Calcium Efflux from Frog Twitch Muscle Fibers

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ABSTRACT An apparatus is described which collects the effluent from the center 0.7 cm of a single muscle fiber or bundle of muscle fibers. It was used to study the efflux of ^{45}Ca from twitch muscle fibers. The efflux can be described by three time constants 18 ± 2 min, 300 ± 40 min, and 882 ± 172 min. These kinetics have been interpreted as those of a three-compartment system. The fastest is thought to be on the surface membrane of the muscle and of the T system. It contains 0.07 ± 0.03 mM Ca/liter of fiber and the Ca efflux is 0.11 ± 0.04 pM Ca/cm^2. sec. The intermediate rate compartment is thought to represent the Ca in the longitudinal reticulum. It contains approximately 0.77 mM Ca/liter. Only the efflux from this compartment increases during stimulation. The most slowly exchanging compartment is poorly defined. Neither Ca-free nor Ni-Ringer solutions alter the rate of loss from the fastest exchanging compartment. Ni apparently alters the rate of loss from the slowest compartment.

From all the available evidence, intracellular calcium has been assigned the role of the activator of the actomyosin contractile system (Podolsky, 1965). This control system has been studied by a variety of methods; the efflux of radiocalcium should provide data on an increased Ca efflux with stimulation and on Ca compartments. Previous studies on calcium efflux (Shanes and Bianchi, 1959; Gilbert and Fenn, 1957), done with sartorius muscles, were difficult to interpret because of efflux from extracellular space and tendon. These difficulties also made study during the early phase of efflux impossible. This paper describes a method for collecting the effluent from the center 0.7 cm of a muscle fiber or bundle. This method eliminates many problems of interpretation encountered in earlier studies. Three distinct Ca compartments are described.

METHODS

Muscle Fibers Single or small bundles of twitch fibers were dissected from the semitendinosus muscle of *Rana pipiens*. The width of the single fibers was measured at 10 places along its length and the average was considered to be the diameter for flux calculations. For the bundles which contained a known number of fibers (three-seven) the individual fibers were considered to have an average diameter of 75 μ which is

the average diameter of the single fibers used in this study and in a previous one (Curtis, 1966).

Solutions The standard Ringer solution had a pH of 7.0–7.2 and contained (in mM): NaCl, 116; KCl, 2.5; $CaCl_2$, 1.0, Na_2HPO_4, 2.5; NaH_2PO_4, 0.85. Other solutions were obtained by replacement and will be noted in the text. The radioactive ^{45}Ca solution was made up as previously described (Curtis, 1966).

The Experimental Chamber The efflux chamber is similar in principle to an apparatus described by Hodgkin and Keynes (1955), and is shown in Fig. 1. The

