

# The Time Course of the Loss and Recovery of Contracture Ability in Frog Striated Muscle Following Exposure to Ca-Free Solutions

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**ABSTRACT** Using area under the contracture curve to quantitate contractures, the diffusion coefficient of calcium ions within the frog toe muscle during washout in a calcium-free solution and subsequent recovery after reintroduction of calcium to the bathing solution was calculated to be about  $2 \times 10^{-6}$  cm<sup>2</sup>/sec. The diffusion coefficient measured during washout was found to be independent of temperature or initial calcium ion concentration. During recovery it was found to decrease if the temperature was lowered. This was likely due to the repolarization occurring after the depolarizing effect of the calcium-free solution. The relation between contracture area and  $[Ca]_o$  was found to be useful over a wider range than that between maximum tension and  $[Ca]_o$ . The normalized contracture areas were larger at lower calcium concentrations if the contractures were produced with cold potassium solutions or if NO<sub>3</sub> replaced Cl in the bathing solutions. Decreasing the potassium concentration of the contracture solution to 50 mM from 115 mM did not change the relation between  $[Ca]_o$  and the normalized area. If the K concentration of the bathing solution was increased, the areas were decreased at lower concentrations of Ca.

## INTRODUCTION

The necessity for the presence of calcium ions for the production of potassium contractures was firmly established by Frank (1958, 1960). It was shown that washing the small toe muscle of the frog in a calcium-free solution reversibly abolished the ability to produce potassium contractures provided that active responses were prevented by the substitution of choline for sodium ions in the bathing solution. Frank calculated from the time course of the loss of the contracture ability during the washout that the calcium ions diffused from the muscle with an apparent diffusion coefficient of the same order of magni-

tude as that for self-diffusion of calcium ions in free solution. Because the muscle was still able to produce caffeine contractures after the ability to produce potassium contractures had been abolished, it was concluded that the contractile "machinery" was intact and therefore the removal of calcium ions interfered only with excitation-contraction coupling.

If the study of coupling in contractures can be considered relevant to the coupling after normal excitation it seems reasonable to conclude that the calcium required comes, in part at least, from a common source. If the calcium responsible for excitation-contraction coupling in the twitch is extracellular it must be present in the transverse tubules, since there is insufficient time for it to diffuse from the sarcolemma surface to account for the rapidity of the establishment of the active state (Hill, 1949).

Because diffusion to and from this restricted space might be expected to be slower than from the extracellular space between the muscle fibers, it was decided to determine the diffusion coefficient of this "washout" calcium using a more accurate, though still indirect method, than Frank used. The temperature and concentration sensitivity of the time course of both the loss of contracture ability during a wash in calcium-free solution and the subsequent recovery on replacement of calcium were also studied since physical processes are relatively insensitive to these changes.

#### METHODS

*General Experimental Procedure* Toe muscles were obtained from grass frogs, *Rana pipiens*, of both sexes. Experiments were done year-round and best results were obtained when the animals were kept at 3–4°C until 24 hours before use, when they were warmed to 20°C.

The muscles, extensor longus digiti IV, were dissected with the aid of a binocular microscope. Often a distinct band of fibers could be distinguished on the medial border of the muscle and these were removed, when possible, since this tended to reduce the amount of response attributed to "slow" fibers. The major and minor diameters of the muscles at their *in situ* length were measured in three places using a calibrated scale in the microscope eyepiece.

The muscle was mounted in a vertical position between a strain gauge and a rigid No. 14 gauge platinum wire using No. 50 mercerized surgical cotton thread. The muscle was stretched to approximately *in situ* length by adjusting the initial tension on it to about  $\frac{1}{4}$  gm with a rack and pinion attached to the strain gauge.

Contracture tensions were measured by the deflections of a thin phosphorus bronze strip with SR-4 strain gauges mounted on each side to form the two active arms of a Wheatstone bridge. The voltage changes were recorded with a Grass polygraph (Grass Instruments, Quincy, Mass.)

The tip of the strip moved about 0.85 mm for each gram of tension applied so that the contractures, which were usually  $\frac{1}{2}$  to 1 gm peak tension, resulted in less than 5 per cent shortening of the muscle and can be regarded as isometric in nature. In-

vestigations of isotonic contractures with two muscles using a kymograph gave similar results to those reported here.

The area under the contracture curve was measured either electronically with a Tektronix type O operational amplifier (Tektronix Inc., Portland, Oregon) or manually with a planimeter.

Bathing solutions, contained in 30 ml glass beakers, were changed manually in less than 5 seconds by removing the beaker and substituting another.

