J. Physiol. (1979), **288**, *pp.* 45–70
With 9 text-figures
Printed in Great Britain

THE EXTRACELLULAR COMPARTMENTS OF FROG SKELETAL MUSCLE

By M. C. NEVILLE

From the Department of Physiology, University of Colorado Medical Center, Denver, Colorado 80262, U.S.A.

With an Appendix by

R. T. MATHIAS

From the Department of Physiology, Rush Medical Center, Chicago, Illinois 60612, U.S.A.

(*Received 15 May 1979*)

SUMMARY

1. Detailed studies of solute efflux from frog sartorius muscle and single muscle fibres were carried out in order to characterize a 'special region' (Harris, 1963) in the extracellular space of muscle and determine whether this 'special region' is the sarcoplasmic reticulum.

2. The efflux of radioactive Na, Cl, glucose, 3-*O*-methylglucose, xylose, glycine, leucine, cycloleucine, Rb, K, inulin (mol. wt. 5000) and dextran (mol. wt. 17,000) from previously loaded muscles was studied. In all cases except dextran the curve had three components, a rapid (*A*) component which could be equated with efflux from the extracellular space proper, a slow (*C*) component representing cellular solute and an intermediate (*B*) component. The distribution space for the *B* component was 8% of muscle volume in summer frogs and 12% in winter frogs and appeared to be equal for all compounds studied. We tested the hypothesis that the *B* component originated from the sarcoplasmic reticulum.

3. The *C* component was missing from the dextran curves. Both dextran and inulin entered the compartment of origin of the *B* component (compartment *B*) to the same extent as small molecules.

4. For all compounds studied, the efflux rate constant for the *A* component could be predicted from the diffusion coefficient. For the *B* component the efflux rate constant was 6–10 times slower than that for the A component but was still proportional to the diffusion coefficient for the solute in question.

5. When Na and sucrose efflux from single fibres was followed, a *B* component was usually observed. The average distribution space for this component was small, averaging 1·5% of fibre volume. There was no difference between the average efflux rate constants for Na and sucrose.

6. In an appendix, the constraints placed on the properties of a hypothetical channel between the sarcoplasmic reticulum and the T-system by the linear electrical parameters of frog skeletal muscle are derived. It is shown that the conductance of such a channel must be less than $0{\cdot}06 \times 10^{-3}$ mhos/cm^2 of fibre membrane.

7. The conductance between compartment B and the extracellular space can be calculated from the efflux rate constants for Na, K and Cl. The value obtained was 5×10^{-3} mhos/cm^2 of fibre membrane or 100 times the limiting value for the conductance of the T-SR junction.

8. The finding that there is a B component in the efflux curves for large molecular weight substances like inulin and dextran and the small size of the B component in efflux curves from single muscle fibres indicate that *the 'special region' of the extracellular space of frog muscle is not the sarcoplasmic reticulum.* This conclusion is confirmed by a calculation of the conductance between the B compartment and the extracellular space. The value obtained is incompatible with predicted electrical properties of the SR-T-tubule junction. These results, in conjunction with the cellular ion concentrations which can be derived from the efflux data on whole muscle, are consistent with data from other laboratories (Somlyo, Shuman & Somlyo, 1977 *a*, *b*) which indicate that the ionic composition of the fluid in the sarcoplasmic reticulum resembles intracellular more than extracellular fluid.

INTRODUCTION

The necessity of an accurate measure of extracellular space in the interpretation of ion and nutrient fluxes has led to extensive studies of solute compartmentalization in muscle. Extracellular space values of 12% or less of the volume of frog sartorius muscles were obtained from the rapidly exchanging Cl (Harris, 1963) and inulin spaces (Boyle, Conway, Kane & O'Reilly, 1941; Desmedt, 1953) and from the poly-l-glutamate space (Ling & Kromash, 1967). On the other hand, the space available to non-metabolizeable sugars such as sucrose and raffinose appeared to vary from 20 to 45% of the tissue depending on the time of incubation and the physical condition of the frog (Johnson, 1955; Tasker, Simon, Johnstone, Shankley & Shaw, 1959; Narahara & Ozand, 1963). Harris (1963) proposed the muscle fibre to be composed of two cellular spaces, a non-specific 'special region' comprising 15–35% of the cell water and freely assessible to Na, Cl and sucrose and a specific region which excluded Na. Dydynska & Wilkie (1963; see also Birks, 1964) suggested that the 'special region' might be the sarcoplasmic reticulum. A morphological study of the effect of hypertonic sucrose on the internal membranes of muscle produced results consistent with this hypothesis (Birks & Davey, 1969). Studies of sucrose efflux from frog sartorius muscle (Vinogradova, 1967, 1968; Vinogradova, Nikolsky, & Trochin, 1967; Sperelakis, Shigenobu & Rubio, 1978) and Na efflux from rat extensor digitorum longus (Rogus & Zierler, 1973) provided clear evidence of the existence of a non-osmotically active 'special region' in both types of muscle. All three groups interpreted their data to indicate that this special region was the sarcoplasmic reticulum which was, therefore, freely accessible to extracellular solute.

A crucial test of this hypothesis is to examine solute flux from single muscle fibres to determine whether the 'special region' persists when the fibres are isolated from the majority of the extracellular elements of the whole muscle. Because of the forbidding technique associated with flux measurements on single fibres, such experiments have not often been performed. Hodgkin & Horowicz (1959) found that the efflux of Na from single muscle fibres followed a single exponential time course.

As these authors were careful to point out, however, their study did not preclude the idea that Harris 'special region' was intrafibrillar because they were unable to obtain experimental points earlier than 10 min after the start of the efflux experiment. On the basis of whole muscle experiments Na would have been lost from the special region within this time interval. Ling (1970) reported Na efflux curves for single fibres which did show an early rapid component. However, his technique was such that the tendonous region of the fibres was not excluded from the efflux chamber. Our preliminary studies suggested that this could lead to errors. For these reasons we developed a new technique which allowed the study of the early time points in solute efflux from the centre portion only of single fibres. Here we report the results of these single fibre studies along with a detailed study of solute efflux from sartorius muscles. The results of these studies will be shown to be inconsistent with the hypothesis that the 'special region' is the sarcoplasmic reticulum. The results are thus in accord with recent evidence from electron microprobe studies (Somlyo et al. 1977 *a*, *b*) which indicated that the composition of the fluid within the sarcoplasmic reticulum resembles that of the intracellular space. Preliminary accounts of this work have been presented (Neville, 1977, 1978).

METHODS

Rana pipiens (northern or southern) were obtained from NASCO, Fort Atkinson, Wisconsin. They were maintained in running tap water at 60 °F and force-fed cow's liver twice weekly. Carrier-free ^{22}Na was obtained as the chloride salt from New England Nuclear. ^{36}Cl, 10 μc/mg was obtained from Amersham-Searle as HCl. Sugars and amino acids labelled with ^{14}C or ^{3}H were obtained from New England Nuclear and Amersham-Searle at the lowest specific activity available. The composition of the Ringer solution was NaCl, 104 mM; KCl, 2·5 mM; $CaCl_2$, 1 mM; $MgSO_4$, 2 mM; $NaHCO_3$, 6 mM; NaH_2PO_4, 1 mM; Na_2HPO_4, 1·2 mM. For nitrate Ringer $NaNO_3$ was substituted for NaCl.

Experiments with whole sartorius muscles

Efflux experiments. Sartorius muscles (20–50 mg, 0·4–0·6 mm thick) were dissected from small northern *Rana pipiens* with all fibres intact. For loading, 8–12 muscles were shaken at 0 °C overnight or longer in 3–5 ml. solution containing 0·5–7 μc isotope/ml. and a suitable concentration of the corresponding non-labelled solute. Frog muscle has been shown to maintain its normal ion and water concentrations under these conditions for up to 12 days (Neville, 1972). Occasionally muscles were loaded at 25 °C, in which case 15 ml. incubation solution was used. Following incubation, the muscles were drained on moistened filter paper, weighed and a small piece of silk thread was tied to the tendon of the distal end. To avoid damage to muscle fibres, the tissues were not blotted. For efflux studies (washout) the muscle was placed for precisely timed intervals in a series of 6 ml. scintillation vials containing 1 ml. Ringer solution each and shaken vigorously in a water-bath maintained within 0·5 °C of the stated temperature (usually 0 °C). During the transfer from tube to tube fine forceps were used to hold the muscle by its thread, drawing it up the side of the tube to minimize transfer of solution from vial to vial. At the end of the total washout period, usually 2–4 hr. the radioactivity remaining in the muscle was extracted overnight in 2 ml. 5 % trichloroacetic acid. In order to ensure that all radioactivity was recovered during the washout process, muscles were similarly analysed directly after loading. In no case reported did the total radioactivity recovered from the washed muscles differ significantly from that extracted from muscles analysed directly. Both muscle extracts and washout solutions were counted with 5 ml. Bray solution (Bray, 1960) in a Picker liquimat β-scintillation counter. Quench correction, when necessary, was carried out with internal standards.(In certain experiments the efflux of both a ^{14}C-labelled and a ^{3}H-labelled compound were determined simultaneously. In this case the windows of the scintillation counter were set so that spillover from the ^{3}H-channel to the ^{14}C channel was less than 0·5 % of the counts in the

^{3}H-channel. Efflux curves were constructed from the data by successively summing the counts remaining in the muscle at the end of the experiment with the counts in the preceding washout vials after subtraction of the background. This process as well as the data analysis described below were carried out on a CDC 6400 computer located in the computing centre of the University of Colorado. The. The data were plotted as log solute remaining in the muscle *vs.* time.

It was possible to discern three components with quite different rate constants in the efflux curves for all solutes studied in this series (see Fig. 4, for example): a fast component (A) with a rate constant of the order of 1 min^{-1}, an intermediate component (B) with a rate constant about 0·15 min^{-1} and a slow component (C) which appeared after 20–40 min and had a rate constant of the order of 10^{-3} min^{-1}. We found that the entire curve could be fit satisfactorily by an equation of the form

$$[X]_t = \alpha \frac{8}{\pi^2} \sum_{n=0}^{3} \exp -[(2n+1)^2 k_1 t]/(2n+1)^2 + \beta e^{-k_2 t} + \gamma e^{-k_3 t}, \quad (1)$$

where $[X]_t$ is solute remaining at time t; n is an integer; α, β, and γ are the ordinate intercepts of the three component curves and k_1, k_2 and k_3 their respective rate constants. If the rate of passage of solute through the extracellular space is determined by diffusional processes,

$$k_1 = \pi^2 D/\lambda^2 r^2, \quad (2)$$

where D is the diffusion coefficient of the compound in question; r is the thickness of the muscle: λ, a factor for the tortuosity of pathway traversed through the extracellular space which is equal to 1·5 (Harris & Burn, 1949; Ling & Kromash, 1967). Thus, the first term in eqn. (1)

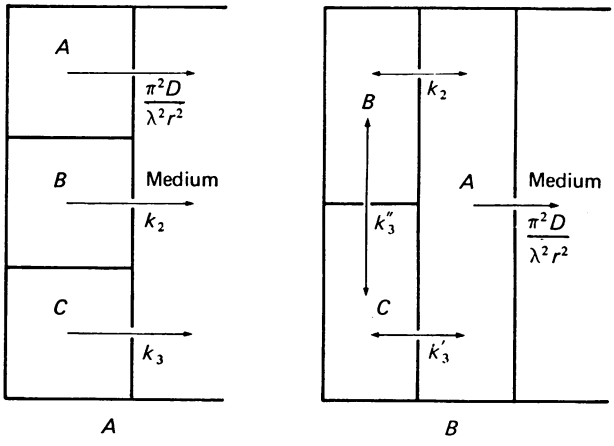

Fig. 1. Pathways for solute efflux from skeletal muscle. A, parallel efflux from three compartments into medium. B, series efflux with exchange between all compartments. In both cases the concentration of labelled solute in the medium is assumed to be sufficiently low that back flux is effectively zero. See text for meaning of symbols.

represents diffusion-limited efflux through the extracellular space considering the sartorius muscle to be a flat sheet (Harris & Burn, 1949; Jost, 1960). The next two terms represent exponential fluxes from two additional compartments (B and C) with slower time constants.

