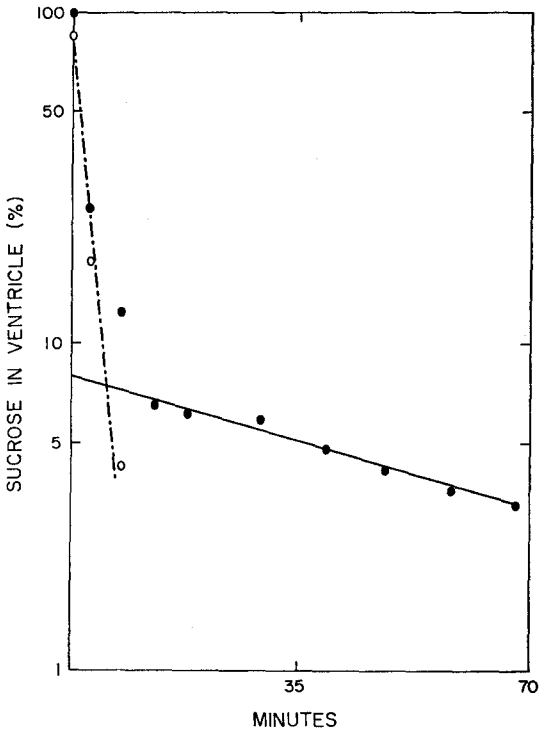

FIGURE 1. An integrated curve for C^{14} sucrose in a ventricle as a function of time. The ventricle was soaked for 150 minutes in Ringer's solution containing C^{14} sucrose. At time zero, efflux into sucrose solution was begun. The lines show an analysis into two exponentials.

Efflux of C^{14} Sucrose

Since the starting point of the experiments was the prolonged contractility of cardiac muscle soaked in sucrose solutions, the first efflux measurements were made on ventricles which had been previously soaked for 150 minutes in a Ringer's solution containing C^{14} sucrose.

Fig. 1 shows the results of a typical experiment. Apparently the sucrose is distributed in two compartments in the muscle: the largest compartment contains about 92 per cent of the sucrose and the washout of sucrose is extremely rapid; the time constant is about 3 minutes. The remaining 8 per cent washes out much more slowly, with a time constant of almost 80 minutes. It seems most likely that the first, large phase represents sucrose washing out of the extracellular fluid while the second, small phase may represent sucrose emerg-

ing from within the cells. The penetration of sucrose into the cells of cardiac muscle was demonstrated by Krogh and Lindberg (1944). Our measurements suggest that sucrose can rapidly exchange with the extracellular fluid surrounding the muscle fibers.

Na^{24} Efflux

Before describing the experiments on the efflux of ionic tracers it should be pointed out that the ventricle is rather unsuitable for these measurements, because pieces of cardiac muscle are liable to contract spontaneously. Contraction changes the efflux rates of some of the ions (unpublished data), and also might increase the exchange rate of the extracellular space; so spontaneous contractions probably account for some of the divergent points on the curves.

Ventricles soaked for 16 hours in a Ringer's solution containing Na^{24} reached a level of radioactivity equivalent to 93.3 ± 3.0 (SE) μeq Na/gm H_2O; it is therefore likely that there was an almost complete exchange of Na^+ between the muscle and the Ringer's solution.

When ventricles loaded with Na^{24} are transferred through a series of sucrose solutions, the Na^{24} is lost quite rapidly from the muscle, whether the solution is at 5 °C or at 25 °C. A plot of the logarithm of the Na remaining in the muscle as a function of time gives a complex curve which cannot be resolved into two exponentials (Fig. 2). The curves could be separated reasonably well into three exponentials; this analysis favors a three-compartment model. The justification for plotting the logarithm of the radioactivity remaining in the tissue against time is the assumption that the efflux rate is directly proportional to the amount of isotope in the tissue. In skeletal muscle, however, the simple relationship does not hold true; the efflux rate of Na^{24} is a function of the cube of the tissue concentration over much of the range studied (Keynes and Swan, 1959; Mullins and Frumento, 1963).

Therefore we plotted the log efflux to sucrose solution at 25 °C as a function of the log Na^{24} in the ventricle (Fig. 3). The points fit a straight line with a slope of 1.6. Consequently the efflux rate of Na^{24} to sucrose solution will be given by:

$$-\frac{dC_i}{dt} = K(C_i)^n \tag{1}$$

where K is a rate constant, C_i is the concentration in the tissue (expressed as a percentage), and n is found by measuring the slope of the line on a plot like Fig. 3. Therefore the part of the efflux curve which fits these kinetics can be calculated by integrating equation (1):

$$K = \frac{1}{t(n-1)}\left(\frac{1}{(C_t)^{n-1}} - \frac{1}{(C_0)^{n-1}}\right) \tag{2}$$

where C_0 is the percentage of the total tracer at $t = 0$ which obeys these kinetics, and C_t is the percentage of the tracer remaining at time t.

The efflux of Na^{24} from a ventricle to sucrose solution at 25°C is shown as Fig. 2. The curve was calculated by Equation 2. C_0 was estimated by assuming

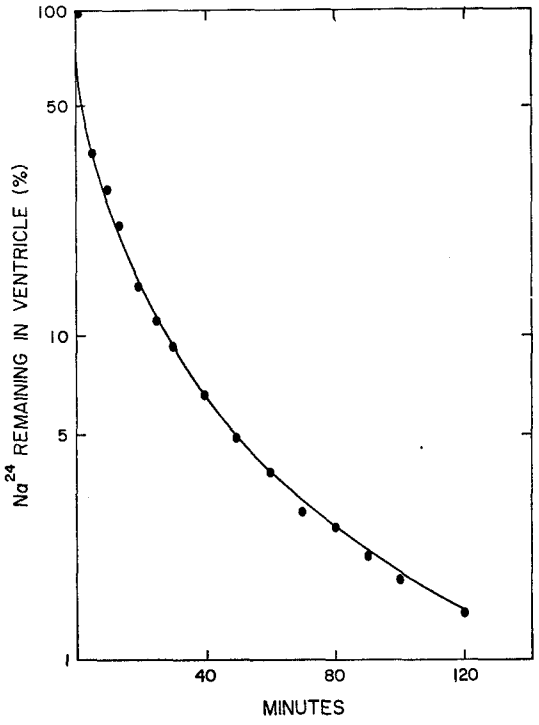

FIGURE 2. An integrated plot of the loss of Na^{24} from a ventricle to sucrose solution at 25°C. The curve was calculated by the method described in the text.

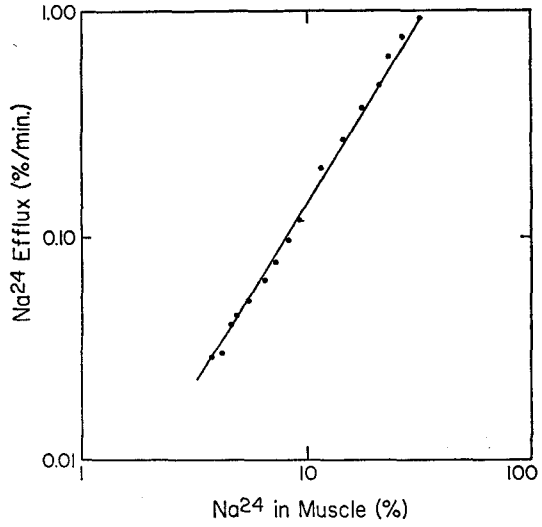

FIGURE 3. The efflux of Na^{24} from a ventricle to sucrose solutions at 25° as a function of the Na^{24} remaining in the tissue.

an extracellular space of 19.9 per cent (Table I), n was taken from Fig. 3, and K was calculated from Equation 2 by using the level of Na^{24} in the ventricle at 60 minutes as C_t. The calculated curve fits the data well, except for the first efflux period, during which most of the Na^{24} in the extracellular space must leave the ventricle.