The room temperature was controlled at either 18° or 20°C.

*Bathing Solution Composition* The composition of the various bathing solutions is given in Table I. All the solutions had the following common characteristics. (a) The  $[K] \times [Cl]$  product was about 300 mm<sup>2</sup>. This prevents swelling due to entry of

TABLE I  
RECIPE FOR SELECTED SOLUTIONS

	Tris	HCl	Methane sulfonic acid	KCl	K methane sulfonate	Ca gluconate	CaCl <sub>2</sub>
	mM/liter	mM/liter	mM/liter	mM/liter	mM/liter	mM/liter	mM/lite
Altered Ca							
0 × Ca	127	114		2.6			
1 × Ca	127	114		2.6		1.8	
16 × Ca	78	59.5	11.5	2.5			28.8
Increased K							
2 × K	124	55	57	5.0		*	
8 × K	104		93.5	15.0	5.0	*	
Contracture solution							
116 K	6.3		5.7	2.6	114	‡	
50 K	73.8		66.4	6.0	44	‡	

\* The appropriate amount of Ca-gluconate was added to give the desired concentration.

‡ 1.8 mM Ca-gluconate if Ca is present.

KCl when the potassium concentration is increased (Boyle and Conway, 1941). (b) The large, impermeant anions used to replace Cl in elevated K solutions (gluconate and methane sulfonate) do not precipitate Ca ions in the concentrations used and since they are monovalent, the substitution did not change the ionic strength of the solutions. (c) Na ion in the solutions was replaced with 90 per cent neutralized tris [Sigma brand of purified tris(hydroxymethyl)aminomethane]. This substance does not support active responses, and therefore twitching of the muscle at low concentrations of Ca ion is prevented. In addition it served as a buffer to keep the pH at about 7.2. (d) The osmolality of all solutions was 230 mosm ± 10 per cent.

Methane sulfonate and tris were shown to be functionally non-permeating by noting the permanence of the weight loss induced by placing frog sartorius muscles in hypertonic solutions composed of either 120 mM Na methane sulfonate and 120 mM K methane sulfonate or 120 mM NaCl and 240 mosm tris Cl. A similar method was used by Hutter and Noble (1960) to show that methyl sulfate was non-permeating.

Special precautions were taken to ensure that the Ca-free solutions contained mini-

mal Ca due to contamination (Frank, 1958, 1960). Deionized water having a conductivity of less than 0.1 ppm NaCl from a Bantam Demineralizer (Barnstead Still Co., Boston, Mass.) was used to rinse all glassware 3 to 6 times and to make all solutions. Analysis of the calcium-free solutions by the EDTA-calcein calcium method (Appleton *et al.*, 1959), as modified by Dr. Ellis Benson of the University of Minnesota Hospitals, showed that there was less than 18  $\mu\text{M}$ /liter calcium present.

*Measurement of Membrane Potentials* Transmembrane potentials were measured with glass pipettes filled with 3 M KCl. Blunt, stiff pipettes with a resistance of 7 to 12 megohms were the most satisfactory. Reversible Ag-AgCl electrodes prepared after the method of Brown (1934) were used to attach the pipette to a Biotronics (Bioelectric Instruments, Hastings-on-Hudson, New York) preamplifier and to connect the bathing solution to ground *via* a Biotronics low impedance calibration unit.

The muscle was tied at rest length between two stainless steel pegs in a gently sloping groove in a lucite block. A well at the lower end of the chamber with a hole in its bottom allowed complete replacement of solution within the groove in less than 5 sec. as measured by dye washout.

Rapid, consecutive, impalements of different individual muscle fibers were made over a 6 mm length of muscle using a micromanipulator. The resting potentials were displayed on a Tektronix 502 oscilloscope (Tektronix Inc., Portland, Oregon) and photographed on continuously moving film with a Grass camera (Grass Instruments, Quincy, Mass.). When reading the film only penetrations which gave step changes in potential and which were stable over the period of impalement (usually 1 to 2 seconds) were used.

*Method of Calculating D* The diffusion coefficient,  $D$ , may be calculated for a particular substance and system, provided the concentration of that substance is known within the system as a function of time and distance. With this information the experimental findings can be related to an appropriate theoretical model which gives  $D$  as a function of these variables. It was not possible to measure calcium concentrations in this way in the toe muscle and an indirect method similar to that followed by Hill and Macpherson (1954) was used.