Although it was possible to resolve the efflux curves into their component curves by hand, the process proved to be tedious and somewhat subjective. For most curves we therefore used a non-linear least-squares analysis developed by Marquardt (1963) for digital computer, weighting the data as suggested by Dell, Sciacca, Lieberman, Case & Cannon (1973). Satisfactory results were obtained when n (eqn. (1)) was taken from 0 to 3. The program in FORTRAN is available from the author.

In using the coefficients α, β, and γ from eqn. (1) as a measure of the size of the compartments of origin of components A, B and C, we assume parallel efflux from three compartments into the medium (Fig. 1A). If compartment A represents solute loss from the extracellular space, it is

more realistic to assume a series model (Fig. 1*B*) in which labelled solute from the compartments of origin of components *B* and *C* enters the extracellular space before diffusing into the medium; there may also be exchange between *B* and *C*. For this pattern of efflux use of intercepts α, β, and γ overestimates the solute content of *B* and *C* and underestimates the solute content of *A*. We can find a lower bound for the solute content of compartments *B* and *C* and an upper bound for the solute content of compartment *A* by assuming series flux from *C* to *B* to *A* (equivalent to the assumption that $k'_3 \approx 0$, Fig. 1) and applying a method developed by Huxley (1960) to obtain compartment size from the coefficients of exponential resolution of a two component efflux curve. To apply this method to our data the slow and intermediate components were corrected for series flux using the expression

$$\gamma' = \beta.\gamma\,(k_2-k_3)^2/(\beta k_2^{\,2}-\gamma k_3^{\,2}), \tag{3}$$

where γ' is the lower bound for the solute content of compartment *C*. The lower bound β' for the solute content of compartment *B* was then obtained by correcting for series flux from *B* to *A*:

$$\beta' = \alpha.\beta''\,(k_1-k_2)^2/(\alpha k_1^{\,2}-\beta'' k_2^{\,2}), \tag{4}$$

where $\beta'' \equiv \beta+\gamma-\gamma'$. Finally an upper bound α' for the solute content of compartment *A* was obtained from the expression

$$\alpha' = \alpha+\beta''-\beta'. \tag{5}$$

The sequential correction should be valid for present purposes because the difference between γ and γ' was small in every case reported here. In this correction, flux from *A* is treated as an exponential rather than a diffusional process; this may result in a slight underestimate of the size of the *B* compartment.

MacDonald, Mann & Sperelakis (1974) have given an alternate means of analysing a two compartment system which leads to much smaller corrections for pool size (see also Kirby, Lindley & Picken, 1975). Rogus & Zierler (1973) analysed two component Na^+ efflux curves from rat soleus muscle using a method which takes loading time into account in deriving bounds in fraction sizes. If loading time is long enough so that the tissue specific activity equals that of the bathing medium, their analysis is equivalent to the series analysis used here. Because it is mathematically simpler and the magnitude of the series correction is always small, only average values for the series model are given. As we were interested only in comparing rate constants obtained from one compound with those for another compound, no attempt was made to obtain rate constants for the series model.

In order to compare results from experiments in which the extracellular concentration of solute was varied, the compartment size was calculated as a *distribution space* from the expression.

$$\text{distribution space (\%)} = \frac{\text{amount of solute per gram muscle in a given component}}{\text{solute concentration in medium } (\mu\text{mole/g})} \times 100. \tag{6}$$

All space given here are thus *weight* percentages rather than volume percentages. To change to volume percentages we assume that water occupies 1 ml./g and that the 19% of the dry weight of the tissue occupies about 13% of the muscle volume (Blinks, 1965). The reported distribution spaces should thus be divided by 0·94 to obtain tissue volume percentages.

Solute efflux from single fibres

Single fibres were dissected from the semitendenosus muscle of large northern or southern *Rana pipiens* in such a way that the entire fibre length could be visualized under the dissecting microscope for damage. Dissecting pins were used to affix the tendenous ends of the fibres to a flat bed of transparent encapsulating resin (Sylgard®). The fibres were allowed to rest for 1 hr or more in Ringer solution during which time we found that damaged fibres developed opacities visible under the dissecting microscope. Fibres which showed no such damage and which gave a twitch in response to an electrical stimulus applied through the bath were used in the reported experiments. Such fibres appear normal under Nomarski microscopy and have resting potentials of -80 to -90 mV. The average diameter of each fibre was measured using a calibrated graticle with the Nomarski microscope. In some cases sarcomere length was estimated by counting the number of striations in a calibrated interval.

Isotope loading and efflux were carried out in such a way that only the centre portion of the fibre was exposed to the incubation and wash solutions. To achieve this, the fibres were pinned

under light mineral oil or silicone oil in special chambers made of Sylgard® (S-chamber) or Plexiglass® (P-chamber) (Fig. 2). For incubation a depression under the fibre held a 200 μl. 'bubble' of Ringer solution containing ^{22}Na or [^{14}C]sucrose at a concentration of 100 μc/ml. This 'bubble' surrounded a 0·8–1·5 cm length of the centre portion of the fibre. After a 1 hr loading period the fibre was drawn through the oil and repositioned over a second depression in the

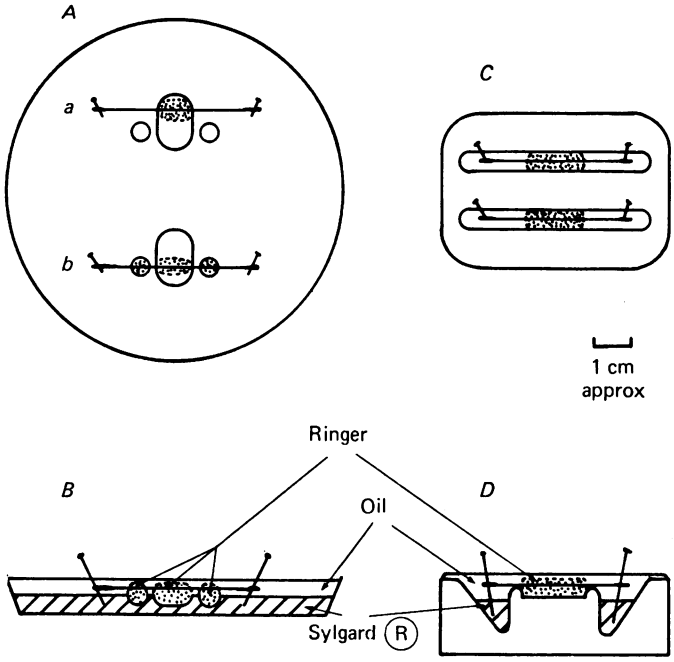

Fig. 2. Chambers for studying solute efflux from single muscle fibres. *A, top view of S-chamber.* A 5 mm layer of Sylgard® in the bottom of a 9 cm Petri dish contains a 2 mm deep depression of the shape shown. The fibre is stretched across the chamber as shown in *a* for incubation with isotope or as shown in *b* for efflux. A 5 mm deep layer of mineral oil covers all. In general, to keep the fibre from adhering to the oil water interface it must be stretched to a sarcomere length of 2·8–2·9 μm in this chamber. *B, side view of S-chamber* with fibre arranged for efflux portion of experiment. Note the 50 μl. side 'bubbles' which prevent diffusion of isotope back to the central washout 'bubble' along the fibre. *C, top view of P-chamber.* The fibre is contained in a 2 mm wide slot in a solid block of Plexiglas®. Its tendonous ends are affixed with pins to Sylgard® pads in the side wells which are separated from the incubation-washout chamber by ridges 1·5 cm apart. A layer of silicone oil covers fibre and incubation solution. It is possible to study a fibre at resting length in this chamber. *D, side view of P-chamber.* During the washout, tendons were washed at intervals with 50 μl. Ringer solution. The fibre was incubated in one chamber, then moved under oil to the adjacent chamber for washout.

chamber and the chamber placed in an ice bath. The portions of the fibres which had not been exposed to isotope-containing solution were washed free of any contaminating isotope with two successive 50 μl. 'bubbles' of non-radioactive solution. A Pipetteman® microlitre pipettor with plastic tip was used to place a 200 μl. drop of ice-cold Ringer solution around the isotope-loaded portion of the fibre. When necessary a small glass hook was used to bring the fibre through the oil-water interface. The first drop was immediately removed as completely as possible in order to wash away isotope adherent to the surface of the fibre. A second drop of the same volume was placed around the fibre. At intervals, 100 μl. solution were removed from the chamber for counting and immediately replaced with an equal volume of fresh ice-cold Ringer. The solution around the fibre was gently stirred between samples by pumping the wash solution in and out

of the tip of a 50 μl. Eppendorfer® micropipettor. In some experiments the portions of the fibre adjacent to the isotope-loaded portion were continuously bathed in 50 μl. drops of Ringer. In experiments in the P-chamber we found that an occasional wash of the fibre ends with non-radioactive Ringer was sufficient to show that lateral diffusion of isotope was negligible. After 1 hr the washout was terminated by snipping out the washed segment with microdissection scissors. Radioactivity was extracted from this segment with 5% trichloroacetic acid. The extract and the bathing solution samples were counted with 10 ml. Budget-Solve® (Research Products, Inc.) in a liquid β-scintillation counter. The counts remaining in the fibre at a given time were calculated from the sum of the activity extracted from the muscle and the counts entering the wash solution after that time. The resulting curves were plotted and analysed as described in the text. Fibre volume was calculated from the measured diameter and the exposed length of fibre (0·8 cm in the S-chamber, 1·5 cm in the P-chamber).