Unfortunately, calculating the curve does not help with the further treatment of the data, because the meaning of the rate constant, K, varies with n.

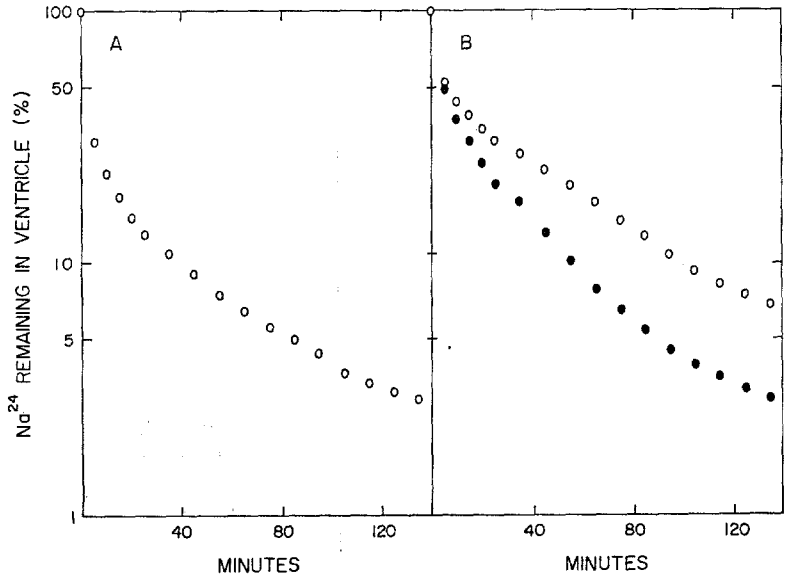

FIGURE 4. A, an integrated plot of the loss of Na^{24} from a ventricle to sucrose solution at 5°C. B, an integrated plot of the loss of Na^{24} from a ventricle to Ringer's solution at 25°C (filled circles) and at 5°C (open circles).

For example, the K for the curve in Fig. 3 is 0.53 per cent$^{-0.65}$ hr.$^{-1}$. If n varies from one experiment to the next, the K's cannot be used to indicate the relative rates of efflux.

Fig. 4A shows the efflux of Na^{24} from a ventricle to sucrose solution at 5°C. A plot like that of Fig. 3 shows that n is 1.8, K then is 0.22 per cent$^{-0.8}$ hr.$^{-1}$. The relative rates at 5 and at 25°C vary depending on the stage in the efflux measurements at which the comparisons are made. Probably the most useful comparison is the time ($t_{1/2}$) for the loss of 50 per cent of the tracer from the second (intracellular) phase:

$$t_{1/2} = \frac{2^{n-1} - 1}{KC_0^{n-1}(n - 1)} \tag{3}$$

For the examples shown, $t_{1/2}$ at 25°C is 6.5 minutes, $t_{1/2}$ at 5°C is about 8

minutes, so the Q_{10} is low. If the effect of low temperature in prolonging the excitability of ventricle in sucrose solution depends on an action on Na efflux, it involves only a few per cent of the initial Na, since it is only at low Na concentrations that changing temperature has a pronounced effect on efflux rate. At 25°C, 1 per cent of the initial Na^{24} is left after 162 minutes, at 5°C 1 per cent remains after 334 minutes.

In experiments on the efflux of Na^{24} from skeletal muscles in Li- or choline-Ringer's solution, n is about 3 (Keynes and Swan, 1959; Mullins and Frumento, 1963). Perhaps this means that a carrier molecule in the Na pump moves 3 Na^+'s in each transport cycle. For ventricles in sucrose solution at 25°C, the values of n were 1.65, 1.6, and 1.7 in our three experiments. In sucrose solution at 5°C n was 1.9, 1.75, and 1.8. The lower value of n for the ventricle might arise because of the use of sucrose solution instread of choline-Ringer's. This was tested in one experiment in which a ventricle was placed into choline-Ringer's; n was 2.1. Carmeliet (1964) found that n was 2 for the efflux of Na from cat ventricular muscle to Li-Tyrode's solution. This does not mean that cardiac and skeletal muscles are strikingly different, since Keynes (1963) showed that n for skeletal muscles could be shifted to 1.9 to 2.2 in HCO_3–Ringer's solution, which would lower the intracellular pH.

When ventricles are in Ringer's solution, the concentration of Na in the cells should remain almost constant. Therefore total Na efflux should remain at a steady level and Na^{24} efflux should depend directly on the concentration of tracer remaining in the cell; that is n should be 1. In Ringer's solution at 25°C the actual values were 1.0, 1.2, 1.3, and 1.6; close fits to straight lines were obtained. In two experiments using Ringer's solution at 5°C n was 1.4. Further speculation on the significance of n will be postponed until after the data for other ions are presented.

The loss of Na^{24} from ventricles to Ringer's solution at 25 and 5°C is shown in Fig. 4B. The rate of Na^{24} loss to Ringer's solution is somewhat slower than to sucrose solution. For example, the times needed for Na^{24} in the ventricle to fall to 10 per cent of the initial level are: sucrose solution at 25°C, 29 minutes; sucrose solution at 5°C, 40 minutes; Ringer's solution at 25°C, 56 minutes; Ringer's solution at 5°C, 79 minutes. Two other sets of experiments gave similar results. Since Na^{24} efflux is more rapid into sucrose solutions, the efflux rate is probably not limited by the availability of diffusible anions.

Chloride Efflux

During the soak-in period, ventricles incorporate 71.9 ± 5.2 μeq Cl^-/gm muscle H_2O (mean ± SE; No. = 6). A log efflux–log concentration plot for Cl^{36} is shown in Fig. 5. Similar curves were obtained from other ventricles in Ringer's and in sucrose solutions. The points do not fit a straight line. If we assume that 10 per cent of the Cl^{36} is unable to diffuse freely, and then subtract this 10

per cent from all measured tissue concentrations, the points approach a straight line (Fig. 5). With this adjustment, n is between 0.8 and 1, suggesting that the greater part of the Cl^{36} efflux is directly proportional to the tissue concentration.