The muscle was allowed to equilibrate with several different concentrations of calcium ion and a potassium contracture was produced after each equilibration period. The area under each contracture curve was measured and compared to that obtained at the highest calcium concentration. This normalized value of the area,  $\Phi$ , thus obtained was then plotted as function of calcium concentration.

The theoretical model chosen to approximate the frog muscle system was an infinitely long uniform circular cylinder with an initial uniform concentration within it at the start of the diffusion period and a constant concentration outside it at all times. The toe muscle approximates the model fairly well. However, the cross-section is not a circle but is a non-uniform ellipse. Since the product of the major and minor radii of this elliptical cross-section is almost constant over the length of the muscle, this value averaged from three places was used for  $r^2$  in the theoretical model. The values of the product fell between 1.5 and  $7.0 \times 10^{-4}$  cm<sup>2</sup>. The other conditions were met by allowing adequate equilibration periods, by keeping the volume of the external solution much greater than the volume of the muscle, and by stirring.

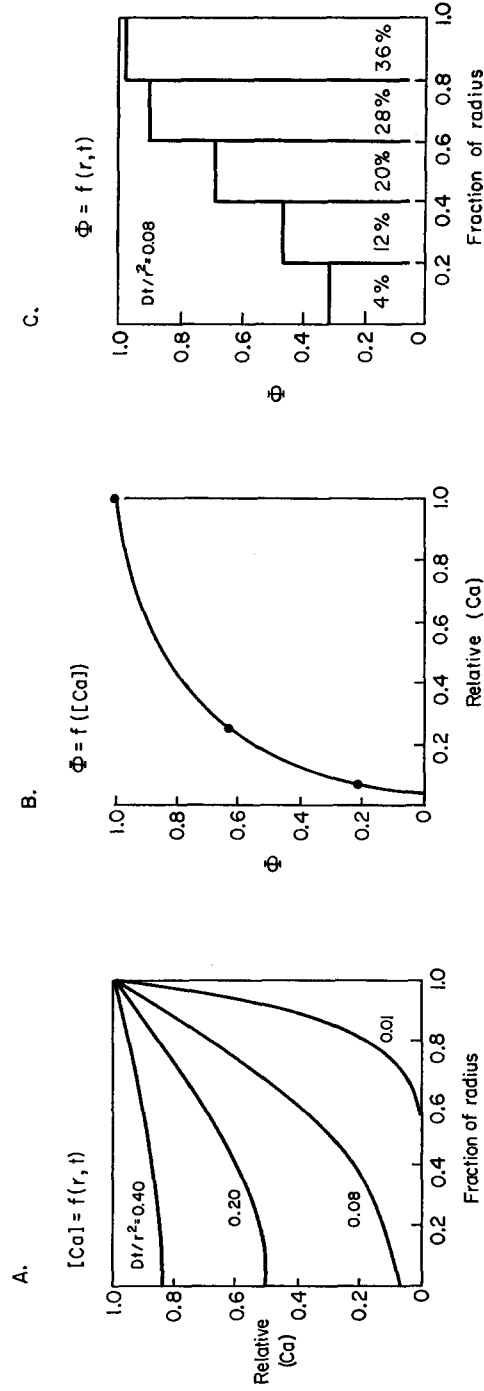


FIGURE 1. Computation of the theoretical value of  $\Phi$  as a function of time ( $Dt/r^2$ ). A. Solution for the relative concentration of a diffusing substance within a cylinder as a function of fraction of the radius and  $Dt/r^2$ . An approximate integration and transformation to obtain  $\Phi$  as a function of  $Dt/r^2$  is accomplished by taking the average value of calcium concentration within each fifth of the radius and transforming this to a value of  $\Phi$  using the relation measured in the whole muscle as is shown in part B. C

The relative concentration of the diffusing substance at each point in the cylinder is shown in Fig. 1A as a function of fraction of the radius and  $Dt/r^2$ , a dimensionless parameter, where  $D$  equals diffusion coefficient in  $\text{cm}^2/\text{sec.}$ ,  $t$  equals time in sec.,  $r$  equals radius in cm (Crank, 1956, chapter V).

Assuming that the response of all the muscle fibers within the whole muscle is qualitatively the same, it is possible to transform the solution of the diffusion equation for relative concentrations into a solution for  $\Phi$ , the relative area under the contracture curve, as a function of fraction of the radius and  $Dt/r^2$ , using the relation calculated between  $\Phi$  and relative Ca ion concentration for the whole muscle (Fig. 1B). If this relation is properly integrated with respect to fraction of the radius,  $\Phi$  can be obtained as a function of  $Dt/r^2$ .