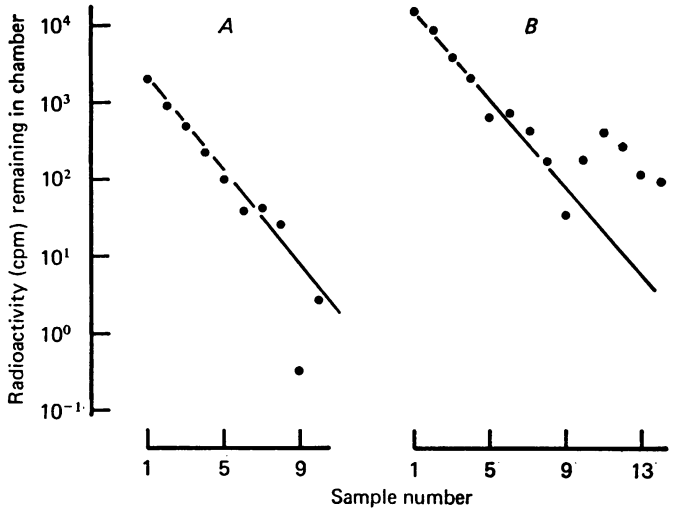

Fig. 3. *Loss of radioactivity from efflux chamber. A*, after loading for 1 h a single fibre was placed in the S-chamber. After addition of 200 μl. wash solution the fibre was removed and samples taken as for a washout experiment (see Methods). The results are expressed as total counts remaining in chamber at the time of removal of a particular sample. *B*, 200 μl. Ringer solution containing 3×10^7 counts per minute [^{14}C]sucrose were allowed to rest in the *S*-chamber for 1 hr. After removal of this solution the usual washout procedure was followed. The washout solution was placed at the identical position in the chamber. Results expressed as in *A*.

In order to ascertain that radioactivity was not retained by the chamber and released in such a way as to affect the efflux curves, we studied the washout of radioactivity from a chamber containing no fibre. In the first experiment a loaded fibre was placed in the chamber as for a washout experiment. After addition of the wash solution the fibre was removed and the wash-out procedure followed as usual. Because one half the radioactivity is removed with each sample, a twofold decrease in the activity in the chamber should be observed. Fig. 3*A* shows the results obtained in the S-chamber; the points represent the observed activity; the line represents the expected result. As can be seen, the deviation from the expected result is small and can be attributed to random sampling and counting error.

In a second, more rigorous test, 200 μl. incubation solution containing [^{14}C]sucrose at a concentration of 100 μc/ml. (3×10^7 counts per minute total) were allowed to equilibrate in the chamber for 1 hr. This solution was removed and the washout procedure followed at this location in the chamber, again with no fibre present. About 10^4 counts or 0·03% of the initial radioactivity remained at the start of the washout. Curve 3*B* shows how this activity was diluted by the sampling procedure. Clearly there was no significant difference between the observed and pre-

dicted counts remaining for the first eight samples. The small amount of residual activity appearing in later samples totalled less than 1000 counts or 0·003% of the activity originally present in the chamber and was, therefore, too small to contribute significantly to the efflux results.

RESULTS

Solute efflux from sartorius muscle

Small molecular weight substances. Fig. 4 shows a typical curve for [^{14}C]sucrose efflux at 0 °C from frog muscle incubated overnight with tracer quantities of [^{14}C]sucrose. The best resolution of the experimental curve gave the three components shown. Two components resolution gave a significantly poorer fit; four component resolution did not result in an improved fit over resolution into three components.

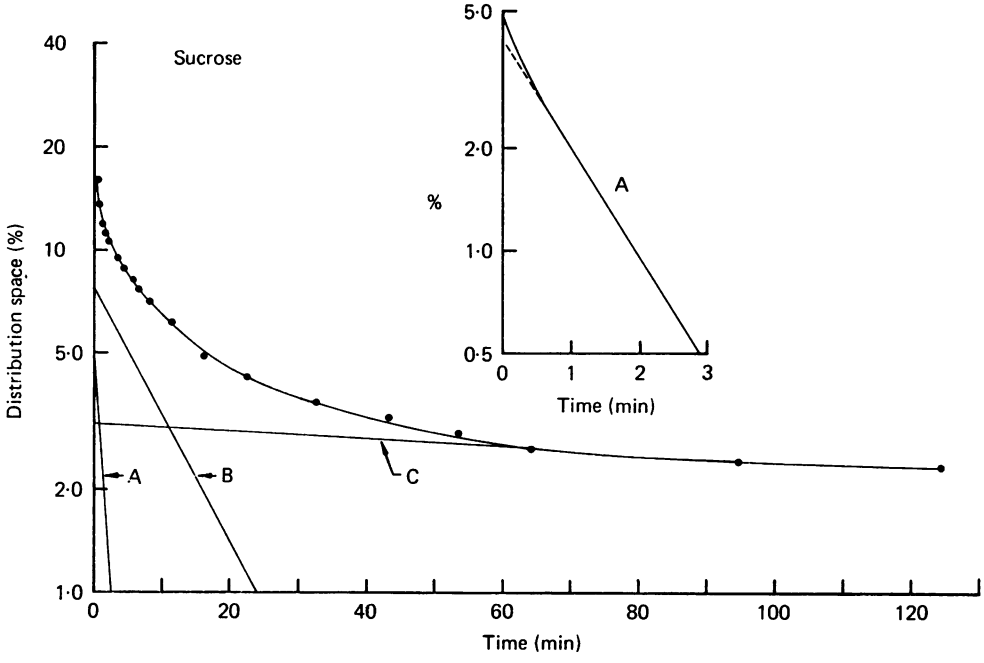

Fig. 4. Resolution of sucrose efflux curve. Washed muscle loaded overnight at 0 °C in Ringer solution containing 7 μc [^{14}C]sucrose/ml. (specific activity 12 μc/mg). Inset: expansion of component A showing diffusion limited nature of curve. Points experimentally determined. Curves theoretical according to eqn. (1).

This was true for all curves whose analysis is discussed in this paper, except for dextran (Fig. 5, see below). Average values for the compartment sizes and rate constants obtained from least-squares analysis of similar curves from four muscles are shown in Table 1 along with comparable data for muscles incubated and washed in Ringer solution made hypertonic by the addition of 200 mM-sucrose. Compartment sizes for both the series and parallel models shown in Fig. 1 are given. The series correction results in an increase in the estimated size of the *A* compartment and a decrease in that of *B* (Table 1).

This analysis suggests that there are two extracellular compartments of approximately equal size available to sucrose; efflux from compartment *A* is extremely rapid; that from compartment *B* is about eight times slower. The origin of com-

ponent *C* in the efflux curves for sucrose is not completely certain; it may represent intracellular sucrose which has penetrated the cell membrane very slowly during the overnight incubation. Alternatively it may represent a very small amount of an isotopic impurity such as glucose which has entered the muscle. Whatever its origin, the component was always small (< 2 % distribution space) and left the muscle very slowly. Quantitatively similar sucrose efflux curves have recently been published by Sperelakis *et al.* (1978).

TABLE 1. Parameters obtained from resolution of sucrose efflux curves. All values are means of results from four muscles ± the S.E. of the mean. Experiment conducted in July

		Compartment size		
Component	Sucrose concentration	Parallel model (%)	Series model (%)	Rate constant (min^{-1})
A-Fast	Trace	5·7 ± 0·6	7·4 ± 0·5	0·79 ± 0·07
	200 mM	8·3 ± 0·7	11·3 ± 1·0	0·65 ± 0·16
B-Intermediate	Trace	8·7 ± 0·7	7·0 ± 0·5	0·09 ± 0·01
	200 mM	13·4 ± 9·6	10·7 ± 0·9	0·08 ± 0·01
C-slow	Trace	2·4 ± 0·5	1·7 ± 0·3	0·0028 ± 0·0004
	200 mM	2·2 ± 0·5	1·5 ± 0·3	0·0035 ± 0·0007

The distribution space for both the *A* and *B* components was increased by loading and washing in Ringer solution made hypertonic by the addition of 200 mM-sucrose (Table 1). Neither the size of the slow fraction nor the rate constants of exchange were altered by this procedure. This behaviour is consistent with the notion that the intermediate and fast components are essentially extracellular and thus osmotically inactive. If the sarcoplasm behaves as a perfect osmometer (Blinks, 1955) in the hypertonic solution and compartments *A* and *B* maintain the same absolute volumes as in isotonic solution, it can be calculated that the distribution spaces for the *A* and *B* compartments (parallel model) should increase to 8·1 and 12·4 % respectively, in the hypertonic solution. The observed values (Table 1) were 8·3 % and 13·4 %, in good agreement with the predicted values.

Efflux curves similar to that of sucrose were obtained for ten other small molecular weight compounds including ^{22}Na, ^{36}Cl, ^{886}Rb, ^{42}K, glucose, 3-*O*-methylglucose, xylose, glycine, leucine and cycloleucine. The results of resolution of those curves obtained from summer frogs are given in Table 2. The distribution spaces for components *A* and *B* were similar to that of sucrose; in no case was the value for a single compound significantly different from the mean value obtained from averaging all the results from eight compounds. This suggests that both compartments *A* and *B* contain extracellular fluid. As for sucrose, correction for series flux between compartments *A* and *B* resulted in a slight increase in the size of compartment *A* and a slight decrease in compartment *B*. Both the rate constant and the size of the *C* component varied from compound to compound. In all cases the *C* component left the muscle at least 14 times more slowly than the *B* component. For this reason a series arrangement of compartments *B* and *C* would not alter the estimated compartment sizes.

TABLE 2. Parameters from resolution of curves for efflux of small molecular weight compounds from sartorius muscles of summer frogs. Loading and efflux carried out as described in methods

Solute	Number of muscles	External concentration (mM)	Washout temperature (°C)	Distribution spaces (%)			Rate constants (min^{-1})		
				$A(\alpha)$	$B(\beta)$	$C(\gamma)$	$A(\pi^2 D/\lambda^2 r^2)$	$B(k_2)$	$C(k_3) \times 10^3$
^{22}Na	15	105	0	10·9 ± 1·6	7·9 ± 0·3	6·2 ± 2·7	0·9 ± 0·3	0·15 ± 0·02	3·09 ± 0·9
^{22}Na	4	105	21	14·1 ± 2·7	8·5 ± 0·5	4·2 ± 0·4	1·6 ± 0·4	0·22 ± 0·04	23·9 ± 5·5
^{36}Cl	8	108	0	7·9 ± 1·0	8·8 ± 0·9	0·65 ± 0·05	1·02 ± 0·15	0·18 ± 0·02	13·1 ± 2·0
[^{14}C]glucose	19	1	0	8·4 ± 1·3	9·5 ± 0·8	21·6 ± 5·0	0·79 ± 0·28	0·10 ± 0·02	2·4 ± 0·6
3-*O*-methyl-[^{14}C]glucose	4	1	0	5·5 ± 0·7	9·3 ± 0·7	8·6 ± 2·1	0·60 ± 0·11	0·08 ± 0·01	1·9 ± 0·2
[^{14}C]xylose	4	1	0	7·2 ± 1·5	7·4 ± 1·5	40·7 ± 2·9	1·26 ± 0·48	0·13 ± 0·04	1·0 ± 0·2
[^{14}C]glycine	8	100	0	8·5 ± 1·3	9·6 ± 1·4	42·7 ± 1·1	0·94 ± 0·30	0·11 ± 0·03	5·3 ± 0·25
[^{14}C]leucine	4	1	0	5·1 ± 2·1	7·5 ± 0·4	49·4 ± 4·4	0·86 ± 0·37	0·11 ± 0·02	1·6 ± 0·1
[^{14}C]cyclo-leucine	6	1	0	6·6 ± 2·0	9·3 ± 1·8	1·55 ± 20	1·00 ± 0·44	0·12 ± 0·03	0·2 ± 0·04
	Average parallel model			7·7 ± 2·0	8·6 ± 0·9				
	Series model			8·7 ± 2·0	8·1 ± 0·8				