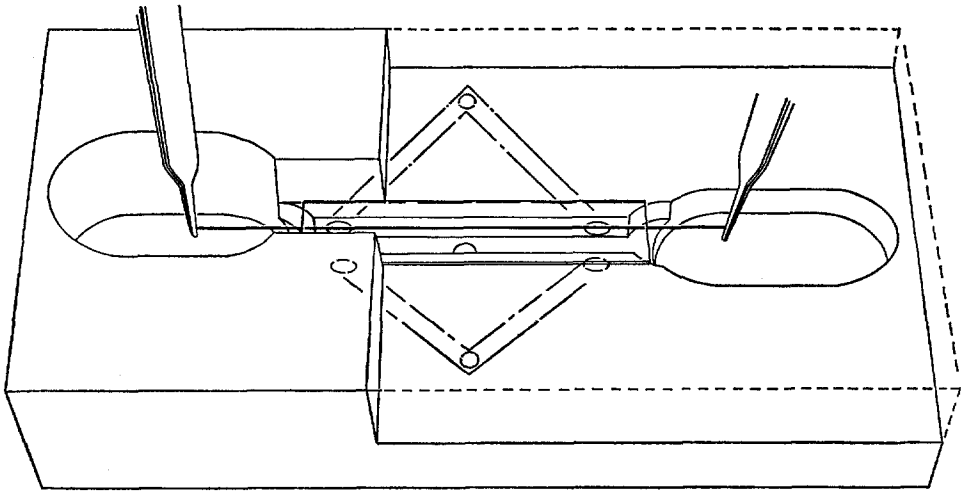

FIGURE 1. A diagrammatic view of the efflux chamber. A single fiber is shown stretched between the forceps. The trough in which the fiber runs is known as the collecting trough. The trough is enclosed by the glass slide on the top. Ringer solution flows in through the four diagonal channels. At the junctions with the collecting trough the flow divides. Most of it flows out into the end pools and carries off the ^{45}Ca from the tendons. About 5% flows through the collecting trough and down the center hole onto a planchet. It carries the effluent from the center of the muscle fiber with it. In this way the efflux from the center of the fiber is separated from the efflux from the tendons.

muscle bundle is lowered into the collecting trough and a cover glass is placed above it to create an enclosed chamber. Ringer solution flows into this chamber via the four diagonal channels or inlet jets. 95% of the flow goes into the large end chambers, is aspirated, and discarded. The other 5% (0.5 ml/min) flows past the center of the fiber, and is collected on a planchet via the hole in the floor of the chamber.

The chamber has been tested in the following ways: (*a*) When a solution containing 10^6 cpm is added to the bottom of either end pool, the counting rate of the solution collected via the center hole is never above background. Therefore, radioactivity from the tendons will not be collected. (*b*) When a fine polyethylene tube is substituted for the muscle bundle, and ^{45}Ca forced out of a side hole in it, the radioactivity is collected only when the side hole is inside the inlet jets. When the hole is moved

200 μ at the inlet jets, a change in the counting rate of the planchets from background to full activity results. This shows that the length of the fiber in the collecting gap is constant and is 6.95 mm. (*c*) When the solution flowing through the inlet jets is suddenly changed from radioactive to nonradioactive, the radioactivity of the collected samples falls to 1% of the initial value in 20 sec and to 0.01% in 60 sec. It is clear that the cell will collect the effluent from the bundle very quickly and this delay is of no significance in relation to the usual 10 min collecting interval. (*d*) When the chambers and procedure were tested for possible damage to the fiber and general reproducibility by carrying out the influx: efflux procedure a second time, the two time constants for efflux were not significantly different. Clearly the fiber is not damaged by the cell.

General Procedure After the fiber or bundle had been dissected, it was tested for excitability by observing the presence of a propagated twitch and its diameter or the number of fibers measured. It was immediately transferred to the influx chamber. About one-half the fluid in the influx trough was removed and replaced with ^{45}Ca-Ringer. The fiber was never stuck to glass or plastic or exposed to an air-Ringer interface. After the influx period (3–4 hr) a sample of the influx solution was obtained and the influx chamber was flooded with cold Ringer. The fiber or bundle was checked visually for damage and tested for its ability to respond to electrical stimulation with a propagated twitch.

The bundle was transferred under Ringer solution to the efflux chamber and lowered into position in the collecting trough. The cover glass was placed above it and the first collection interval of 10 min begun. There were stimulating electrodes in the efflux chamber so that the fiber could be checked for excitability. One tendon was often attached to a force transducer rather than to the forceps shown in Fig. 1. The radioactivity on the planchets was assayed on a low background counter. The counting rate was corrected for background ($\sim$2.5 cpm) and divided by the collection interval. At the end of the experiment, the center 1 cm of the fiber was cut out and assayed in a similar manner.

RESULTS

Resting Efflux A typical efflux curve is shown in Fig. 2. It is a plot of the log of the counts per minute per minute lost by the fiber vs. time. When the values before 60 min are neglected for the moment, the points from 60 min onwards can be adequately described by a single straight line, fitted by the method of least squares, with a time constant of 218 min. This almost certainly represents ^{45}Ca coming out of a single compartment within the muscle fiber. ^{45}Ca has presumably been coming out of this compartment at the same time constant since the bundle was removed from the influx solution. The points before 60 min contain, therefore, radioactivity coming from this compartment. The line is extrapolated to zero and subtracted from each of the data points to produce the open circles. These points fall on a straight line with a time constant of 17 min.