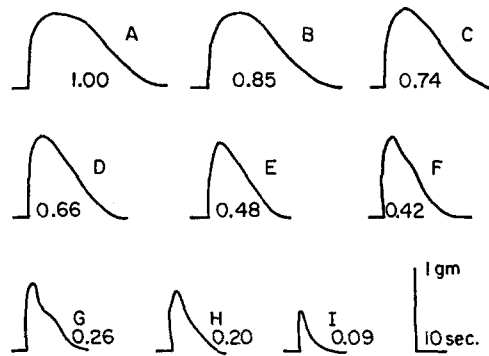


FIGURE 2. Effect of calcium concentration on potassium contractures. Muscles were exposed to the various Ca concentrations for 20 minutes before a contracture was produced with 116 mM potassium. The numbers refer to the area of the contracture at each concentration relative to that produced at A. The calcium concentrations in mM were A—27.5, B—14.8, C—7.4, D—3.75, E—1.95, F—0.95, G—0.47, H—0.23, I—0.105. The experimental sequence was B, I, E, C, G, H, F, D, A.

To simplify this integration the theoretical cylinder was divided into five evenly-spaced concentric cylinders and the average relative concentration within each was calculated for different values of  $Dt/r^2$ . The corresponding value of  $\Phi$  for each average value of calcium was then determined from the measured relation between these in the whole muscle (Fig. 1C). These  $\Phi$  values represent the contribution of each cylinder of muscle, and were multiplied by the fraction of the total cross-sectional area of each cylinder before being added together to give the value of  $\Phi$  for the whole muscle for each respective value of  $Dt/r^2$ .

The experimental values for  $\Phi$  obtained by dividing areas obtained at various recovery times by the control area (see Fig. 8) could be related to the theoretical values calculated by the method described above, by multiplying the values of  $Dt/r^2$  by the value of  $r^2$  calculated from muscle dimensions and dividing it by the value of  $D$  which gave the best fit between experimental and theoretical points. This value of  $D$  is an estimate of the diffusion coefficient for calcium ions within the muscle bundle.

Approximating the elliptical cross-section of the muscle with a circular cross-section and using this coarse integration procedure tend to underestimate the value

of  $\Phi$  for times up to about 20 seconds. However, the first value was neglected if it did not fit with the others and this error was minimized.

Maximum tension was used by Frank (1960) and maximum shortening by Lorković (1962) to quantitate contractures and to determine a value for D in the toe muscle and the sartorius muscle, respectively.

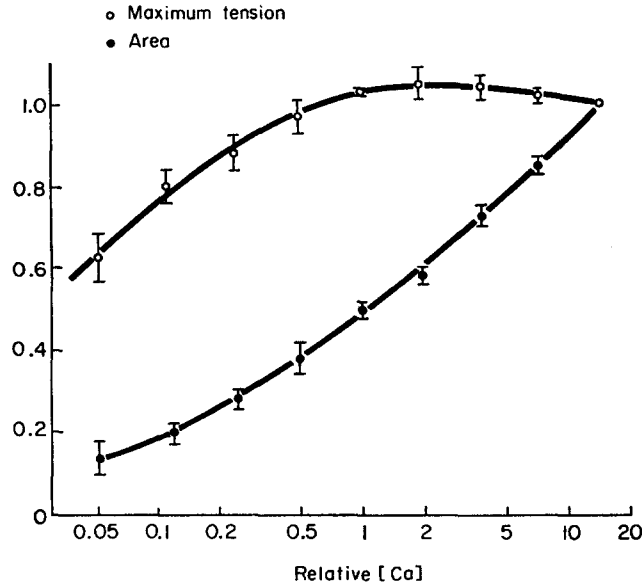


FIGURE 3. The relation between relative maximum tension or relative area under the contracture curve and Ca concentration in the bathing solution. All contractures were produced with 116 mM K. The relative maximum tension and area were calculated using the highest Ca concentration as reference. The abscissa scale is logarithmic and relative values of the Ca concentration should be multiplied by 1.8 mM to get the absolute value. The muscles were allowed to equilibrate in each Ca solution for at least 15 minutes. Each point is the average of 6 muscles. The bars are standard errors.