TABLE 3. Parameters from resolution of solute efflux curves from sartorius muscles of winter frogs. [^{3}H]sucrose efflux was studied simultaneously by loading the muscle at 0 °C with the isotope shown and [^{3}H]sucrose, then washing out by the usual methods. There was no C component in the sucrose curve accompanying ^{86}Rb efflux nor in the [^{14}C]dextran curves. ^{36}Cl efflux was studied in NO_3 Ringer to reduce the exchange component of cellular efflux. [^{14}C]dextran had a molecular weight of 17,000 daltons

Solute	Washout temperature (°C)	Number of muscles	Distribution spaces (%)			Rate constants (min^{-1})		
			$A(\alpha)$	$B(\beta)$	$C(\gamma)$	$A(k_1)$	$B(k_2)$	$C(k_3) \times 10^3$
^{86}Rb	25	8	15·2 ± 1·3	7·6 ± 1·0	128 ± 12	0·79 ± 0·08	0·07 ± 0·01	0·5 ± 0·1
[^{3}H]sucrose			11·1 ± 1·6	11·2 ± 0·6	—	0·68 ± 0·10	0·10 ± 0·01	—
^{42}K	25	4	14·1 ± 2·0	9·1 ± 1·9	352 ± 88	1·26 ± 0·20	0·12 ± 0·03	2·7 ± 0·4
[^{3}H]sucrose			11·0 ± 2·0	10·3 ± 0·7	0·3 ± 0·0	0·94 ± 0·11	0·33 ± 0·10	5·0 ± 1·0
^{36}Cl	0	4	10·2 ± 1·4	12·6 ± 0·3	1·32 ± 0·03	1·9 ± 0·3	0·28 ± 0·02	12·0 ± 1·0
[^{14}C]sucrose			9·1 ± 1·2	14·8 ± 1·5	2·93 ± 0·9	1·1 ± 0·2	0·11 ± 0·01	1·0 ± 0·5
[^{14}C]inulin	0	6	8·6 ± 0·9	12·0 ± 0·7	3·6 ± 0·6	0·33 ± 0·10	0·066 ± 0·009	14 ± 3·0
[^{14}C]sucrose			16·2 ± 1·1	11·9 ± 0·8	0·6 ± 0·1	0·88 ± 0·01	0·11 ± 0·01	1·0 ± 3·0
[^{14}C]dextran	0	4	11·6 ± 2·3	12·4 ± 0·7	—	0·092 ± 0·011	0·013 ± 0·001	—
[^{3}H]sucrose			7·9 ± 0·7	12·6 ± 0·7	1·0 ± 0·5	0·56 ± 0·18	0·126 ± 0·04	0·31 ± 0·15
[^{14}C]dextran	25	4	11·6 ± 2·5	11·8 ± 0·4	—	0·187 ± 0·026	0·035 ± 0·004	—
[^{3}H]sucrose			10·0 ± 0·7	11·7 ± 2·5	1·1 ± 0·3	1·22 ± 0·08	0·23 ± 0·07	0·39 ± 0·22
[^{3}H]sucrose	Average		9·9 ± 1·2	12·0 ± 1·5	1·0 ± 1·0			

The results shown in Tables 1 and 2 were obtained from studies on summer frogs. Table 3 gives the results of a study of ^{42}K, ^{86}Rb and ^{36}Cl efflux from sartorius muscles of winter frogs. [^{3}H]sucrose efflux was studied simultaneously in these muscles in order to monitor seasonal variations in compartment sizes. In these winter muscles the sucrose distribution spaces for compartments *A* and *B* were somewhat larger than the values obtained from summer frogs. This was a consistent finding.

In studying ^{86}Rb and ^{42}K efflux, we did not find it possible to obtain adequate resolution of the curves if the muscles were fully equilibrated with isotope, because the very large cellular component totally obscured the much smaller extracellular components. For this reason the muscles were incubated only 2–4 hr at 0 °C with

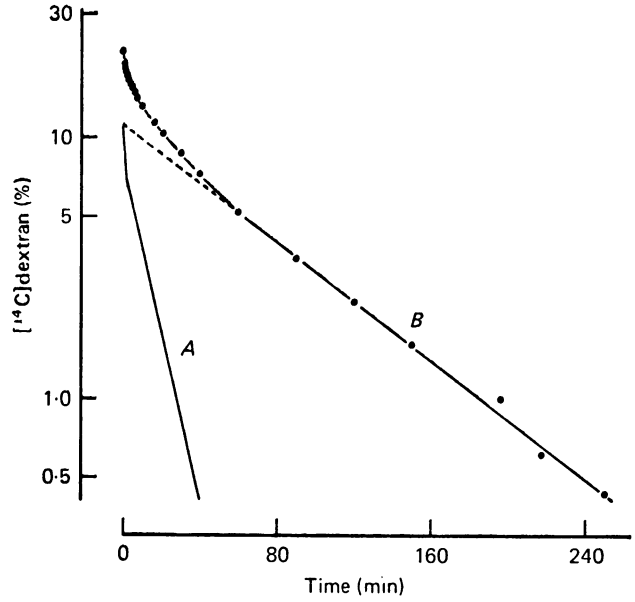

Fig. 5. [^{14}C]dextran (mol. wt. 17,000) efflux from frog sartorius muscles. Muscles were loaded with isotope by incubation at 0 °C in the presence of [^{14}C]dextran (0·5 μc/ml.) at 0 °C overnight. They were then washed by the usual procedure at 0 °C.

isotope before the efflux experiment. Under these conditions three components were obtained from both the ^{86}Rb and the ^{42}K experiments. For these isotopes the *A* component had a slightly larger distribution space than the corresponding sucrose space. A close examination of the data in Table 2 suggests that the same is true of ^{22}Na. This result may stem from non-specific ion binding to extracellular elements. On the other hand the *B* component was smaller for ^{86}Rb and, unlike the results presented in Table 2, the rate constant of efflux was smaller for ^{42}K and ^{86}Rb than for sucrose. Although the quantitative argument is too complex to present here, these differences probably arise from the fact that, in the non-equilibrated muscle, there is a net transport of solute from compartment *B* into the muscle fibres during the washout experiment as well as into the extracellular space.

For the ^{36}Cl experiment shown in Table 3 the muscle was washed in a solution in which 100 mM of the extracellular Cl was replaced by NO_3 to reduce exchange diffusion with the cellular compartment. Under these conditions the cellular com-

ponent was increased (see also Adrian, 1961) to a distribution space of 1·32%, corresponding to a Cl concentration in the cell water of 2·4 mM or a Cl equilibrium potential of −96 mV. This is not much different from the membrane potential of frog sartorius muscles measured with a micropipette of −90 mV.

Larger molecular weight substances. We also studied the efflux of [^{14}C]inulin (mol. wt. 5000) and [^{14}C]dextran (mol. wt. 17,000) from sartorius muscles of winter frogs. The inulin efflux curves resembled the sucrose curves shown in Fig. 4 except that both components *A* and *B* had slower efflux rates. A typical curve for [^{14}C]dextran efflux is shown in Fig. 5. The curve could be resolved into 2 components,

TABLE 4. Effect of various treatments on component *C*. For ^{22}Na, muscles were incubated overnight at 0 °C then washed out at either 0 or 21 °C. Ouabain, when present, was added at a concentration of 10^{-4} M to the 21 °C wash solution. Insulin treatment was accomplished by pretreating muscles with insulin (0·1 μ/ml.) for 6 hr at 25 °C before incubation at 0 °C overnight for 6 hr. *Significantly different from control $P > 0{\cdot}01$

Solute	Treatment	Washout temperature (°C)	Number of muscles	Component *C* Distribution space (%)	Component *C* Rate constant ($min^{-1} \times 10^3$)
^{22}Na	Control	0	15	6·2 ± 2·7	3·0 ± 0·9
		21	4	4·2 ± 0·4	23·9 ± 5·5*
	Ouabain	21	4	3·7 ± 0·6	8·4 ± 1·0*
3-*O*-methyl[^{14}C]glucose	Control	0	4	8·6 ± 2·1	1·9 ± 0·2
	Insulin	0	3	64·5 ± 2·3*	6·7 ± 0·8*
[^{14}C]xylose	Control	0	4	10·6 ± 0·9	1·6 ± 0·1
	Insulin	0	3	43·7 ± 4·4*	0·93 ± 0·01*

an exponential one (labelled *B*) and one which follows the pattern of bulk-phase limited diffusion (labelled *A*). We were unable to discern a *C* component. Parameters obtained from resolution of both the inulin and dextran curves, along with parameters obtained for simultaneous [^{3}H]sucrose efflux are given in Table 3. There are no significant differences between the distribution space for the *A* and *B* components and the corresponding sucrose components. This indicates that both compartments *A* and *B* are accessible to compounds with molecular weights of as much as 17,000. There appears to be an effect of temperature on the rate constants observed in the dextran experiments. This will be discussed in the next section.

The origin of component C. The problem now was to relate components *A*, *B*, and *C* in the efflux curves to anatomical spaces within the muscle. The sucrose, dextran and inulin spaces for component *C* were small or non-existent, consistent with the notion that these molecules penetrate muscle cells poorly or not at all (Johnson, 1955; Birks & Davey, 1969). The ^{22}Na efflux rate constant for component *C* was increased eightfold by increasing the temperature from 0 to 21 °C and was significantly decreased by ouabain (Table 4). Treatment with insulin before isotope loading significantly increased 3-*O*-methylglucose and xylose entry into component C (Table 4). With the exception of the temperature increase (see below), none of these treatments significantly altered either the distribution space or the rate constant for components *A* and *B*. All these data confirm the notion that component *C* arises from the intracellular compartment.

The origin of component A. In Fig. 6 the efflux rate constants for component A for all compounds studied at 0 °C have been plotted as a function of the diffusion coefficient. The shaded-hatched area shows the range of values, k_1, predicted from eqn. (2) for muscles varying in thickness from 0·3 to 0·5 mm. The rate constants for all the muscles used in these experiments fell within this range, as expected if component A originates from the extracellular space and is diffusion-limited in its passage out of the muscle.