Loss curves for Cl^{36} are shown in Fig. 6. Cl^{36} is lost at about the same rate in either Ringer's or sucrose solutions, and temperature has a minor influence on

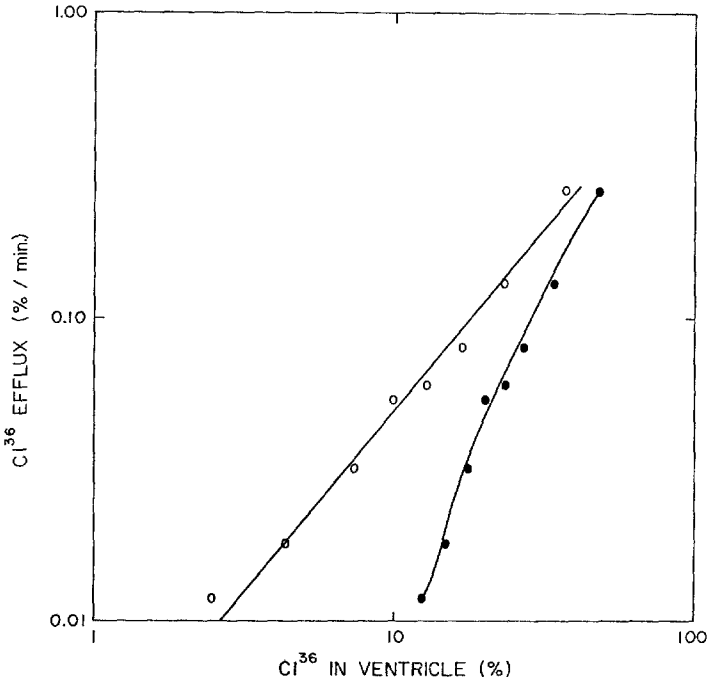

FIGURE 5. The efflux of Cl^{36} from a ventricle to sucrose solution at 25°C as a function of the Cl^{36} remaining in the ventricle (filled circles). The efflux replotted on the assumption that 10 per cent of the Cl^{36} in the ventricle is in a slowly moving fraction (open circles).

the rates. In sucrose the efflux becomes slow when 10 per cent of the initial concentration is reached, which agrees with the assumption that there is a fraction unable to diffuse at a rapid rate. In Ringer's solution the slowing at 10 per cent of the initial concentration is less marked; perhaps the slow fraction of the Cl^{35} exchanges with Cl^{35} from the Ringer's solution.

K^{42} Efflux

During the 16 hour soak-in period, ventricles exchanged 40.7 ± 4.8 μeq K/gm muscle H_2O (mean ± SE; No. = 9). The log-log plots of efflux to sucrose solution as a function of tissue concentration are complex and difficult to interpret. Fig. 7 shows a plot for a ventricle in sucrose solution at 25°C; pro-

bably the best interpretation is that the efflux is independent of tissue concentration ($n = 0$) until the 70 per cent level is reached, and then n becomes slightly greater than 1 as the concentration falls to 60 per cent. The scatter in the points from a single experiment makes this interpretation unconvincing,

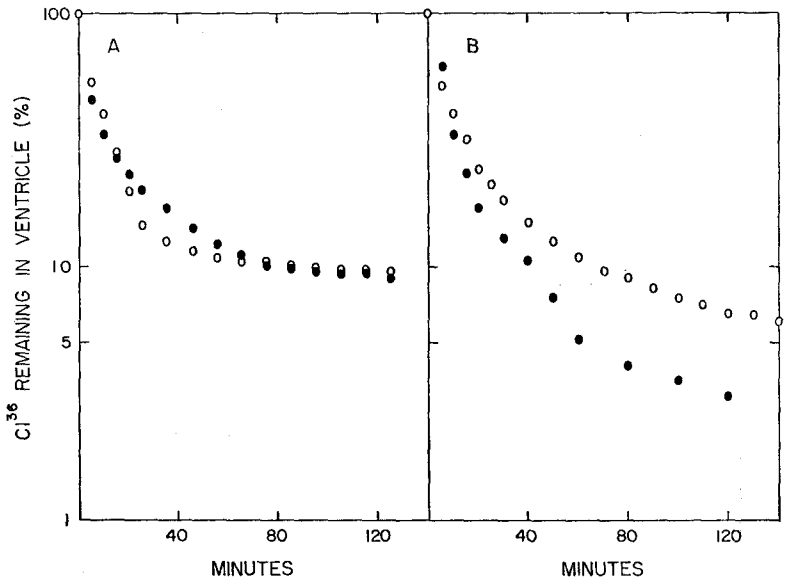

FIGURE 6. Integrated plots of the loss of Cl^{36} from ventricles. A, to sucrose solution at 25°C (filled circles); to sucrose solution at 5°C (open circles). B, to Ringer's solution at 25°C (filled circles); to Ringer's solution at 5°C (open circles).

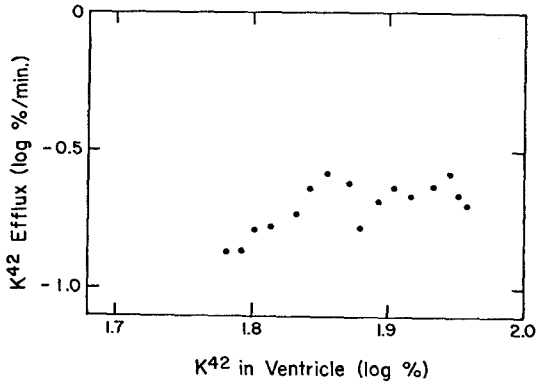

FIGURE 7. The efflux of K^{42} from a ventricle to sucrose solution at 25°C as a function of the K^{42} remaining in the ventricle.

but this is the trend in all the experiments on efflux to sucrose solution. Similar results were obtained in two other experiments with sucrose solution at 25°C and in two experiments with sucrose solution at 5°C. In Ringer's solution, at either 5 or 25°C, n varied between 1.2 and 1.6, though the data were never as linear as the Na^{24} efflux data.

Loss curves for K^{42} appear in Fig. 8. K^{42} efflux is almost the same in Ringer's and in sucrose solutions at 25°C. When the Q_{10}'s for K^{42} efflux were estimated from the rates; in Ringer's solution the Q_{10} was about 1.3. In the example shown the Q_{10} for efflux to sucrose solution is about 2.0; this was higher than Q_{10}'s in two other experiments; the mean value was 1.6. Of the ions studied so far, changing temperature has most effect on the efflux of K^{42}.

Ca^{45} Efflux

During the soak-in period ventricles incorporated 1.82 ± 0.15 μmoles Ca^{+2}/gm muscle H_2O (mean ± SE. No. = 8). Efflux as a function of concentration

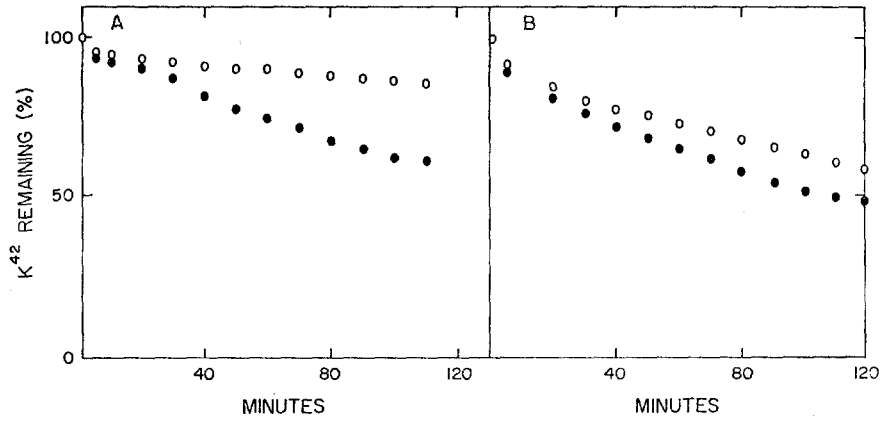

FIGURE 8. Integrated plots of the loss of K^{42} from ventricles. An arithmetic scale is used for convenience in seeing the points. A, to sucrose solution at 25°C (filled circles); at 5°C (open circles). B, to Ringer's solution at 25°C (filled circles); at 5°C (open circles).