## RESULTS

*Area Under the Contracture Curve* Area under the contracture curve has previously been used by several other investigators to quantitate contractures (Hodgkin and Horowicz, 1960a, Curtis, 1963). Its relation to external calcium concentration is different from that of maximum tension (Fig. 2). The average values for 6 muscles of both maximum tension and area under the contracture curve are shown in Fig. 3. A similar relation in the single muscle fiber was reported by Lüttgau (1963). The values were normalized before being averaged, by dividing them by the value obtained at the highest external calcium concentration of 27.5 mM. The relative maximum tension increases rapidly and shows saturation for concentrations greater than about 900  $\mu\text{M}$  while the relative area under the contracture curve has a gradually

increasing value over the entire range of calcium concentrations used. Thus, the area under the contracture curve is the more useful parameter to quantitate contractures, although its physical significance is not obvious.

#### FACTORS AFFECTING THE CALCIUM-AREA RELATION

*Temperature and Potassium Concentration* The absolute area under a potassium contracture curve can be increased 2 to 3 times by either reducing the concentration of potassium in the contracture solution from 116 to 50 mM (Hodgkin and Horowicz, 1960a) or by cooling the muscle before producing

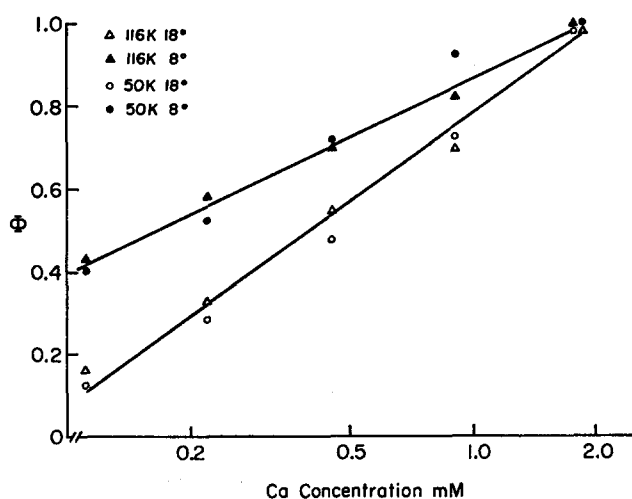


FIGURE 4. Relative area under the contracture curve as a function of external Ca concentration. Contractures were produced with either 116 or 50 mM K at both 8° and/or 18°C. The abscissa scale is logarithmic. The contractures produced at 1.8 mM Ca were used for reference to obtain the  $\Phi$  values. Two muscles were used and the points represent their average values.

the contracture (Lorković, 1963). The effects of these treatments upon the relation between relative area and calcium ion concentration in the range of 125  $\mu$ M to 1.8 mM are shown in Fig. 4. The values were normalized by dividing each area by that obtained for 1.8 mM Ca under the same circumstances in this and in the  $\text{NO}_3$  result.

Reducing the potassium concentration in the contracture solution from 116 to 50 mM did not change the slope of the relation between relative area and calcium concentration. However, if the contractures were produced with either 50 or 116 mM potassium solutions at 8°C. rather than 18°C., the relative areas were much greater at the lower concentrations of calcium, making the slope of the line less steep. There was no interaction between the temperature and the concentration of the potassium solution. These results imply that the mechanisms responsible for the increase in contracture area produced by ex-



posure to cold potassium solutions are different from those produced by reducing the concentration of potassium.

*Nitrate* The enhancing effect of  $\text{NO}_3$  upon twitch or contracture height has been reported by many investigators (references in Kahn and Sandow, 1950), and Hodgkin and Horowicz (1960*b*) showed that  $\text{NO}_3$  also lowered the