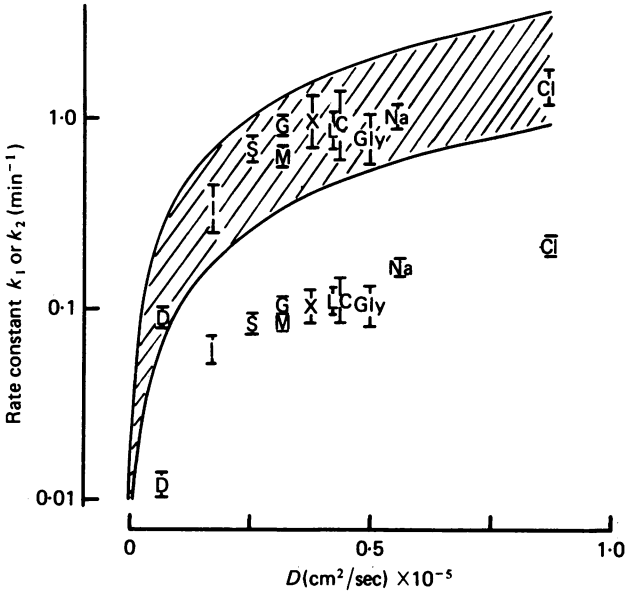

Fig. 6. The observed rate constants for components A and B as a function of the diffusion coefficient for all compounds studied at 0 °C. Data from Tables 1–3. Points between curves are observed rate constants (k_1) for the A component. Points below the curves are the observed rate constants (k_2) for the B component. Upper curve: theoretical value for the quantity $\pi^2 D/\lambda^2 r^2$ for $r = 0{\cdot}25$, $\lambda = 1{\cdot}5$. Lower curve: theoretical value for the same quantity with $r = 0{\cdot}5$, $\lambda = 1{\cdot}5$. Rate constants taken from data in Tables 1–3. Diffusion coefficients were taken from the *Handbook of Chemistry and Physics* (Weast, 1969) for sucrose, glucose, glycine, Na, and Cl. For other compounds D was extrapolated from the molecular weight (Ling & Kromash, 1967). D, [^{14}C]dextran; I, [^{14}C]inulin; S, [^{14}C] and [^{3}H]sucrose; G, [^{14}C]glucose; M, 3-*O*-methyl[^{14}C]glucose; X, [^{14}C]xylose; L, [^{14}C]leucine; C, [^{14}C]cycloleucine; Gly, [^{14}C]glycine; Na, ^{22}Na; Cl, ^{36}Cl. Where more than one value was obtained for the same compound at 0 °C the results were averaged.

The effect of temperature on the efflux rate constant for the A component was that expected for diffusion in aqueous solution. This can be seen most clearly from the [^{14}C]dextran experiment shown in Table 3. Here a group of muscles of approximately the same size and from the same batch of frogs were washed at 0 and 25 °C. The increase in temperature produced a twofold increase in the rate of dextran efflux from compartment A. The expected ratio R would be 2·2 as calculated from the relationship

$$R = \frac{\eta_o}{\eta_t}\frac{T_t}{T_o}, \tag{7}$$

where η_0 and η_t are the viscosity of water at 0 °C and at temperature t and T_0 and T_t are the respective temperatures in degrees Kelvin. A similar trend can be seen in the [^{3}H]sucrose data from this experiment as well as the ^{22}Na data in Table 1. In other cases this temperature effect was probably obscured by the variability in muscle thickness. In any case the rate constants are consistent with the notion that component A represents efflux from the extracellular space proper.

The problem of the origin of component B. The observed rate constants for the B component (k_2) are also shown in Fig. 6. In every case the observed rate constant fell substantially below the expected range of variation for extracellular diffusion. The rate constants do appear to be a function of the diffusion coefficient, however. Thus the compartment of origin of component B must allow diffusional exchange of solute with the extracellular compartment, but clearly does not itself represent unrestricted extracellular space. The size of the B component, equivalent to about 10 % of the fibre volume, is close to the value of 9·1 % obtained by Mobley & Eisenberg (1975) using stereological techniques to estimate the volume of the sarcoplasmic reticulum. Its osmotic characteristics, as well as the efflux rate, are similar to results obtained by Rogus & Zierler (1973) for an analogous component in rat extensor digitorum longus muscle.

Efflux of ^{22}Na and [^{14}C]sucrose from single muscle fibres

If component B arises from the sarcoplasmic reticulum, it should be present in efflux curves from single fibres and its characteristics should resemble those found in the whole muscle studies, e.g. the fraction should increase in size in hypertonic solution and the rate constant should be proportional to the diffusion coefficient of the substance in question.

The technique we evolved allows solute efflux from a segment of fibre to be studied under conditions which exclude the tendons from the washout process. We found this to be absolutely essential if accurate assessment of the early time points was to be obtained. Fig. 7 shows ten of the twenty-four efflux curves for ^{22}Na and [^{14}C]-sucrose obtained from single fibres. Three curves were similar to ^{22}Na curve 77154 which shows a single exponential time course from 1·5 min onward. Most curves resembled ^{22}Na curve 77126 or [^{14}C]sucrose curve 77112 in which there was a small rapid or B component complete after 10 min. Only in the case of [^{14}C]sucrose efflux from two fibres, 77192 and 77165, both shown in Fig. 7, was the distribution space for the B component larger than 3 % of the fibre volume. In these cases the distribution spaces were 10·8 and 4·6 %, respectively. In most other fibres the space ranged between 1·5 and 2·5 %.

The results are summarized in Table 5. For ^{22}Na we tried several variations of the technique in order to ascertain that neither the degree of stretch on the fibre nor the chamber design altered the characteristics of the efflux curves. There was no significant difference between the distribution spaces for components A, B, or C in either of the two chambers (see Methods) nor in stretched (sarcomere length 2·8 μm) or slack (sarcomere length 2·2 μm) fibres. The amount of adhering solution (component A) varied widely, averaging 20 % of fibre volume. The C component had an average distribution space of 6 % which corresponds to a cellular sodium concentration of about 9 mM. This is not much different from the intracellular Na content of whole

muscles (Table 2) and suggests that these single fibres have normal ionic permeability properties.

All but one of the [^{14}C]sucrose curves were obtained in the S-chamber. The average distribution space of the *A* component was 44 %, larger than that for ^{22}Na. That for the *B* component was 2·2 % not significantly different from the *B* component for ^{22}Na. The *C* component probably represents cellular accumulation of an isotopic

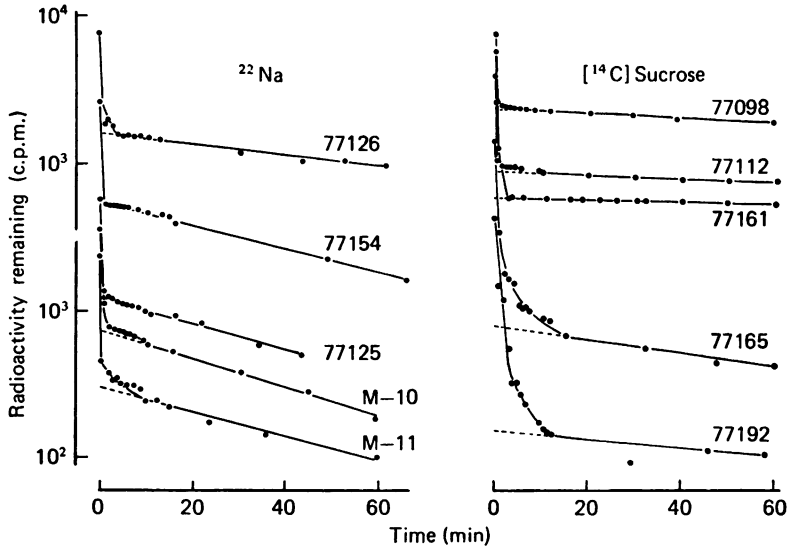

Fig. 7. ^{22}Na and [^{14}C]sucrose washout from single fibres. Fibres were dissected, loaded and washed as described in methods. Fibres 77126, 77112, 77125 were stretched to a sarcomere length of 2·8–2·9 μm in the S-chamber; fibres 77154, 77161 and 77165 were loaded and washed in the S-chamber at slack length (sarcomere spacing, 2·1–2·3 μm). Fibres M-10, M-11 and 77192 were loaded and washed at slack length in the P-chamber. Fibre 77098 was stretched to a sarcomere length of 2·8 μm in the S-chamber and loaded and washed in Ringer solution made hypertonic by the addition of 200 mM-sucrose. Fibres were loaded at room temperature to hasten equilibration, but washed at 0 °C to decrease the rate of efflux from the cellular compartment.

TABLE 5. Components of efflux curves from single muscle fibres. Values are expressed as mean ± S.E. of the mean if results from three or more fibres were averaged

Isotope	Chamber	Sarcomere length (μm)	Number	Distribution space (%) *A*	*B*	*C*	Rate constant (min^{-1}) *B*
^{22}Na	S	2·8–2·9	5	17·2 ± 2·1	1·6 ± 0·6	5·1 ± 0·7	
	S	2·1–2·3	3	27·8 ± 8·3	1·2 ± 0·7	6·1 ± 0·8	
	P	2·1–2·3	4	15·9 ± 2·7	1·7 ± 0·2	6·2 ± 0·6	
^{22}Na	Over-all average		12	20·3 ± 3·2	1·5 ± 0·3	5·9 ± 0·4	0·24 ± 0·04
[^{14}C]sucrose	Over-all average		11	44·7 ± 8·5	2·2 ± 1·0	7·8 ± 3·0	0·26 ± 0·07
[^{14}C]sucrose	In hypertonic solution		1	5·4	1·2	14·8	0·23

contaminant. This fraction became somewhat larger after the isotope had been stored for a period of time. Because of the necessity for using very high isotope concentrations in substrate-free solutions, any contaminating sucrose metabolite, e.g. glucose, would tend to be accumulated by the fibre.