plots are shown in Fig. 9. For the example in Ringer's solution at 25°C, n was 2.2; other experiments gave n as 1.2, 1.4, and 1.4. In Ringer's solution at 5°C the values were 2.2 and 2.2. Ventricles in sucrose solution show a higher n; in the example at 5°C, n is 6.3, the other value obtained was 5.1. At 25°C in sucrose solution, n was 5.1, 8.1, and 9.5. The figure also shows that the efflux to sucrose solution was only about 20 per cent of the rate to Ringer's solution (the greater part of this difference is not due to the different temperatures). If ventricles which had been in sucrose solution were transferred to Ringer's, the Ca^{45} efflux almost immediately rose to the level characteristic of Ringer's solution. A similar increase is found when ventricles are transferred from sucrose solution to 5 mM NaCl, KCl, or choline Cl (made isomotic with sucrose). The example in Fig. 9 shows that in the first 10 minutes in 5 mM NaCl the efflux increased 15-fold and n decreased to 2.5.

Loss curves for Ca^{45} appear in Fig. 10 which emphasize the slowness of efflux to sucrose solution and the relatively small effect of changing the tem-

perature on the amount of Ca^{45} remaining in ventricles soaked in sucrose solution. The Q_{10} for efflux to sucrose and to Ringer's is about 1.3. There are, therefore, striking differences between the efflux rates to sucrose and to Ringer's solution, but changing temperature does not have a pronounced effect.

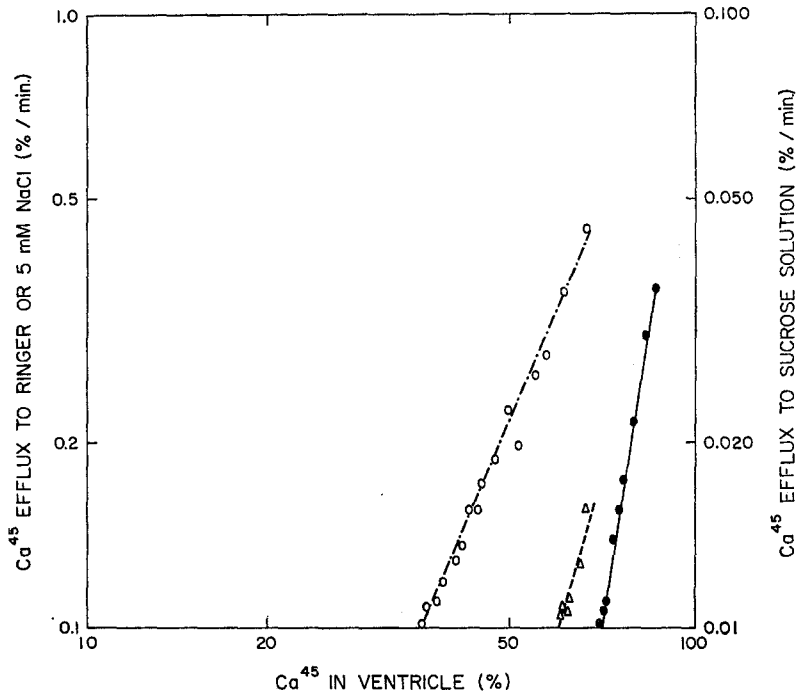

FIGURE 9. The efflux of Ca^{45} as a function of the Ca^{45} remaining in the ventricle. To sucrose solution at 5°C (closed circles). The same ventricle after transfer to 5 mM NaCl (open triangles). To Ringer's solution at 25°C (open circles).

P^{32} Efflux

The experiments with P^{32} were like those with the other isotopes, except that the first 70 minutes of efflux was always into Ringer's solution at 25°C. Fig. 11A shows efflux concentration plots for the efflux of P^{32} into Ringer's solution at 25°C. The data fall close to a straight line with an n of 14. The second example (Fig. 11B) shows the points for a ventricle in Ringer's at 25°C ($n = 6$) which was then transferred to sucrose solution at 25°C (n falls to 3). The transfer to sucrose solution obviously produces a prompt increase in the rate of P^{32} efflux.

Fig. 12 shows efflux from ventricles in sucrose and Ringer's solutions at 5 and 25°C. In this case rates were plotted instead of amounts remaining because only a fraction of the total P of the ventricles is labeled in the experiments

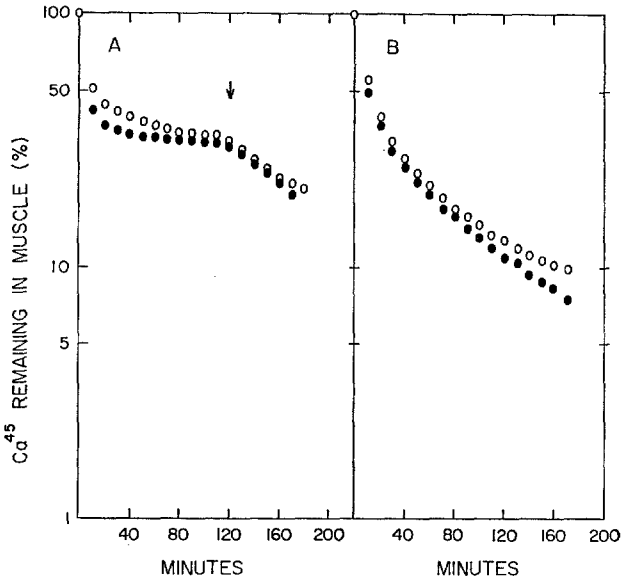

FIGURE 10. Integrated plots of the loss of Ca^{45} from ventricles. A, to sucrose solution at 25°C (open circles); at the arrow transferred to Ringer's solution at 25°C. To sucrose solution at 5°C, at the arrow transferred to Ringer's solution at 5°C (filled circles). B, to Ringer's solution at 25°C (closed circles); to Ringer's solution at 5°C (open circles).

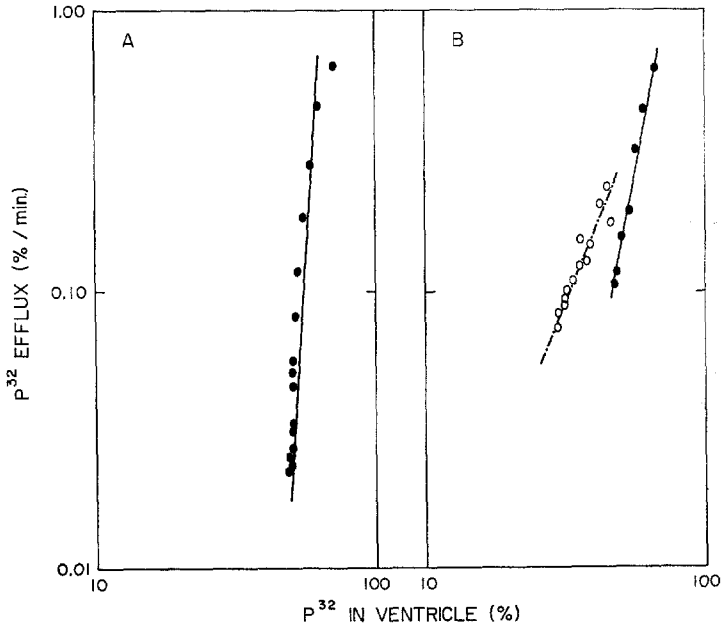

FIGURE 11. The efflux of P^{32} as a function of the P^{32} in the ventricles. A, to Ringer's solution at 25°C. B, to Ringer's solution at 25°C (closed circles). The same ventricle after transfer to sucrose solution at 25°C (open circles).

and the label is distributed among many organic compounds. When ventricles are transferred to sucrose solution at 25°C, P^{32} efflux promptly increases and after 20 minutes reaches a rate which is five times that of control muscles in Ringer's solution (compare the closed circles in Fig. 12A and 12B). At 5°C, the efflux in sucrose levels off at about five times the rate in Ringer's. The Q_{10} for P^{32} efflux to Ringer's is about 1.7 and the Q_{10} for efflux to sucrose solution is about 2.2. Almost identical results were obtained in two similar experiments.