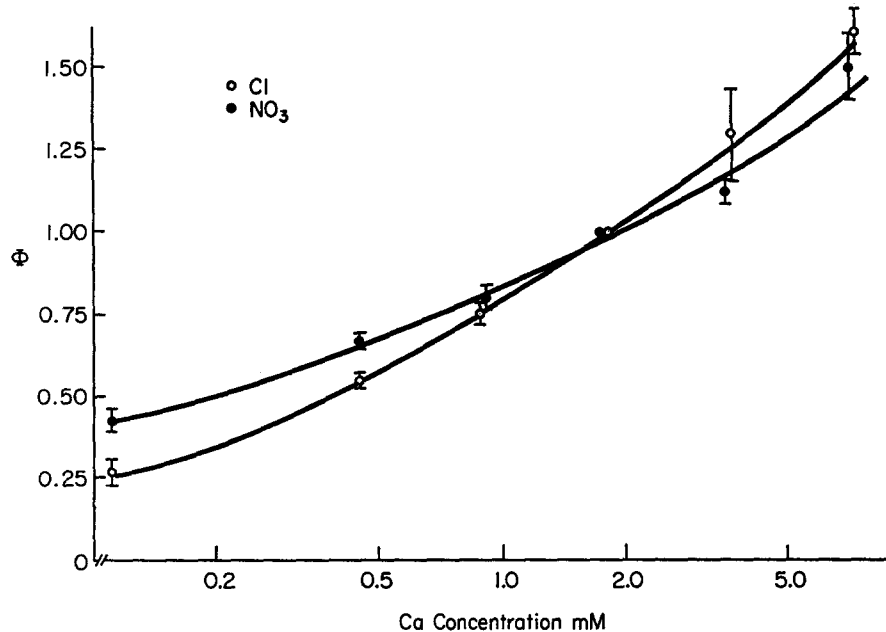


FIGURE 5. Relative area under the contracture curve as a function of external Ca concentration for either Cl or  $\text{NO}_3$  as the anion in all solutions. The averages are significantly different from each other at 0.12 and 0.45 mM, and the P value is less than 0.10 at 3.6 and 7.2 mM using the paired "t" test. The abscissa scale is logarithmic. The contractures produced at 1.8 mM Ca were used as reference to obtain the  $\Phi$  values. Six muscles were used and the vertical lines show the standard errors.

threshold concentration for potassium contractures. Nitrate changed the relation between contracture area and calcium concentration in the same way as using cold potassium solutions for the contractures (Fig. 5). This is despite the different character of the two contractures. Contractures following  $\text{NO}_3$  treatment have the same absolute area as Cl contractures, but have up to 40 per cent greater maximum tension whereas contractures in cold potassium solutions are 2 to 3 times larger in absolute area. In both cases, however, the relative areas are larger at low Ca ion concentrations by approximately the same amount.

*Increased Potassium Concentrations* Increasing the extracellular potassium concentration from the normal value of 2.5 to 20 mM does not produce a con-

tracture, even though the muscle fibers are substantially depolarized (Hodgkin and Horowicz, 1960a). To study the effect of this treatment upon the ability of the muscle to produce a contracture, muscles were allowed to equilibrate for 10 minutes in a tris Cl solution containing either 0.9, 1.8, or 7.2 mM calcium and 2.5, 5.0, 10, 15, or 20 mM potassium before treatment with 116 mM potassium. All contracture areas were compared to the value obtained at 1.8 mM calcium and 2.5 mM potassium (Fig. 6). The slopes of the curves are con-

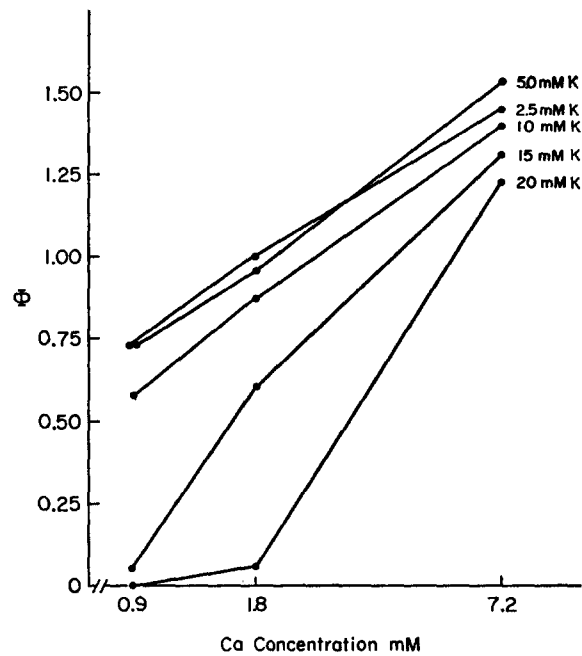


FIGURE 6. Relative area under the contracture curve as a function of calcium concentration for 5 different concentrations of K. The abscissa scale is logarithmic. All areas were compared to that obtained with the normal concentrations of 1.8 mM Ca and 2.5 mM K to obtain the  $\Phi$  values. Two muscles were used and the points represent the average of these.

stant for concentrations of potassium of 10 mM or less, although the absolute area was reduced slightly at this concentration. At higher potassium concentrations the relative area decreased much more at the lower concentrations of calcium than at the higher levels.

**TIME COURSE OF WASHOUT** The ability to give a contracture in response to potassium is lost rapidly during washing of the muscle preparation in Ca-free solution. The time courses for three different muscles are shown in Fig. 7. Parts a and b show calcium washouts after equilibration for 10 min. in normal calcium concentrations of 1.8 mM.

The  $D$  values required to fit the experimental to the theoretical points are smaller than that given for the free diffusion of calcium ions from a chloride solution of low concentration (Harned and Owen, 1958). There are, however, corrections which should be applied before such a comparison can be made, and these are discussed below.