At the end of the experiment, the center of the fiber was cut out, dissolved,

and counted. The final rate constant, the final efflux per minute divided by the fiber-counting rate, is 825 × 10^{-6} min^{-1}. The reciprocal is 1215 min which is much larger than the time constant fitted to the data points; therefore, there must be another ^{45}Ca compartment or compartments within the fibers. This t_c is so slow that it does not materially alter the earlier values.

We have evidence, therefore, for three time constants of efflux. The simplest model compatible with this data is a three-compartment one. This model will be assumed for the rest of the paper.

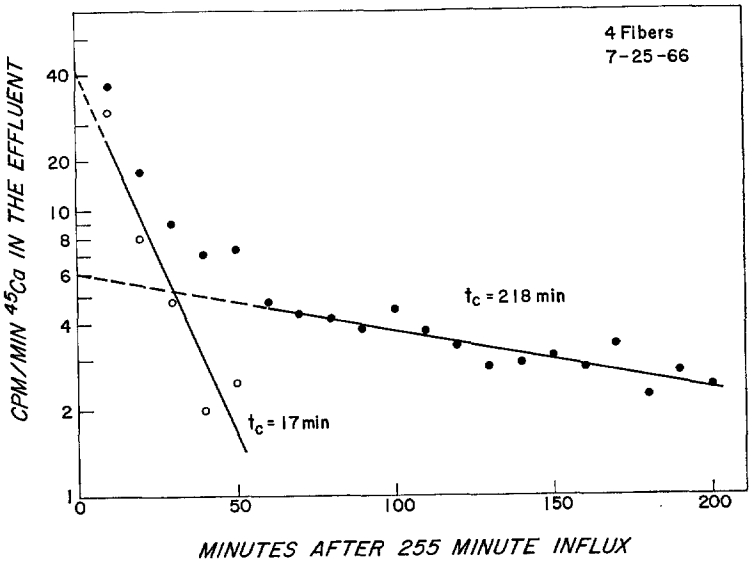

FIGURE 2. Efflux of ^{45}Ca into normal Ringer solution. The open circles are obtained by extrapolating the 218 min time constant line back to zero (dashed line) and subtracting it from the earlier solid circles. The lines are fitted by the method of least squares. Note that the vertical axis is the rate of loss of radioactivity from the bundle. The specific activity of the influx solution was 1.94 × 10^{-14} moles Ca/cpm. The reciprocal of the final rate constant is 1215 min.

Fig. 3 shows the ^{45}Ca efflux from a single fiber. Since it shows the same pattern of efflux as seen in Fig. 2, it is clear that this pattern is a property of the muscle fiber and not of the small extracellular space between the small bundles of muscle fibers. Consequently, the results from single fibers and small bundles (three–seven fibers) will be used interchangeably. The bulk of the results are from bundles of three–five fibers.

The compartment with the fastest time constant has reached isotopic equilibrium during the influx phase since the influx time is greater than five times the average time constant of *18 ± 2 min* ($\bar{X} \pm s/\sqrt{n}$) (21 muscles). The size of the compartment can be determined by graphic analysis of a plot of the

reconstructed counts per minute ^{45}Ca within the fiber. A simpler method is obtained from a time differential of the standard equation for loss of radioactivity, $P = P_o e^{-kt}$: $dp/dt = -kP_o e^{-kt}$. If this is evaluated at zero time one obtains $dp/dt_o = -kP_o$. Since the plot seen in Fig. 2 is of dP/dt, i.e. the rate of loss, the zero intercept of the line formed by the circles gives dp/dt_o and the reciprocal of the time constant gives k. It is well to note that the rate constants