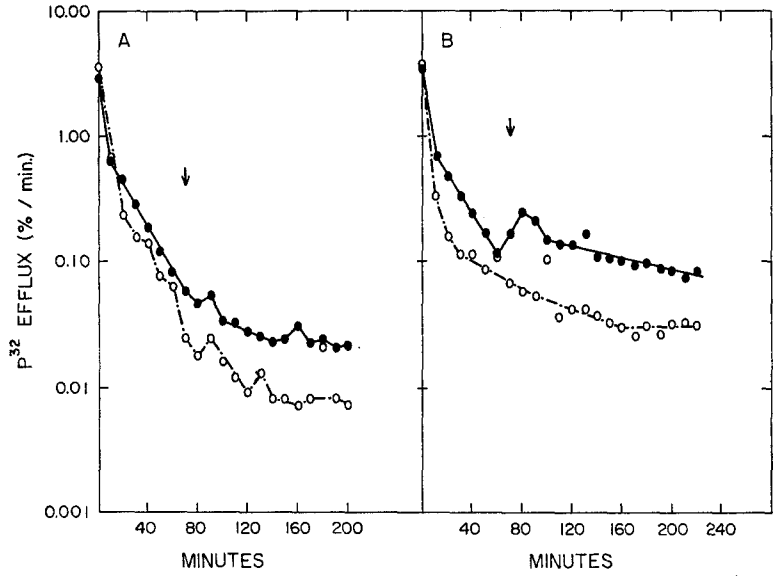

FIG. 12. Efflux of P^{32} from ventricles. A, to Ringer's solution at 25°C (filled circles). To Ringer's solution at 25°C, transferred at the arrow to 5°C (open circles). B, to Ringer's solution at 25°C, transferred at the arrow to sucrose solution at 25°C (filled circles). To Ringer's solution at 25°C, at the arrow transferred to sucrose solution at 5°C (open circles).

P^{32} has the highest Q_{10} for efflux into sucrose solution of the isotopes studied, and therefore seems to offer the best clue uncovered so far for explaining the substantial difference in the physiology of ventricles in sucrose solutions at 5 and at 25°C. An immediate question then is the chemical form in which P^{32} leaves the ventricles. The other inorganic ions are assumed to leave the ventricles in the same chemical state as that in which they entered; P^{32} might be coming out as any of a variety of organic phosphates.

To estimate the extent to which organic compounds containing P^{32} contribute to the isotope efflux, five column chromatographic separations were run. To take a typical example, ten ventricles were soaked overnight in Ringer's containing 0.1 mc/ml of P^{32} at 7°C. The next morning the ventricles were rinsed for 5 minutes in a large volume of sucrose solution and then kept

for 3 hours in 100 ml of sucrose solution at 25°C. The anions in the 100 ml of sucrose solution were then absorbed in a 10 cm × 0.6 cm^2 column of Dowex 1-formate. The column was rinsed with distilled water and then eluted with 100 ml of each of the solutions shown in Fig. 13 (Abood and Goldman, 1956). The figure shows that most of the P^{32} was eluted from the column by 0.15 M

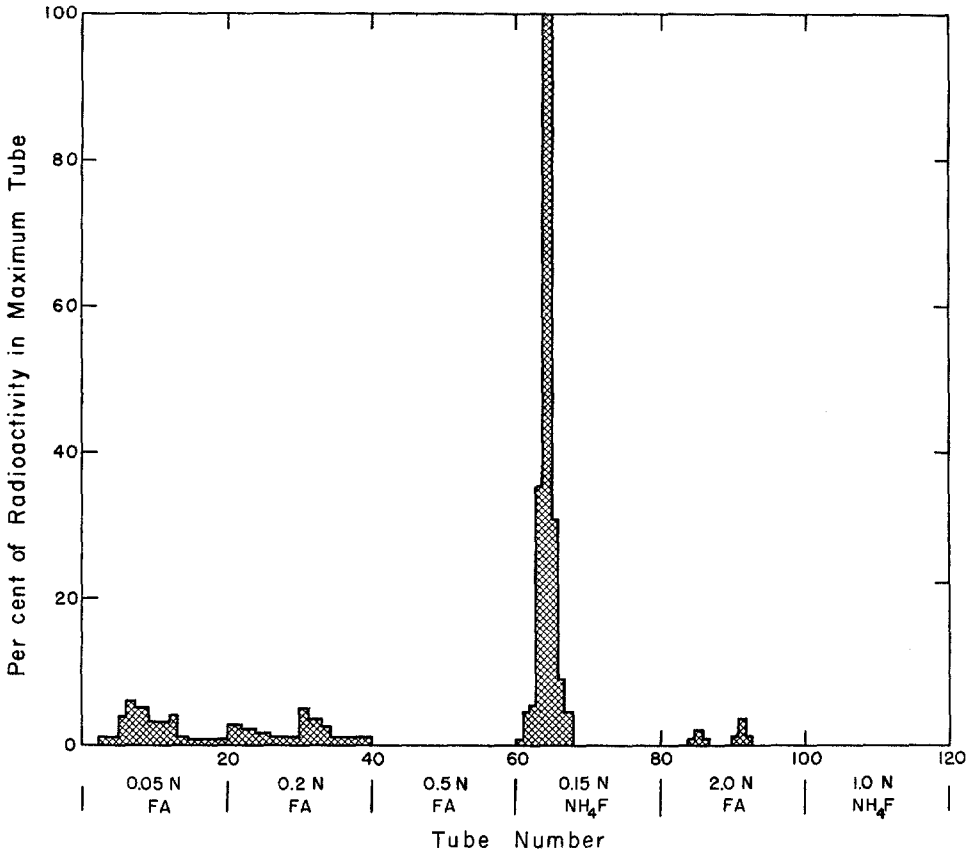

FIGURE 13. The column chromatography of the P^{32} in a sucrose solution in which P^{32}-loaded ventricles soaked for 3 hours at 25°C. The sequence of eluting solutions is shown under the axis of abscissas. FA, formic acid. NH_4F, ammonium formate.

ammonium formate as a single peak. This is the stage at which PO_4^{-3} should be eluted from the column (this point was checked in a separate experiment using $P^{32}O_4^{-3}$). Each of the column separations was performed using a different elution sequence, including gradient elution from Dowex 1-formate by H_2O (500 ml) and 1.0 N formic acid (500 ml), which is useful for separating PO_4^{-3} from organic phosphates with almost identical chromatographic properties (Wyatt, 1959). Therefore the chromatographic evidence suggests that almost

all the P^{32} leaves the fibers as $P^{32}O_4^{-3}$. The chromatography of Ringer's solutions in which P^{32}-loaded ventricles had soaked gave similar results.