The temperature of the washout solution appears to have little effect upon the rate at which the contracture ability is lost (Fig. 7*a* and 7*c*) which is as would be expected for a system where calcium ions leave by diffusion alone.

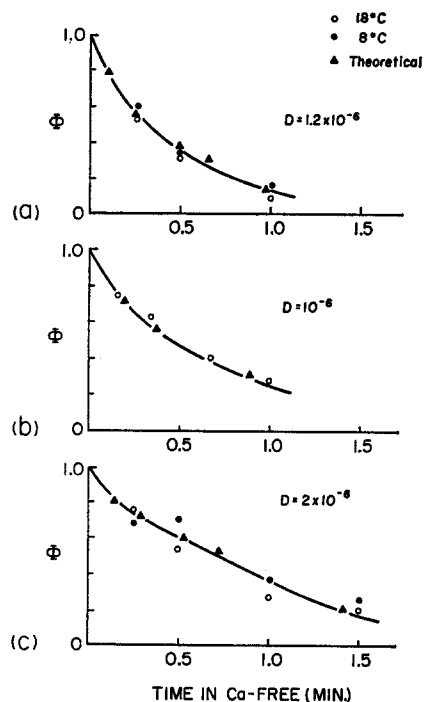


FIGURE 7. The time course of loss of ability to produce potassium contractures during washout in Ca-free solution. *a* and *b* controls were in 1.8 mM Ca and *c* was in 7.2 mM Ca.  $\Phi$  is the ratio of areas referred to a control contracture taken after at least 10 minutes recovery in the respective control solution. The  $D$  values were those required to give the best fit between theoretical and experimental values.

The  $Q_{10}$  for free diffusion of calcium ions is about 1.3 (Höber, 1945) and a change in  $D$  of this magnitude would be difficult to determine from this data.

In the experiment shown in Fig. 7*c*, the muscle was allowed to equilibrate in 7.2 mM calcium before it was placed in the calcium-free solution. The absolute washout time is slower than for the other two muscles, due to the non-linear relation between contracture area and calcium concentration, but the experimental points are adequately fitted with a  $D$  value of similar magnitude.

**TIME COURSE OF RECOVERY AFTER WASHOUT** Muscles which have been soaked in calcium-free solutions for as long as 15 to 30 minutes fully recover the ability to produce potassium contractures if they are returned to a solution containing calcium ions (Fig. 8). This recovery is quite rapid and the average

time course for 6 muscles at two different temperatures is shown in Fig. 9. The D value required to fit experimental and theoretical points is  $2 \times 10^{-6}$  cm<sup>2</sup>/sec. at 18°C. which is about the same as that required for the washout pro-

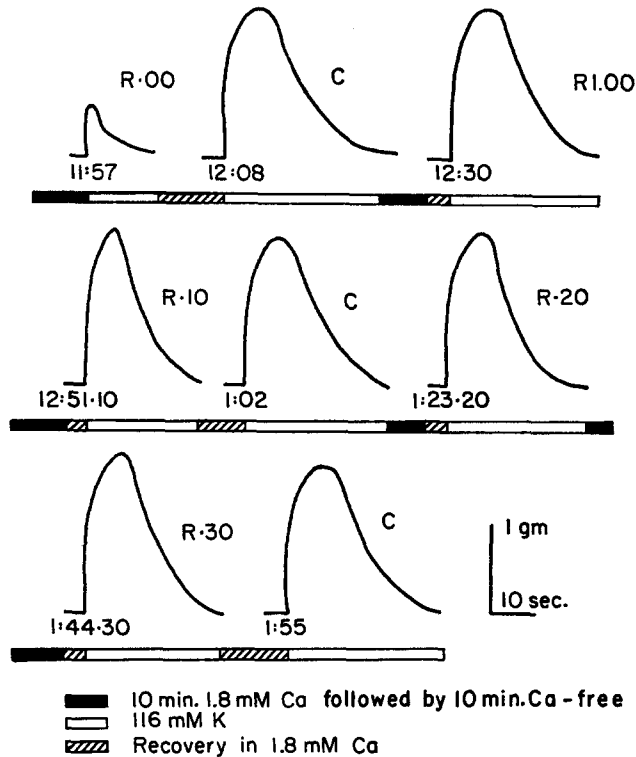


FIGURE 8. Time course of recovery of contracture ability following restoration of Ca after 10 minute washout in Ca-free. The numbers below the beginning of each contracture indicate the time the muscle was placed in 116 mM potassium. The period of recovery is noted in minutes and seconds by the times following R, and C is a control contracture where the muscle had recovered in 1.8 mM Ca for 10 minutes. The filled bar represents a 10 minute recovery followed by a 10 minute washout; the hatched bar is the appropriate recovery time and the open bar is an exposure to 116 mM K lasting until the muscle spontaneously relaxed. The average dimensions of the muscle were  $0.65 \times 0.32$  mm.

cedure. If, however, the muscle is allowed to recover in a solution at 8°C., a D value of  $8 \times 10^{-7}$  gives the best fit.