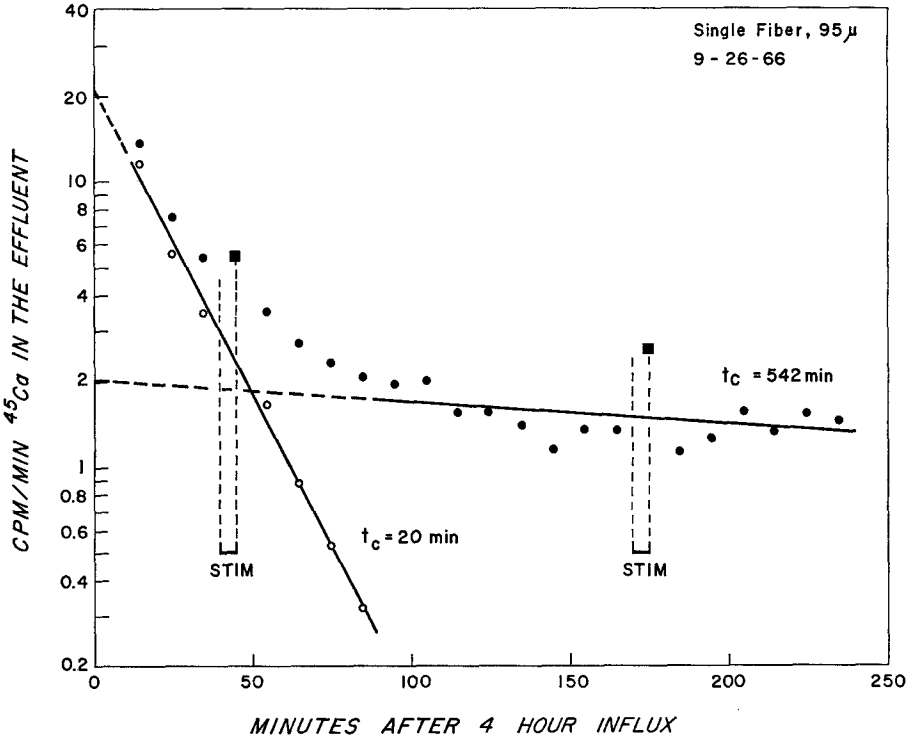

FIGURE 3. The effect of stimulation on ^{45}Ca efflux. The lines were obtained in the same manner as in Fig. 2. The reciprocal of the final time constant is 20,800 min. The fiber was stimulated twice at 40/sec for 5 min. The specific activity of the influx solution was 6.4×10^{-15} moles Ca/cpm. The first compartment contained 0.14 mM Ca/liter fiber. The Ca efflux from the first compartment was 0.06 pM Ca/cm² sec.

are the same whether determined from the plot of dP/dt or from the counting rate of the whole fiber. The method used here is much more sensitive to changes in rate and is a plot of the original data. The first or fast compartment has a size of 0.07 ± 0.03 mM Ca/liter of fiber or 4% of the total muscle Ca. The rate of loss of ^{45}Ca from this compartment is 0.11 ± 0.04 pM Ca/cm².sec.

The second compartment has an average time constant of 300 ± 40 min (22 muscles). Since this compartment has not reached isotopic equilibrium,

determining its size is more difficult and subject to greater error. One method is to take the value (0.84 mM/liter of fiber) given for total exchangeable Ca as determined from short influx periods (Curtis, 1966) and subtract the known value (0.07 mM/liter) for the size of the first or fast compartment to obtain a value of 0.77 mM/liter of the total muscle Ca in this compartment. A second method is to estimate the specific activity within this compartment on the basis of the equation $P = P_\infty (1 - e^{-t/\tau})$, which does not take backflux into account. Then the size of the compartment can be determined from $dP/dt_o = kP_o$. While this method is time-consuming and not very accurate, it provides a useful check on the previous estimate. The value obtained in this manner is 0.87 mM Ca/liter which is in good agreement.

The time constant of the third, or slow compartment, is obtained from the reciprocal of the final rate constant. This establishes the existence of a third compartment or compartments, but very little more can be determined. The average value is 882 ± 172 min (11 muscles).

Stimulated Efflux The effect of electrical stimulation on the ^{45}Ca efflux is shown in Fig. 3. When the fiber is stimulated at 40/min for 5 min after 160 min of efflux, the rate of loss increases *1.75*-fold. The average value for nine muscles is *2.51* ± *0.78*-fold increase. Since the slowest compartment has very little ^{45}Ca in it the extra efflux must come from the intermediate compartment.