The efflux of $P^{32}O_4^{-3}$ from the ventricles to sucrose solution suggested that this might be a significant fraction of the total anion lost by the tissue. To check on this, ventricles were soaked for 90 minutes in 5 ml of solution and the solutions were then analyzed for PO_4^{-3} (Table II). Substantial amounts of PO_4^{-3} are lost by the ventricles and, as in the P^{32} experiments, more P_i is lost to sucrose than to Ringer's solution. The results are not directly comparable to the P^{32} efflux data, because the ventricles placed in sucrose solution did not have a presoak in Ringer's solution. Unlike the P^{32} data, the Q_{10} for total P_i efflux is low, only about 1.3. This difference probably arises from the dif-

TABLE II

PHOSPHATE CONTENTS OF BATHING SOLUTIONS AFTER 90 MINUTES

Solution	Temperature	PO_4^{-3} wet weight ± SE; No. = 5
	°C	*μmoles/gm*
Ringer's	25	1.06±0.09
Ringer's	5	0.69±0.12
Sucrose	25	2.03±0.18
Sucrose	5	1.25±0.09

ferent procedures used in the two types of experiments; to reach levels of P_i adequate for chemical analysis a small volume of soak solution was used, so appreciable concentrations of P_i appear in the solution, and some may even be picked up again by the ventricles.

Efflux of Ultraviolet-Absorbing Substances

Our experiments were limited by the isotopes which we decided to incorporate into the ventricles. We can work with all the normal constituents of Ringer's solution, but there is no assurance that only these substances are important. For this reason, we measured the absorbance in the ultraviolet of solutions in which ventricles had soaked for several hours. As Fig. 14 shows, the solutions have considerable absorbance in the ultraviolet. Ventricles at 25° lose more ultraviolet-absorbing substances to sucrose solution that to Ringer's solution. Considerably more is lost to sucrose solutions at 25°C than at 5°C. It seemed worth while to identify the ultraviolet-absorbing substances.

The ultraviolet-absorbing substances remained in solution when basic organic phosphates were precipitated by adding an excess of barium acetate, bringing the pH to 8.2, and adding 4 volumes of ethyl alcohol. The substances

were almost completely absorbed on a column of Dowex 50-H^+ (0.79 cm^2 × 2 cm) and were eluted by 15 ml of 2 N NH_4OH. The eluate was evaporated to dryness and the residue was dissolved in a small volume of 0.1 N HCl and spread on a strip of Whatman No. 1 filter paper. The chromatogram was developed in an ascending system with isopropanol:HCl:H_2O (65:16.7:18.3). The dried chromatogram was examined with an ultraviolet lamp. Two absorbing bands were seen, one at R_f 0.41 (unknown I) and the second at R_f

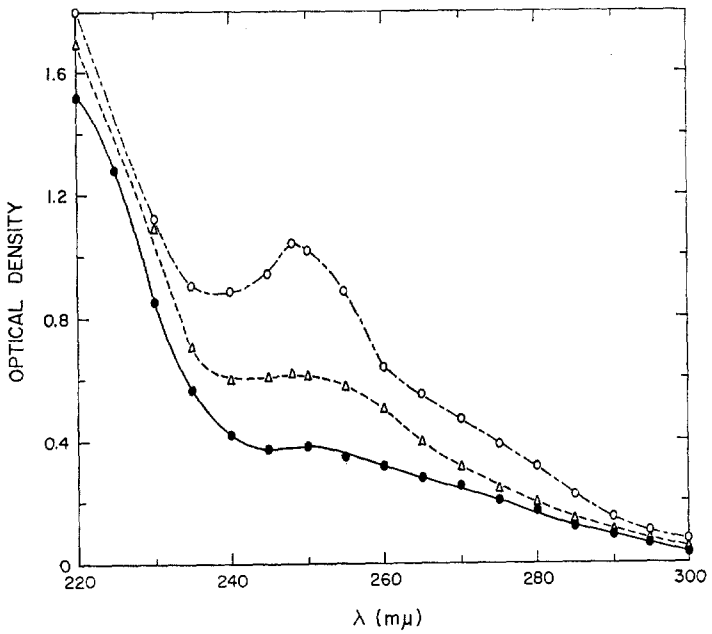

FIGURE 14. The absorbance (in OD units) in the ultraviolet of solutions in which ventricles had soaked for 3 hours. Sucrose solution at 25°C (open circles). Ringer's solution at 25°C (open triangles). Sucrose solution at 5°C (closed circles). The curves have not been adjusted for the differing weights of the ventricles.

0.57 (unknown II). These areas were cut from the chromatogram and the substances were eluted from the paper with 5 ml of 0.1 N HCl. The absorption spectra of the eluates were measured using HCl eluates from a blank chromatogram to zero the spectrophotometer.

First consider unknown I. In 0.1 N HCl the maximum absorbance is at 248.5 mμ; in 0.1 N NaOH the maximum shifts to 254 to 256 mμ. The absorption spectrum resembles that of inosine (pH 1, maximum 248.5 mμ; pH 11, maximum 255.5 mμ), but possible confusion with hypoxanthine still seemed possible.

Therefore unknown I was chromatogramed on Whatman No. 1 paper alongside spots of inosine and hypoxanthine. The results strongly support the

identification as inosine (Table III). As a further check, a sample of unknown I was evaporated to dryness and dissolved in 3.0 ml of 0.1 M phosphate buffer at pH 7.5. The maximum absorbance of this solution was at 248 mμ. Then 10 μl of xanthine oxidase was added. If unknown I were hypoxanthine, uric acid would be formed, the absorbance at 248 mμ would decrease, and a new absorbance band peaking at 290 mμ would appear. However, after 5 minutes, the absorbance at 248 mμ was unchanged.

A second sample of unknown I was dissolved in 1 N HCl, kept at 100°C for 1 hour, and then evaporated to dryness. This treatment would cleave the N-glycosidic bond in inosine and convert it to hypoxanthine and ribose. The

TABLE III
ASCENDING CHROMATOGRAPHY OF UNKNOWN I ON WHATMAN NO. 1 PAPER

Solvent system	R_f of inosine	R_f of hypoxanthine	R_f of unknown I
Isopropanol:HCl:H_2O (65:16.7:18.3)	0.47	0.43	0.48
5 per cent Na_2 H PO_4 in isoamyl alcohol	0.05	0.20	0.04
n-Butanol saturated with 5 per cent urea	0.91	0.82	0.89

hydrolyzed sample was dissolved in phosphate buffer and xanthine oxidase was added. The absorbance decreased at 248 mμ and increased at 290 mμ, showing that hypoxanthine was converted to uric acid. Unknown I could also be converted to hypoxanthine by treatment with a liver protein fraction containing nucleoside phosphorylase, which converts inosine to hypoxanthine and ribose phosphate. We conclude that unknown I is inosine.

Unknown II was eluted from the chromatogram with 3 ml of 0.1 N HCl and the absorption spectrum was measured: the maximum was at 278 mμ and there was a minimum at 240 mμ. The data suggest that unknown II is cytosine (max. 274) or cytidine (max. 280). The chromatographic properties of unknown II are summarized in Table IV. The results, taken together with the absorption spectrum, suggest that it is cytidine.