The origin of the *B* component in these single fibres is uncertain. There are a number of reasons for thinking that it is not the sarcoplasmic reticulum. First, it is too small, having a distribution space of about 2 % rather than the 7–11 % seen in our whole muscle studies and expected on morphological grounds (Mobley & Eisenberg, 1975) if compartment *B* is the sarcoplasmic reticulum. Secondly, the rate constant for efflux, about 0·25 min^{-1}, was similar for both Na and sucrose. In whole muscles the ^{22}Na rate constant was significantly faster than that for sucrose. Finally, one fibre (77098) was loaded with [^{14}C]sucrose and washed in Ringer solution made hypertonic

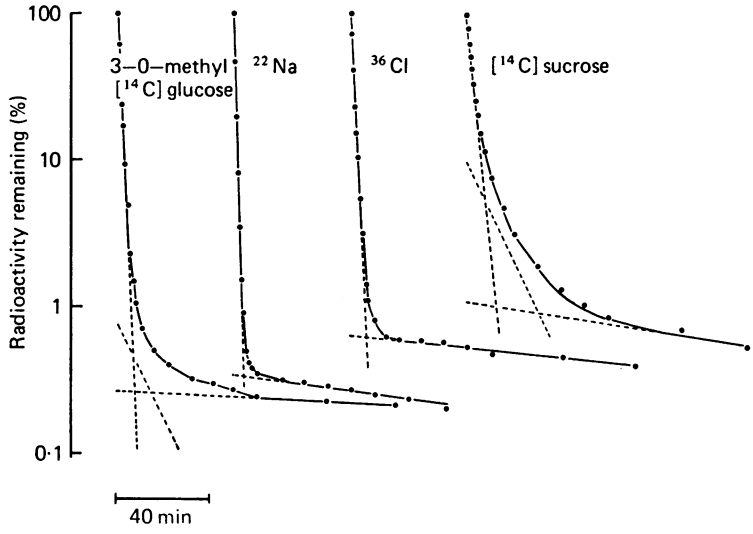

Fig. 8. Solute efflux from connective tissue. Pieces of fascia from the legs of large *Rana pipiens* were incubated overnight at 0 °C with the isotopes shown. The isotopes were washed out at 0 °C using the technique described in Methods for sartorius muscles. The results are plotted as per cent initial radioactivity remaining. The ^{22}Na and ^{36}Cl curves could be resolved into the two components shown (interrupted lines). The 3-*O*-methylglucose and sucrose curves were best fit by the three component curves shown (interrupted lines).

by the addition of 200 mM-sucrose. The distribution space for the *B* component remained small, 1·2 %, suggesting that this *B* component in single fibres does not have the same osmotic characteristics as the *B* component in whole muscles. For all these reason the data on single muscle fibres lead to the conclusion that component *B* in the efflux curves from whole muscle is physically located in the extracellular space rather than within the muscle fibres.

Solute efflux from connective tissue

In order to determine whether component *B* resulted from anomalous efflux of of solute from the connective tissue elements of the muscle, pieces of connective tissue taken from leg fascia were loaded with isotope by overnight incubation and

washed out using the same technique as for sartorius muscles. Fig. 8 shows some typical results for 3-*O*-methylglucose, ^{22}Na, ^{36}Cl and [^{14}C]sucrose. In some cases, as for the Na and Cl curves shown here the efflux curve had only two components, a slow component comprising less than 1 % of the radioactivity in the tissue and a fast component with a rate constant of the order of 0·3–0·8 min^{-1}, probably representing pure diffusion-limited efflux from the extracellular compartment. This compartment had a distribution space between 70 and 80 % of the tissue volume. Some samples (see the 3-*O*-methylglucose and sucrose curves in Fig. 8) had an intermediate component with a rate constant of about 0·08 min^{-1}. The distribution space of this component was highly variable, values as small as 1 % of the tissue volume and as high as 20 % being observed for sucrose.

These results suggest that the majority of solute in the connective tissue space of muscle is freely diffusible and that the connective tissue *per se* does not give rise to the *B* component in solute efflux from whole muscle.

DISCUSSION

The curves for the efflux of a large series of solutes from frog sartorius muscle were found to possess a component too slow to represent solute loss from the extracellular space and too fast to represent loss of cellular solute. The distribution space of this component was about 10 % for all solutes examined, a value similar to morphological estimates of the size of the sarcoplasmic reticulum (Peachey, 1965; Mobley & Eisenberg, 1975). Rogus & Zierler (1973) interpreted a similar component in the curves for the efflux of Na from rat extensor digitorum longus muscle as indicating that the sarcoplasmic reticulum is freely accessible to extracellular solute.

An intermediate component has often been observed in efflux curves from frog muscle. Harris (1963) found such a component in data for Cl efflux. Ling (1966, 1970) and Sjödin & Beauge (1973) reported it in sucrose efflux curves. Kirby *et al.* (1975) observed two intermediate components in their curves for Ca efflux. In mammalian muscle, in addition to the report of Rogus & Zierler, Case, Creese, Dixon, Massey & Taylor (1977) reported an intermediate component in the efflux of decamethonium from rat diaphragm. Thus it appears that an intermediate component is a consistent and widespread finding for solute efflux from muscle.

The data presented in this paper show that this component must arise from a compartment equally accessible to all substances of molecular weight 17,000 daltons or lower. The rate constants for this component suggest that access to this compartment, termed compartment *B* for convenience, is gained by a diffusive process. Is compartment *B* the sarcoplasmic reticulum? If so, our results show that the sarcoplasmic reticulum must contain fluid similar in composition to the extracellular fluid and the T-SR junction must allow relatively free passage of solute from T-tubule to terminal cisterna. If not, our results are consistent with other findings (Somlyo *et al.* 1977 *a*, *b*) indicating that the fluid in the SR is similar to intracellular fluid with respect to its concentration of the major monovalent cations. Three lines of evidence emerge from the work in this paper which allow a definitive answer to be given to this question.

First, the data presented here show that compartment *B* is accessible not only to small molecules, but to larger substances such as inulin (mol. wt. 5000) and dextran

(mol. wt. 17,000). Most studies in the literature suggest that substances which can be shown morphologically to enter the T-tubules are excluded from the sarcoplasmic reticulum. Thus, in an early light microscopic study of single muscle fibres, Endo (1966) showed that the dye, lissamine rhodamine B200 (mol. wt. 440) was confined to the region of the T-tubules and had a distribution space of 1–2 % of the fibre volume. This was too small to represent even the terminal cisternae of the muscle fibres. Hill (1964) made a similar observation for serum albumin. At the electron microscope level, La (Revel & Karnovsky, 1967) and horseradish peroxidase (Eisenberg & Eisenberg, 1968; Peachey & Franzini-Armstrong, 1977) are usually confined to the T-tubule system. Some apparent exceptions have appeared in the literature especially when the tissue has been treated with hypertonic solutions (Rubio & Sperelakis, 1972; Sperelakis, Valle, Orozco, Martinez-Paloma & Rubio, 1973; Kulczycky & Mainwood, 1972.) The possibility that these anomalous results are related to hypertonic sucrose treatment or to staining artifacts has not been ruled out. Thus, our finding that large molecules consistently gain entry to compartment *B*, suggests that this compartment is not the sarcoplasmic reticulum.

The second critical observation is that no efflux component comparable in size to that seen in the whole muscle studies was present in efflux curves from single fibres. The origin of the small *B* component in the single fibre curves is unknown. It may be significant that the fibres were not entirely devoid of connective tissue elements, including capillaries. (Attempts to remove these elements with collagenase invariably left us with fibres which were too fragile to survive the washing procedure.) In any case the size of component *B* in single fibres was not increased by hypertonic solutions and the rate constants for sucrose and sodium were similar. All these observations suggest that the *B* component in the curves for solute efflux from whole muscle arises from an extrafibrillar space.

A third test of the hypothesis is provided by an analysis of the conductance between compartment *B* and the extracellular space. It has been shown that the capacitance of frog skeletal muscle arises primarily from the plasma and T-tubule membranes, the extensive membranes of the sarcoplasmic reticulum making an insignificant contribution (see Appendix). In the Appendix, Mathias shows that this is possible only if the conductance of the T-SR junction is less than $0{\cdot}06 \times 10^{-3}$ mho/cm^2 of fibre membrane.

In order to make the argument clear, let us, for the moment, accept as valid the hypothesis that compartment *B* is the sarcoplasmic reticulum. The conductance of the T-SR junction could then be calculated from the flux of the major current-carrying species, Na, K and Cl, across this junction, i.e. from the rate constants for efflux of these ions from compartment *B*.

The calculation is as follows: The flux M_n (in mole.sec^{-1}.cm^2) of ion n across the junction is calculated from

$$M_n = k_2.[X_n]_o/60.H, \tag{8}$$

where k_2 is the effiux rate constant from compartment *B* as before and $[X_n]_o$ is the extracellular concentration of ion n, equal to its concentration in compartment *B*. *H* is the plasma membrane area per ml. muscle water, estimated to be 560 cm^2 if the fibres have an average diameter of 50 μm. The factor 60 converts k_2 from minutes to

seconds. The results obtained are given in Table 6. The fluxes are converted to specific ion conductances, G_n, using the expression, valid for equilibrium conditions only (Hodgkin, 1951):

$$G_n = z_n^2 F^2 M_n / RT, \quad (9)$$

where z_n is the valence of ionic species n and F, R, and T are the Faraday, the gas constant and the absolute temperature, respectively. The specific ion conductances are also given in Table 6 in mho per cm^2 plasma membrane.

TABLE 6. Conductances between compartment B and the extracellular space. Conductances were calculated from the ionic flux using eqn. (9). The total conductance represents the sum of the three specific ion conductances shown. The rate constants were taken from Table 2 (0 °C data) for Na, from Table 3 for Cl. For K, the 0 °C rate constant for component B was calculated from the 25 °C rate constant in Table 3 making the assumption that the decrease in temperature decreased the efflux rate by a factor of 2·2 (see eqn. 7).

Ion	Rate constant (k_2) $(min)^{-1}$	Flux $(mole.sec^{-1}.cm^{-2})$	Conductance $(mho.cm^{-2})$
K	0·06	$4{\cdot}0 \times 10^{-12}$	$1{\cdot}5 \times 10^{-5}$
Na	0·15	$4{\cdot}7 \times 10^{-10}$	$1{\cdot}8 \times 10^{-3}$
Cl	0·28	$9{\cdot}3 \times 10^{-10}$	$3{\cdot}6 \times 10^{-3}$
		Total conductance	$5{\cdot}4 \times 10^{-3}$

From the results in Table 6 it can be seen that movement of Na and Cl would provide most of the total calculated conductance of $5{\cdot}4 \times 10^{-3}$ mho/cm^2 outer membrane. This value is nearly 70 times larger than the limiting value calculated in the Appendix for the conductance of the T-SR junction of $0{\cdot}08 \times 10^{-3}$ mho/cm^2. *Thus the hypothesis that compartment B is the sarcoplasmic reticulum must be rejected.*

Our results further suggest that extracellular species do not gain ready access to the sarcoplasmic reticulum across the T-SR junction. It is important to note that this conclusion may not be valid for Ca ion. Kirby *et al.* (1975) found that there are two intermediate components in the calcium efflux curves from sartorius muscle. They found that these components persist when the experiment is carried out on single fibres suggesting that calcium fluxes may have to be interpreted differently from those of other molecules. Indeed, the autoradiographic studies of Winegrad (1970) suggest that Ca may cross the T-SR junction rather rapidly.

The question of the anatomical location of compartment B remains. There appear to be three possibilities. (1) The connective tissue near the fibres or in some other region may impede flux in such a way as to produce a second compartment. Against this interpretation is the finding that connective tissue itself possesses only a small and inconsistent compartment B (Fig. 8). (2) There may be a population of dead or damaged fibres whose membranes have lost their selective permeability. Against this interpretation is the relatively constant size of compartment B and the fact that it can be found in muscles loaded *in vivo* (Neville & White, 1979). (3) The vascular space may provide a compartment with the properties of compartment B. Although estimates of the volume of the vasculature of frog muscle are generally smaller than the observed distribution space for compartment B (Boyle *et al.* 1941) it is possible that the vascular spaces swell *in vitro* due to retained plasma proteins. However, the

fact that the distribution space for compartment B for muscles loaded *in vivo* is similar to that for muscles loaded *in vitro* (Neville & White, 1979), suggests that this does not happen. A new approach will be necessary to solve this problem.