When the stimulus occurs at 40 min the rate also increases, but there is a question as to which compartment releases the additional Ca, the fast or the intermediate. If the extra efflux is coming from the second compartment, then the ordinary efflux from the fast compartment must be subtracted before a percent increase can be calculated. In this case the increase is 1.1-fold, which is scarcely significant. The average value, however, obtained by this method is 1.74 ± 0.29 (eight muscles) fold. Since the two average values are not statistically different, it is concluded that the fast compartment does not contribute any extra efflux during stimulation.

Effects of Ca Removal After two or three 10 min collection intervals, 1 mM EDTA was substituted for the $CaCl_2$ in the Ringer for the next three 10 min collection intervals; then normal Ringer was restored. The points representing the first time constant were obtained by subtraction as before. A straight line, fitted to the points in normal Ringer solution, had a time constant of 17.8 ± 1.1 min, which is not significantly longer than the average time constant. When the EDTA points were compared to this line, the first EDTA point was 723 ± 2.9% greater than expected. This probably represents ^{45}Ca coming from the "sticky principle" referred to in an earlier paper (Curtis, 1966). While the per cent increase in the efflux is large, it represents a tiny fraction of the total ^{45}Ca in the bundle. The second and third points of the

EDTA treatment were 106 ± 6 and 99 ± 8% of the expected value. The second time constant following the EDTA exposure is normal, 462 ± 108 min.

It is clear that ^{45}Ca continues to leave the first compartment at the same rate as before; the Ca-free condition does not increase the rate. Since there is neither Ca nor any other divalent ion in the external solution, it is clear that the calcium in this compartment is not being replaced and it is very likely the Ca loss from this compartment which brings about the very rapid alteration in membrane functions associated with Ca-free solution (Frank, 1960; Curtis, 1963; Jenden and Reger, 1963).

In a few experiments the introduction of 1 mM EDTA-Ringer during the second time constant period had no obvious effect on the rate of ^{45}Ca loss.

The Effect of Nickel Ions on the Efflux of Calcium

It has been shown that the addition of nickel and other divalent ions to Ringer solution prevents the mechanically refractory state which occurs in calcium-free solution (Frank, 1960; Curtis, 1963; Fishman and Swan, 1967). It seemed of interest to examine the effect of these ions on Ca efflux.

When the calcium in Ringer solution is replaced by nickel during the time when the first compartment is being washed out (the first hour of the efflux), the rate of calcium efflux does not change markedly. The points all fall on a straight line whether they come from ^{45}Ca efflux into nickel-ringer or into Ringer following the Ni-Ringer. The average time constant for the first compartment is, however, slightly decreased and has an average value of 13.6 ± 1.4 min. The average for controls is 18.0 ± 2 min.

The first compartment is assumed to represent a Ca fraction bound to the sarcolemmal and T system membranes and probably largely involved in membrane stability. The presence of nickel in the Ringer solution does not prevent loss of Ca ions. With a time constant of 14 min all the calcium would be lost from this compartment within an hour, yet muscle continues to function and to maintain a resting potential for many hours in nickel-Ringer (Fishman and Swan, 1967). It is only after 5 or 6 hr that muscle function begins to fade and then not as a result of falling resting potential (Jenden and Reger, 1963). It would appear that this membrane function is not specific for Ca, but that Ca may be replaced by a number of divalent ions.

After an exposure to nickel-Ringer early in the washout, the second time constant is significantly reduced; with an average of 177 ± 60 min. The reciprocal of the final time constant is 1147 min; not significantly different from the average.

When the nickel is applied during the washout phase of the second compartment (Fig. 4), that is from 2 to 3 hrs, there is a gradual linear increase in calcium efflux with time. After the nickel is removed and replaced by

calcium, the efflux remains higher than expected and is described by a straight line on semilogarithmic plots. This new straight line has much the same time constant as the initial second compartment did. This can be seen very nicely in Fig. 4.