Separation of Inosine and Cytidine

Apparently the two principal ultraviolet-absorbing substances coming from the muscle to the bathing solutions are the nucleosides cytidine and inosine. To estimate the amounts of these substances lost by the ventricles, a simple method was developed to separate the two nucleosides. The solution which

had bathed the ventricles was taken and 0.1 gm of acid-washed charcoal was added. The surface of the solution was layered with 0.1 ml of ethanol (Crane and Lipmann, 1953) and the charcoal was spun down. The nucleosides were eluted from the charcoal with four washes, each of 2 ml of 50 per cent ethanol containing 1 per cent of concentrated NH_4OH.

The ethanol-NH_3 solution was evaporated to dryness in a current of warm air and dissolved in 0.5 ml of H_2O. The aqueous solution was placed on a

TABLE IV
ASCENDING CHROMATOGRAPHY OF UNKNOWN II ON WHATMAN NO. 1 PAPER

Solvent system	*Rf* of cytidine	*Rf* of cytosine	*Rf* of unknown II
Isopropanol:HCl:H_2O (65:16.7:18.3)	0.55	0.56	0.57
n-Butanol saturated with 10 per cent urea	0.16	0.31	0.14

TABLE V
NUCLEOSIDES RELEASED BY VENTRICLES INTO THE SOLUTION

Solution	Temperature	Inosine	Cytidine
	°C	*μmole/gm wet weight*	*μmole/gm wet weight*
Ringer's	25	0.46	0.08
	5	0.22	0.08
Sucrose	25	1.12	0.13
	5	0.19	0.06

column of Dowex 1-Cl (40 mm × 7.6 mm^2). The column was eluted with 40 ml of 0.025 M NH_4Cl at pH 10.6 and then with 25 ml of 3 N HCl; 5 ml fractions of the eluate were collected. The absorption of the fractions at 250, 270, and 300 mμ was measured. Cytidine comes out in the first 10 ml of 0.025 M NH_4Cl and inosine with the first 10 ml of 3 N HCl.

Ventricles were dissected and rinsed for 30 minutes in large volumes of oxygenated Ringer's. They were then weighed, placed in 10 ml volumes of Ringer's or sucrose solutions, and incubated at 25 or at 5°C for 3 hours. The tissues were removed, the solutions were placed for 10 minutes in boiling water, and the precipitate, if any, was spun down and discarded. The inosine and cytidine were separated by the method just described and the amounts estimated by measuring absorbance. The results of a representative experiment are given in Table V. The results show that the ventricles lost much more inosine than cytidine; indeed the cytidine levels are so low that the results can

only be considered roughly quantitative. The inosine data are more reliable. Much more inosine is released into sucrose solution than into Ringer's solution at 25°C. The Q_{10} for inosine release into sucrose solution is about 2.4, which parallels the difference in contractility in sucrose solution at the two temperatures.

Amino Acid Efflux

Possibly the increased efflux of inosine with sucrose solution at 25°C merely means that the cell membrane is becoming increasingly permeable to all sorts of organic molecules. To check this possibility, amino acids and peptides in the bathing solution were estimated. The results of two experiments expressed as micromoles glycine per gram wet weight were: Ringer's solution, 25°C, 8.0 and 9.5; sucrose solution, 25°C, 10.2 and 9.8; Ringer's solution, 5°C, 7.7 and 4.4; and sucrose solution, 5°C, 10.9 and 6.4. There is no systematic difference in the amounts of amino acids lost from the ventricles to the solutions and therefore the loss of inosine seems to reflect significant differences in the metabolism of the tissues rather than a non-specific leaking by cell membranes.

Lactate Efflux

From the results so far, we are missing a sizable amount of the anions which must accompany the cations lost from the ventricles to the sucrose solution. Therefore, the lactate lost by the ventricles to sucrose solutions at 25°C in 90 minutes was measured. The result was 17.6 ± 0.5 (No. = 5) micromoles per gram muscle H_2O.

DISCUSSION

Diffusion into the Extracellular Space

The experiments were undertaken to try to find out how frog ventricles remain excitable while soaking in sucrose solution. The first possibility is that in our experiments a portion of the extracellular fluid is not replaced by the sucrose solution as long as the ventricle remains excitable. Some observations by Van der Kloot and Rubin (1962) suggested that the extracellular fluid was rather rapidly replaced by the sucrose solution and this conclusion is borne out by the present experiments. In evaluating the tracer experiments, it should be recalled that the ventricles were prepared and mounted in the same way as the ventricles used for recording contractile force. Our electrical measurements were made on similar pieces of tissue pinned in a wax-bottomed dish, so diffusion was probably limited somewhat from the side of the tissue facing the wax. On the other hand, most microelectrode penetrations were of superficial fibers.

The experiments with C^{14} sucrose show a rapid exchange between extra-

cellular fluid and the soak solution. The rapid changes in the efflux rates of Ca^{45} and $P^{32}O_4^{-3}$ with altered soak solutions are also impressive demonstrations of the rapid permeation of the extracellular space by sucrose solution.

A Multicompartment Model

Another possibility is that the muscle fibers are surrounded by a compartment containing a high concentration of Na^+ and Cl^- and a low concentration of K^+. If this compartment exists, it would account for excitability in sucrose solutions. Furthermore, if the extracellular space of ventricles in Ringer's solution is permeated by I^{131} serum albumin, the calculated intracellular Na^+ and Cl^- concentrations are disconcertingly high. The calculated equilibrium potentials at 25°C are:

$$E_{Na} = +7 \text{ mv} \quad E_K = -95 \text{ mv} \quad E_{Cl} = -21 \text{ mv}$$

E_K is close to the resting potential of the ventricle, but E_{Cl} is off by 70 mv. Moreover, E_{Na} is almost 23 mv below the overshoot of the resting potential. Part of this difficulty might be accounted for by the binding of some of the Na^+ and Cl^-, which was not allowed for in the calculations. Another possibility is that most of the Na^+ and Cl^- are in a separate compartment, outside the excitable membrane but bounded by a membrane impermeable to serum albumin. This compartment would have to be large, almost 40 per cent of the fiber volume in ventricles soaked for 90 minutes in Ringer's solution.

At first we favored the two compartment idea because the Na^{24} and Cl^{36} efflux curves could not be resolved into two exponentials. However, the curves can be fit by assuming that about 10 per cent of the Cl^{36} is bound, and that Na^{24} efflux has the kinetics of a higher order reaction. If Cl^- and Na^+ are in a separate compartment, the volume of the compartment should decrease when the ventricle is in sucrose solution. As the volume of the compartment decreased, the total amounts of Na^+ and Cl^- would also decrease, but the concentrations in the compartment should remain close to the original levels. Therefore the efflux should remain nearly constant until almost all the Na^+ and Cl^- are gone from the tissue if the surface area of the compartment remained constant. On the contrary, the efflux rates of Na^+ and Cl^- decline rapidly and fall almost 50-fold in the course of the experiment.

An alternative is that the compartment is kept at close to its original volume because other ions replace Na^+ and Cl^- as they leave, so that the concentrations of Na^+ and Cl^- in the compartment fall. The only reasonable replacement for Na^+ is K^+, and if this happened the fibers should depolarize and become inexcitable.