When the compartmental distributions of Na and Cl derived from the efflux experiments in whole muscle (Table 2) are considered, it is clear that the majority of both these ions is extracellular. The C or slow component must be composed of solute in both sarcoplasmic and sarcoplasmic reticulum; its size thus sets an upper limit to the amount of these or any other solute within the sarcoplasmic reticulum. Because it is likely that both the 5 μmole/g muscle of intracellular Na and the 1–2 μmole/g muscle of intracellular Cl are largely contained in the sarcoplasmic compartment (see succeeding paper for further discussion of this point), the data presented here are consistent with the conclusion of Somlyo *et al.* (1977*a*, *b*) that ionic composition of the fluid within the sarcoplasmic reticulum is similar to that of the sarcoplasm.

The author would like to express her most sincere appreciation to J. Walsh, S. White and K. Mauer for their competent assistance with the efflux experiments and to P. Hamilton for invaluable help in developing the techniques used with single fibres. A. R. Martin and R. S. Eisenberg provided valuable discussion of the manuscript. This work was supported by N.I.H. grant AM-15807. The author is recipient of Research Career Development Award 1K04-00038.

APPENDIX

An analysis of the consequences of electrical continuity between the T-system and sarcoplasmic reticulum

By R. T. MATHIAS

The electrical data from frog skeletal muscle are well described by models in which the sarcoplasmic reticulum is not considered an extracellular space, (Mathias, Eisenberg & Valdosiera, 1977; Schneider & Chandler (1976). While this finding does not preclude an electrical connexion between the T-system and sarcoplasmic reticulum, it does place some constraints on the properties of such an hypothetical channel. The purpose of this Appendix is to investigate the conditions under which micro-electrode or voltage clamp experiments could induce substantial current flow into the sarcoplasmic reticulum without noticeably altering the linear electrical parameters from skeletal muscle.

Fig. 9 is a lumped equivalent circuit of a muscle fibre in which there is a conductive channel, represented by g_x, connecting the lumen of the T-system and the interior of the sarcoplasmic reticulum. (A lumped equivalent circuit may not be a valid model of a muscle fibre under all conditions of interest (see Mathias *et al.* 1977 Fig. 4 and the related discussion). The conductance and capacitance of the membranes which delineate the compartments of interest in a fibre are represented by (g_m, c_m) for the sarcolemma, (g_w, c_w) for the T-system walls, and (g_{SR}, c_{SR}) for the sarcoplasmic reticulum membranes. The lumen of the T-system, because of its long path length and small cross-sectional area, provides a significant resistance to radial current flow which is modeled by the conductance g_L. The surface areas of the membrane systems associated with a unit area of fibre surface are approximately:

1 cm² for the sarcolemma, 5 cm² for the T-system walls, and 50 cm² for the membranes of the sarcoplasmic reticulum (Peachey, 1965; Mobley & Eisenberg, 1975). The capacitances c_m, c_w and c_{SR} should have approximately the same respective values in microfarads assuming a capacitance of about 1 μF /cm² for a typical biomembrane. The conductance of a biomembrane is a much more variable quantity and we can not realistically assume relative values for g_m, g_w and g_{SR}. The total input or effective conductance of a fibre has been experimentally measured, however, so we can numerically bound the appropriate combination of all the conductances in Fig. 9.

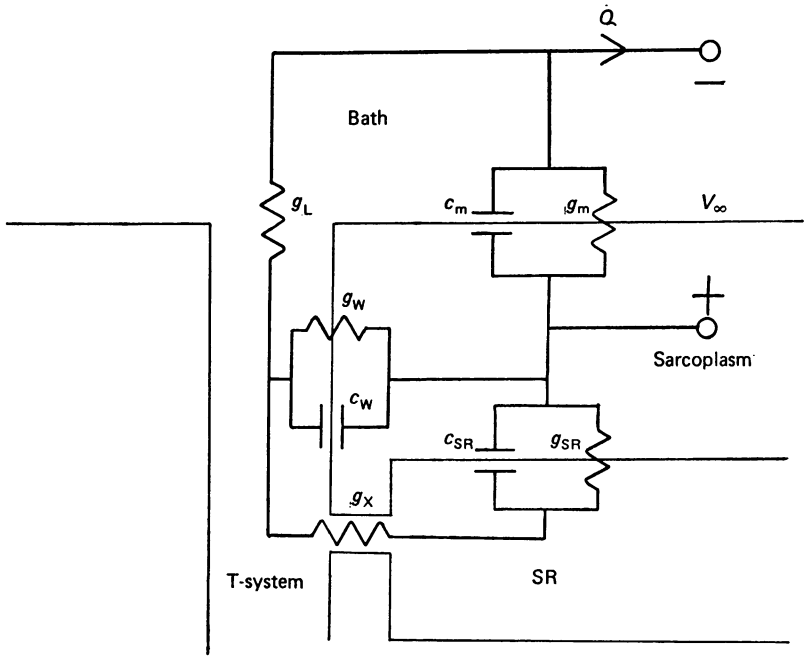

Fig. 9. A lumped circuit representation of a muscle fibre. The circuit elements are referred to a unit area of outer surface of the fibre. The conductance g_x represents an hypothetical channel between the T-system and sarcoplasmic reticulum (SR). The other circuit elements represent structures which are known to exist and, with the exception of (g_{SR}, c_{SR}) have been measured.

If the transient current following as step change in potential across the sarcolemma is integrated, we obtain the total charge deposited on all fibre membranes. When this charge is divided by the potential change, one obtains an effective capacitance. The value of effective capacitance obtained in this manner is model independent and so any proposed model must be consistent with this value. It was shown by Adrian, Almers and Chandler in an Appendix to Adrian & Almers (1974), that the total charge or effective capacitance could be related to the fibre admittance by

$$V_\infty \frac{dy(s)}{ds}\bigg|_{s=0} = V_\infty C_{eff} = Q, \qquad (1a)$$

where s is the Laplace transform variable, $y(s)$ is the Laplace transform representation of the fibre admittance and V_∞ is the amplitude of the step change in potential

across the sarcolemma. The Laplace transformed admittance of the circuit in Fig. 9 is

$$y(s) = g_m + sc_m + g_L \frac{g_x(g_{SR}+sc_{SR}) + (g_w+sc_w)(g_x+g_{SR}+sc_{SR})}{g_x(g_{SR}+sc_{SR}) + (g_L+g_w+sc_w)(g_x+g_{SR}+sc_{SR})}. \quad (2a)$$

Since extracellular markers freely enter the T-system but do not cross into the sarcoplasmic reticulum or the sarcoplasm (Costantin, 1975; Nakajima & Bastian, 1976) the conductances g_x and g_w must be much smaller than g_L, so that

$$g_L + g_w \simeq g_L, \quad (3a)$$

and

$$g_L + g_x \simeq g_L, \quad (4a)$$

The approximations in (3*a*) and (4*a*) allow a simple approximate evaluation of the effective capacitance for the circuit in Fig. 9.

$$c_{eff} \simeq c_m + c_w + c_{SR}\left(\frac{g_x}{g_x+g_{SR}}\right)^2. \quad (5a)$$

Another model independent parameter which has been experimentally measured in skeletal muscle is the effective conductance of a fibre. The effective conductance for the circuit in Fig. 9 is obtained by setting $s = 0$ in eqn. (1*a*). If we again use the approximations given in (3*a*) and (4*a*), then

$$g_{eff} \simeq g_m + g_w + \frac{g_x g_{SR}}{g_x+g_{SR}}. \quad (6a)$$

The effective capacitance measured in muscle fibres is about 7 μF/cm^2 of outer surface area (Adrian & Almers, 1974; Schneider & Chandler, 1976; Mathias *et al.* 1977). This number is consistent with the morphometrically measured values of the surface areas of the T-system and sarcolemma, and is inconsistent with the 50 μF/cm^2 associated with the large surface of the sarcoplasmic reticulum. Thus, the expression for c_{eff} in eqn. (5*a*) must not admit values of g_x and g_{SR} which produce a significant contribution from c_{SR}. Since glycerol shock removes about 40% of c_{eff} (Chandler, Rakowski & Schneider, 1976), if we make the limiting assumption that all of the removed capacitance is due to the sarcoplasmic reticulum, we can use the above capacitance numbers to bound the ratio $g_x/(g_x+g_{SR})$ and, therefore, bound g_x.

$$g_x < 0{\cdot}3\, g_{SR}. \quad (7a)$$

Eqn. (5*a*) is now consistent, within experimental error, with the measured values of c_{eff}. Since we have no idea of the value of g_{SR}, this result alone places no real constraints on g_x.

Eisenberg & Gage (1969) experimentally measured the quantity $(g_{eff} - g_m) \simeq 0{\cdot}06$ mmho/cm^2 by first measuring g_{eff} then detubulating the fibre with glycerol and measuring g_m. If we use their result and also make the limiting assumption that g_w is negligible compared to the ratio $g_x g_{SR}/(g_x+g_{SR})$, then eqn. (6*a*) and (7*a*) give

$$g_x < 0{\cdot}08 \text{ m-mho/cm}^2. \quad (8a)$$

In the text Neville calculates the conductance between compartment *B* and the bathing solution and finds 5 mmhos/cm^2. This number is much too large to represent g_x, particularly since the resting conductance almost certainly depends on g_w and so

(8*a*) is a conservative bound. We can therefore conclude from this analysis that compartment *B* is not the sarcoplasmic reticulum; however, the possibility remains that a channel such as g_x may exist.

In view of the recent findings of Somlyo (1978), and Rakowski (1978) it is important to describe the general consequences of this analysis. An electrical connexion may exist between the T-system and sarcoplasmic reticulum without noticeably altering the measured linear electrical parameters of a muscle fibre if (1) it has a resting conductance which is less than the membrane conductance of the sarcoplasmic reticulum (see eqn. (5*a*)), and (2) it has a resting conductance which is consistent with resting conductance measured for the fibre (see eqn. (8*a*)). We may also infer something about the voltage induced in the sarcoplasmic reticulum; if g_x is less than 30 % of g_{SR}, the voltage induced across the reticular membranes will be less than 30 % of the T-system voltage. In order to account for the known sensitivity of the Ca release, either the voltage sensitivity of the sarcoplasmic reticulum is unusually high or else g_x must have voltage dependence. Non-linear versions of Fig. 9 have been considered in computer simulations; however, our ignorance of the mechanism of Ca release from the sarcoplasmic reticulum precludes the simple calculations and conclusions which can be made in a linear analysis.

This work was partially supported by N.I.H. grant HL20230. I am grateful to my colleagues, R. Eisenberg and R. Levis, for their perceptive comments and their collaboration in the formulation and computing of non-linear extensions of this work.