There are three possible interpretations of the slow increase in calcium efflux when the nickel is applied to the muscle. (*a*) The rate of total exchange may be increasing as both influx and efflux from the second compartment

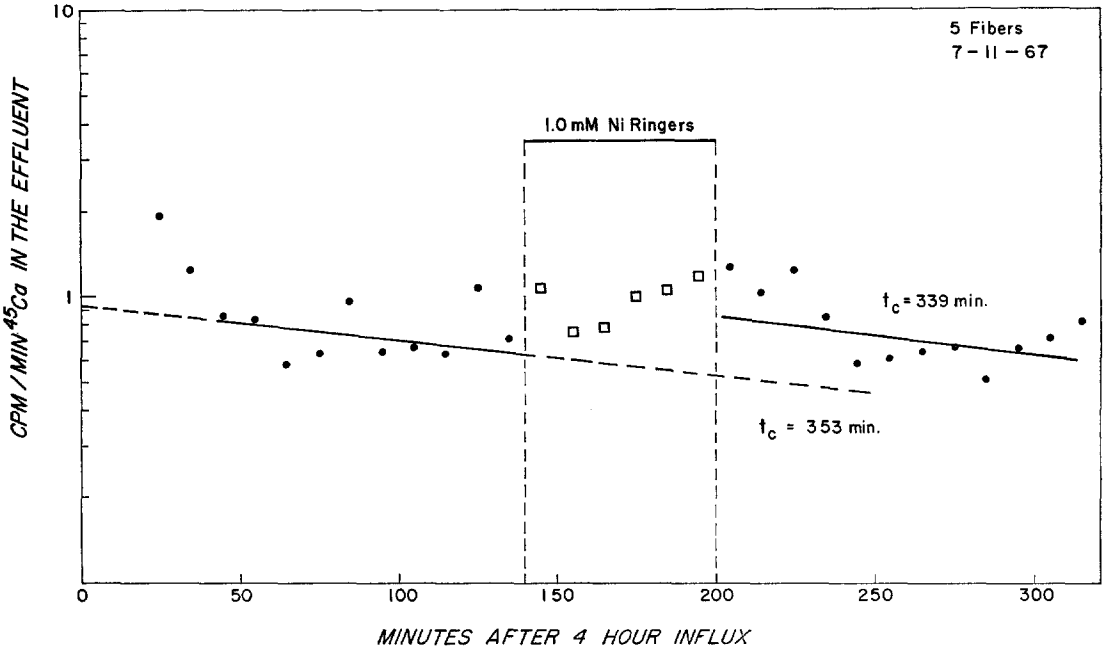

FIGURE 4. The effect of nickel ions on the efflux of ^{45}Ca. The lines were fitted as described in the text and in Fig. 2. Note that during the application of Ni-Ringer the efflux increases and afterwards it declines at much the same time constant as before but at a higher level.

increase. (*b*) There may be an increase in efflux rate. (*c*) The specific activity of the compartment may be increased by the influx of more radiocalcium from the third, and normally much less exchangeable, compartment. This would lead to an increased counting rate at a constant efflux rate. This third alternative is supported by the points obtained after the return to calcium-Ringer solution. These subsequent points are described by a second straight line which has the same time constant as the first portion of the efflux from the second compartment. This argues for an increase in the specific activity of the second compartment. The same sort of response could be gained by putting the muscle back into hot calcium for a short influx. Nickel ions apparently increase the turnover of the calcium in the third compartment.

DISCUSSION

In this paper evidence has been presented for three Ca compartments within the muscle fiber. An earlier paper (Curtis, 1966) described by one exponential the efflux of ^{45}Ca from a single fiber, after a short efflux. These data were obtained by counting the whole fiber, not the effluent as was done in this paper. When the average values for compartment size and time constant found in this present work are used to calculate the counting rate of a fiber after a short influx, the points, plotted semilogarithmically, describe a slightly curved line. When these points were fitted by the method of least squares, the time constant was 77 min and the scatter was not excessive. This value is clearly very close to the average value (125 min) found in the previous paper. The multicompartmental nature of the efflux was not observed previously because of the error inherent in counting the whole fiber and the short counting period.

The finding of more than one compartment does not invalidate any of the previous findings since the efflux was used primarily to estimate the counting rate at zero time. If anything, the total influx will be underestimated.

The total influx calculated in the previous paper was 0.26 pM Ca/cm² sec. Since the fibers are in a steady state at 1.0 mM Ca (Gilbert and Fenn, 1957), the influx must be equal to the efflux. The efflux from the first compartment is 0.11 pM Ca/cm² sec. The second compartment must have an efflux very near 0.15 pM Ca/cm² sec. since the third compartment has such a slow turnover. The efflux from the third compartment can be estimated as 0.007 pM Ca/cm² sec and can be neglected.