The idea that Na^+ is retained outside the excitable membrane when

ventricles are in sucrose solution could only be finally rejected if *all* Na^+ left the tissue; in our experiments some Na^+ is always retained. Casteels (1962) has recorded 80 to 90 mv action potentials in frog ventricles in 4.8 mM $NaHCO_3$ made isotonic with sucrose, which again shows that remarkably low Na concentrations are adequate to preserve conduction. Brady (1964) reports that excitability is lost immediately if isotonic sucrose solution is perfused directly through the wall of the ventricle, which suggests that there is a definite lower limit for extracellular ion concentrations compatible with excitability. The problem in working out the lower limits is that ion balance is obviously important (in Casteels experiments the action potential is inhibited by normal Ringer's solution levels of Ca^{++} or K^+ added to the 4.8 mM Na^+); and the profound effects of abnormal solutions in cellular metabolism (Van der Kloot and Dane, in preparation).

Temperature and Efflux Rates

A second approach to the continued excitability in sucrose solution was to examine effluxes at 25 and 5°C. Excitability persists much longer at the lower temperature, so the processes leading to the loss of excitability must have a high Q_{10}. The Q_{10}'s were calculated from a rather small series of experiments, but agreement between individual runs was good. The effluxes of Na^{24} and Cl^{36} are probably relatively unimportant for retaining excitability since the Q_{10}'s are low for most tissue concentrations. The Q_{10} for K^{42} efflux is about 1.6, so the retention of tissue K^+ may be important for maintaining excitability. K^+ efflux may be limited by the availability of metabolically produced anions, like lactate and phosphate, which in turn could account for the sizable Q_{10}.

The Q_{10} of Ca^{45} efflux is low, so changes in Ca^{++} efflux rate would not be likely to account for the retention of excitability at low temperature. The effect of sucrose solution itself on Ca^{45} efflux is more noteworthy. The efflux rates of Na^{24}, K^{42}, and Cl^{36} are almost the same in sucrose and in Ringer's solutions. But Ca^{45} efflux to sucrose solution is only one-fifth as fast as to Ringer's solution. The slowing of Ca^{45} efflux seems to depend on the low ionic strength of the sucrose solution, since efflux is raised to the Ringer's level by 5 mM NaCl, KCl, or choline Cl. A rate-limiting step in the efflux of Ca^{++} from the ventricle may be the displacement of Ca^{++} from binding sites on the membrane by cations from the solution. Ventricles which are inexcitable after a brief exposure to isotonic NaCl regain excitability when transferred to sucrose solution (Van der Kloot and Rubin, 1962). The sucrose solution slows the rate of Ca^{++} loss and may increase the amount of membrane-bound Ca^{++}; a scheme like this could account for the results.

The highest Q_{10}, 2.2, for efflux to sucrose solution is that of $P^{32}O_4^{-3}$. As soon as ventricles at 25°C are transferred from Ringer's to sucrose solution,

the rate of $P^{32}O_4^{-3}$ loss increases fivefold. There is little free PO_4^{-3} in most cells, so it seems likely that there is an increased breakdown of organic phosphate esters in ventricles in sucrose solution. This agrees with the observation that the other substance with a high Q_{10} for efflux is inosine. The inosine is probably formed from adenosine by adenine deaminase, found in most tissues. Inosine compounds are formed by contracting skeletal muscles (Parnas, 1929; Wajzer and Nekhorocheff, 1952; Wajzer, Nekhorocheff, and Dondon, 1958). Substantial amounts of inosine are released by ventricles in sucrose solution at 25°C, so the adenosine compounds in the cell must fall substantially. Moreover the $P^{32}O_4^{-3}$ data also suggest that ventricles in sucrose solution lose an important fraction of their high energy phosphate compounds. It is worth mentioning that ventricles have been analyzed for ATP and CrP; these esters fell drastically in ventricles in sucrose solution at 25°C and the fall correlates with the decline in the force of contraction (Van der Kloot and Dane, in preparation). A significant metabolic change goes hand in hand with the loss of excitability in sucrose solution.

Cytidine is also lost by ventricles to the bathing solutions, but the amounts lost are so small that our analytical methods cannot show whether the efflux changes with the physiological state of the tissue. Cytidine phosphates take part in phospholipid synthesis, so an investigation of phospholipid synthesis in relation to the activity of the ventricle might be worth while.

The Significance of n

The rates of efflux to sucrose solution of the ions studied never seemed to be directly proportional to the concentration in the tissue; *i.e.*, that is, n was not 1. For Na^{24} efflux it has been suggested that n can be interpreted as the order of the efflux reaction; for example, when n is 3 the efflux processes involve the combination of 3 Na^+ with a carrier molecule. Our results suggest that this interpretation should be made with considerable caution.

Cl^{36} efflux does not fit a straight line in a log efflux–log concentration plot, unless we assume that 10 per cent is a slower fraction. With this assumption the data fit a line with a slope of 1, so the efflux of the rapidly exchanging 90 per cent is directly proportional to the concentration.

Ca^{45} gives a straight line in the log efflux–log concentration plot (and n may be as high as 9), but we know that most of the tissue Ca^{++} is bound to organic phosphates and to myosin (Nanninga, 1961). Therefore, the high value of n reflects the small fraction of the Ca^{45} which is free to diffuse from the cells. The situation with $P^{32}O_4^{-3}$ efflux is similar. In view of these examples, we hesitate to take the n for Na^{24} efflux to sucrose solution as a direct measure of the kinetics of the Na pump.

The efflux of K^{42} to sucrose solution is virtually independent of tissue concentration for the greater part of the efflux period. This was interpreted as due

to the lack of available anions, but other interpretations are possible and this question will be studied further.

Cation-Anion Balance

When in sucrose solution, ventricles must lose equivalent amounts of anions and cations. After 90 minutes they lose 45 μeq of Na^+, 37 μeq K^+, about 2 μeq Ca^{++} (judging from Ca^{45} incorporation and efflux data) per gm muscle H_2O. The total cation loss is about 85 μeq/gm H_2O. The anions lost are about 63.7 μeq Cl^-, 17.6 μeq lactate, and about 3.8 μeq PO_4^{-3} (assuming a valence of 1.89 at pH 7.0): a total of about 85 μeq/gm H_2O. While the balance sheet is surely incomplete, the matching of cations and anions is satisfactory thus far.

CONCLUSIONS

We are still unable to account for the excitability of ventricles soaking in sucrose solution in terms of commonly accepted mechanisms. Na^+ is lost quite rapidly from the ventricles and the amount remaining after 2 hours is unlikely to be sufficient for the operation of a conventional Na mechanism. An Na mechanism which could operate with low extracellular Na concentrations remains a possibility, and alternatives could be suggested, but the evidence does not permit a clear cut decision.

Contraction in a solution without added Ca^{++} may be easier to understand. Our sucrose solutions surely contain some Ca^{++}; moreover in sucrose solution the rate of Ca^{++} loss from the ventricle is markedly reduced. Therefore, the concentration of the membrane Ca^{++}, which seems to be necessary for contraction, may remain at a high level.

Indirect evidence suggests that the energy metabolism of ventricles in sucrose solution is abnormal, but is less affected at 5 than at 25°C. This idea has led to further experiments which suggest that an important reason for the loss of contractility is the loss of the cellular stores of high energy phosphate compounds.

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