REFERENCES

ADRIAN, R. H. (1961). Internal chloride concentration and chloride efflux of frog muscle. *J. Physiol.* **156**, 623–632.

ADRIAN, R. H. & ALMERS, W. (1974). Membrane capacity measurements on frog skeletal muscle in media of low ion content. *J. Physiol.* **237**, 573–605.

BIRKS, R. I. (1964). The sarcoplasmic reticulum of twitch fibres in the frog sartorius muscle. In *Muscle*, ed. PAUL, N. E., DANIEL, E. E., KAY, C. M. & MONKTON, G., pp. 199–215. Oxford: Pergamon.

BIRKS, R. I. & DAVEY, D. F. (1969). Osmotic responses demonstrating the extracellular character of the sarcoplasmic reticulum. *J. Physiol.* **202**, 171–188.

BLINKS, J. R. (1965). Influence of osmotic strength on cross-section and volume of isolated single muscle fibres. *J. Physiol.* **177**, 42–57.

BOYLE, P. J., CONWAY, E. J., KANE, F. & O'REILLY, H. J. (1941). The volume of interfibre spaces in frog muscle and the calculation of concentrations in the fibre water. *J. Physiol.* **99**, 401–414.

BRAY, G. (1960). A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Analyt. Biochem.* **1**, 279–285.

CHANDLER, W. K., RAKOWSKI, R. F. & SCHNEIDER, M. F. (1976). Effects of glycerol treatment and maintained depolarization on charge movement in skeletal muscle. *J. Physiol.* **254**, 285–316.

CASE, R., CREESE, R., DIXON, W. J., MASSEY, F. J. & TAYLOR, D. B. (1977). Movement of labelled decamethonium in muscle fibres of the rat. *J. Physiol.* **272**, 283–294.

COSTANTIN, L. L. (1975). Contractile activation in skeletal muscle. *Prog. Biophys. molec. Biol.* **29**, 197–224.

DELL, R. B., SCIACCA, R., LIEBERMAN, K., CASE, D. B. & CANNON, P. J. (1973). A weighted least square technique for the analysis of kinetic data and its application to the study of renal ^{133}Xenon washout in dogs and man. *Circulation Res.* **32**, 71–84.

DESMEDT, J. E. (1953). Electrical activity and intracellular sodium concentration in frog muscle. *J. Physiol.* **121**, 191–205.

DYDYNSKA, M. & WILKIE, D. R. (1963). The osmotic properties of striated muscle fibres in hypertonic solutions. *J. Physiol.* **169**, 312–329.

EISENBERG, B. & EISENBERG, R. S. (1968). Selective disruption of the sarcotubular system in frog sartorius muscle. *J. cell Biol.* **39**, 451–467.

EISENBERG, R. S. & GAGE, P. W. (1969). Ionic conductances of the surface and transverse tubular membranes of frog sartorius fibres. *J. gen. Physiol.* **53**, 279–297.

ENDO, M. (1966). Entry of fluorescent dyes into the sarcotubular system of frog muscle. *J. Physiol.* **185**, 224–238.

HARRIS, E. J. (1963). Distribution and movement of muscle chloride. *J. Physiol.* **166**, 87–109.

HARRIS, E. J. & BURN, G. P. (1949). The transfer of sodium and potassium ion between muscle and surrounding medium. *Trans. Faraday Soc.* **45**, 508–528.

HILL, D. K. (1964). The space available to albumin within the striated muscle fibre of the toad. *J. Physiol.* **175**, 275–294.

HODGKIN, A. L. (1951). The ionic basis of electrical activity in nerve and muscle. *Biol. Rev.* **26**, 339–409.

HODGKIN, A. L. & HOROWICZ, P. (1959). Movement of Na and K in single muscle fibres. *J. Physiol.* **145**, 405–432.

HUXLEY, A. (1960). Appendix 2 to Solomon, A. K., Compartmental Methods of Kinetic Analysis. In *Mineral Metabolism*, ed. COMIR, C. L. & BONNER, F., pp. 163–167. New York: Academic.

JOHNSON, J. A. (1955). Kinetics of release of radioactive sodium, sulfate and sucrose from the frog sartorius muscle. *Am. J. Physiol.* **181**, 263–268.

JOST, W. (1960). *Diffusion*, p. 6. New York: Academic.

KIRBY, A. C., LINDLEY, B. D. & PICKEN, J. R. (1975). Calcium content and exchange in frog skeletal muscle. *J. Physiol.* **253**, 37–52.

KULCZYCKY, S. & MAINWOOD, G. W. (1972). Evidence for a functional connection between the sarcoplasmic reticulum and the extracellular space in frog sartorius muscle. *Can. J. Physiol. Pharmacol.* **50**, 87–98.

LING, G. N. (1966). Cell membrane and cell permeability. *Ann. N.Y. Acad. Sci.* **137**, 837–859.

LING, G. N. (1970). Studies on ion permeability. I. What determines the rate of Na ion efflux from frog muscle cells. *Physiol. Chem. & Phys.* **2**, 242–248.

LING, G. N. & KROMASH, M. H. (1967). The extracellular space of voluntary muscle tissues. *J. gen. Physiol.* **50**, 677–694.

MACDONALD, R. L., MANN, J. E. & SPERELAKIS, N. (1974). Derivation of general equation describing tracer diffusion in any two-compartment tissue with application to ionic diffusion in any two-compartment tissue with application to ionic diffusion in cylindrical muscle bundles. *J. theor. Biol.* **45**, 107–130.

MARQUARDT, D. W. (1963). An algorithm for least-squares estimation of non-linear parameters. *J. Soc. ind. appl. Math.* **11**, 431–441.

MATHIAS, R. J., EISENBERG, R. S. & VALDOSIERA, R. (1977). Electrical properties of frog skeletal muscle interpreted with a mesh model of the tubular system. *Biophys. J.* **17**, 57–93.

MOBLEY, B. A. & EISENBERG, B. R. (1975). Sizes of components in frog skeletal muscle measured by methods of stereology. *J. gen. Physiol.* **66**, 31–45.

NAKAJIMA, S. & BASTIAN, J. (1976). Membrane properties of the transverse tubular system in amphibian skeletal muscle. In *Electrobiology of Nerve, Synapses, and Muscle*. London: Raven.

NARAHARA, H. T. & OZAND, P. (1963). Studies of tissue permeability. IX. The effect of insulin on the penetration of 3-methylglucose in frog muscle. *J. biol. Chem.* **238**, 40–49.

NEVILLE, M. C. (1972). Solute concentration gradients in frog muscle at 0 °C: Active transport or adsorption? *Science, N.Y.* **176**, 302–303.

NEVILLE, M. C. (1977). Evidence for the extracellular composition of the fluid in the sarcoplasmic reticulum of frog sartorius muscle. *Biophys. J.* **17**, 7*a*.

NEVILLE, M. C. (1978). The extracellular spaces of frog muscle. *Biophys. J.* **21**, 185*a*.

NEVILLE, M. C. & WHITE, S. (1979). Extracellular space of frog skeletal muscle *in vivo* and *in vitro*: relation to proton magnetic resonance relaxation times. *J. Physiol.* **288**, 71–83.

PEACHEY, L. D. (1965). The sarcoplasmic reticulum and transverse tubules of the frog's sartorius. *J. cell Biol.* **25**, 209–231.

PEACHEY, L. D. & FRANZINI-ARMSTRONG, C. (1977). Direct visualization of the network nature of the t-system of frog skeletal muscle and its surface connection by high voltage electron microscopy. *Biophys. J.* **17**, 160*a*.

RAKOWSKI, R. R. (1978). Recovery of linear capacitance in skeletal muscle fibres. *Biophys. J.* **21**, 167*a*.

REVEL, J. P. & KARNOVSKY, M. J. (1967). Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J. cell Biol.* **33**, C7-C12.

ROGUS, E. & ZIERLER, K. (1973). Sodium and water contents of sarcoplasm and sarcoplasmic reticulum in rat skeletal muscle: effects of anisotonic media, ouabain and external sodium. *J. Physiol.* **233**, 227–270.

RUBIO, R. & SPERELAKIS, N. (1972). Penetration of horse radish peroxidase into the terminal cisternae of frog skeletal muscle fibres and blockade of caffeine contracture by Ca^{2+} depletion. *Z. Zellforsch.* **124**, 57–71.

SCHNEIDER, M. F. & CHANDLER, W. K. (1976). Effects of membrane potential on the capacitance of skeletal muscle fibers. *J. gen. Physiol.* **67**, 125–163.

SJÖDIN, R. A. & BEAUGE, L. A. (1973). Analysis of leakage of sodium ions into and potassium ions out of striated muscle cells. *J. gen. Physiol.* **61**, 222–250.

SOMLYO, A. V. (1978). Membrane bridges across the triadic gap after tannic acid fixation. *Biophys. J.* **21**, 56*a*.

SOMLYO, A. V., SHUMAN, H. & SOMLYO, A. P. (1977*a*). Composition of sarcoplasmic reticulum *in situ* by electron probe X-ray microanalysis. *Nature, Lond.* **11**, 556–557.

SOMLYO, A. V., SHUMAN, H. & SOMLYO, A. P. (1977*b*). Elemental distribution in striated muscle and the effects of hypertonicity. Electron probe analysis of cryo sections. *J. cell Biol.* **74**, 828–857.

SPERELAKIS, N., SHIGENOBU, K. & RUBIO, R. (1978). ^{3}H-sucrose compartments in frog skeletal muscle relative to sarcoplasmic reticulum. *Am. J. Physiol.* **234**, *c* 181–*C*190.

SPERELAKIS, N., VALLE, R., OROZCO, C., MARTINEZ-PALOMA, A. & RUBIO, R. (1973). Electromechanical uncoupling of frog skeletal muscle by possible change in sarcoplasmic reticulum content. *Am. J. Physiol.* **225**, 793–800.

TASKER, P., SIMON, S. E., JOHNSTONE, B. M., SHANKLEY, K. H. & SHAW, F. H. (1959). The dimensions of the extracellular space in sartorius muscle. *J. gen. Physiol.* **43**, 39–53.

VINOGRADOVA, N. A. (1967). The distribution of inulin, sucrose and fructose in frog sartorius muscle. *Tsitologia* **9**, 781–790.

VINOGRADOVA, N. A. (1968). Distribution of non-penetrating sugars in the frog's sartorius muscle under hypo- and hypertonic conditions. *Tsitologia* **10**, 831–838.

VINOGRADOVA, N. A., NIKOLSKY, N. N. & TROSHIN, A. S. (1967). Distribution of sugars in the frog sartorius muscle. *Tsitologia* **9**, 658–665.

WEAST, R. C. (1969). In *Handbook of Chemistry and Physics*, 50th edn., pp. F-47, D-12. Cleveland: Chemical Rubber Co.

WINEGRAD, S. (1970). The intracellular site of calcium activation of contraction in frog skeletal muscle. *J. gen. Physiol.* **55**, 77–88.