An efflux time constant of 123 min can be calculated using 0.15 pM Ca/cm². sec for efflux from the second compartment, the Ca content of the second compartment (0.74 mM/liter), and a 60 μ fiber diameter. This compares favorably with an experimental time constant of 300 $\pm$ 40 min for the second compartment. Since the values for efflux and content have been obtained from such different sources, this seems to be quite good agreement.

The three compartments described in this paper are in good agreement with the radioautographic data of Winegrad (1968). After a 5 min soak in 2 mM ^{45}Ca he found the grains localized along the cell border and over the Z line. A 5 min influx of ^{45}Ca would fill the fast compartment to 24% of its capacity ($1 - e^{-5/18}$) and the intermediate compartment to 2% of its capacity ($1 - e^{-5/300}$). Since the volumes of these compartments are 0.07 and 0.80 mM Ca/liter of fiber, they would exchange 0.02 mM Ca/liter and 0.02 mM Ca/liter after 5 min. The total of 0.05 μM/g of Ca exchanged ($1.23 \times (0.02 + 0.02)$) at 1 mM Ca in the Ringer is in good agreement with the 0.08 μM/g calculated by Winegrad.

The compartment with the fastest time constant is thought to be located on the surface of the fiber and on the walls of the transverse reticulum. This

compartment is not well-localized by radioautography due to its small size. The function of the calcium in this compartment is probably membrane stability and it can be replaced by other divalent cations. The calcium content of this compartment is much larger than the Ca content within the transverse tubular system which would contain approximately 0.003 mM Ca/liter of fiber.

After a 5 hr influx, the calcium in the fast compartment is fully exchanged; 0.07 mM Ca/liter. The second compartment is 63% exchanged; 0.50 mM/liter. The slow compartment is 29% exchanged; approximately 0.24 mM/liter. The sum is 0.81 mM Ca/liter or 1.00 μM/g. Once again this is in good agreement with the value calculated by Winegrad (1968) of 0.80 μM/g after a 5 hr influx.

The compartment of intermediate time constant is the one of greatest interest by virtue of its large size and increased turnover with stimulation. It is clear from the localization experiments of Winegrad (1968) that this fraction is localized in the sarcoplasmic reticulum. The sarcoplasmic reticulum has a volume of 13% of the total muscle (Peachey, 1965). Consequently, the true Ca concentration must be about 6 mM/liter since the previous figures were given for the total muscle volume. Since this is a fairly high concentration, it seems reasonable to assume that the Ca is bound in some form. It is clear from the radioautographs taken after tetanus that the sarcoplasmic reticulum is not a single compartment but a series of at least two compartments. They appear to act as a single compartment for resting exchange; probably at the terminal cisternae, transverse reticulum boundary.

The compartment with the very long time constant probably does not represent a single Ca fraction but many intracellular stores. Calcium bound to actin and myosin is almost certainly one of them. The grains over the A band are probably partly from this compartment. Since the exchange rate of this compartment is so slow, the total exchangeable Ca cannot be estimated. Consequently, no statement can be made about nonexchangeable calcium within the muscle fiber.

The data on time constants for exchange and compartment size from this paper and other papers are presented in Table I. The values for concentration in mM/kg were obtained by multiplying the values in mM/liter by

TABLE I

	Fast		Medium		Slow
	T_c	Size	T_c	Size	T_c
	min	*mM/kg*	*min*	*mM/kg*	*min*
Gilbert and Fenn (1957)	—	—	300	0.67	—
Shanes and Bianchi (1959)	3	0.13	500	0.85	—
This paper	18	0.08	300	0.77	2600

1.23 (Hodgkin and Horowicz, 1959). It is clear that the results of these three papers are in gratifying agreement with respect to the medium rate compartment. The slow compartment is not described in the first two papers, but is considered as unexchangeable Ca. The volume and time constants for the fast compartment shown by Shanes and Bianchi are much less reliable because of the multifiber method used; washout from the extracellular space obscures this efflux.

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