The time course of recovery after a washout was also slowed if the calcium concentration of the bathing solution was reduced (Fig. 10). However, this effect is due mainly to the more linear relation between external calcium ion concentration and relative contracture area at the lower concentration. The two D values used to fit experimental and theoretical points give the best fit, but if D equals  $3.2 \times 10^{-6}$  (their average) is used, the resultant theoretical

curves vary only slightly from those plotted and so it seems likely that  $D$  is not significantly changed by concentration, which would be expected for a physical process.

*Rate of Repolarization After a Washout in Ca-free Solution* As early as 1956, Bülbring *et al.* had shown that resting membrane potentials tended to fall in

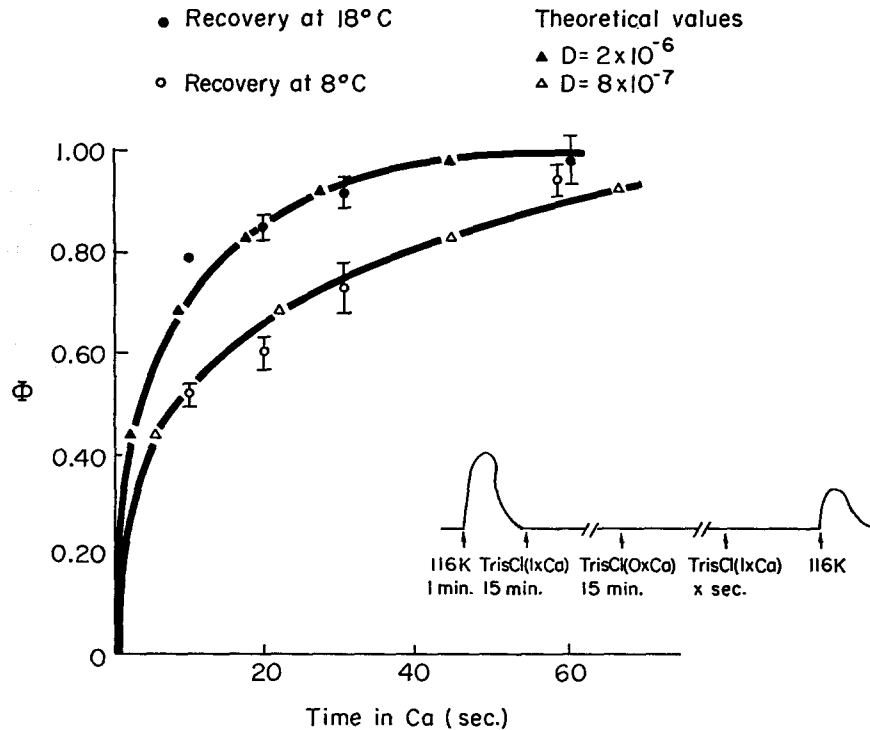


FIGURE 9. Recovery of the ability to give a potassium contracture after a 15 minute washout in Ca-free solution. The recoveries occurred in 1.8 mM Ca at both 8°C, and 18°C.  $\Phi$  is the ratio of the experimental area to a previous control area obtained after 15 minute recovery in normal solution. The low temperature controls were exposed to cold control solution for the last minute of their recovery. Six muscles were averaged and the bar represents the standard error.

frog sartorius muscle fibers exposed to calcium-free solutions. These findings have since been confirmed by many workers (references in Pauschinger *et al.*, 1964).

The time course of repolarization in calcium containing solution after a 20 min. wash in calcium-free solution is shown in Table II. The fall in potential during the 20 minute wash in calcium-free solution is very similar to that reported by Curtis (1963) in the same preparation, although the control values are lower than those reported by him and other investigators. This can be

partly explained by the "slow" fibers present in toe muscle (Gray, 1958). These have a lower average resting potential than "fast" fibers (65 mv compared to about 90 mv) (Kiessling, 1960) and the method employed here of recording potentials would be more likely to record successfully from these muscles than the visual method used by Curtis. Visual observation would re-

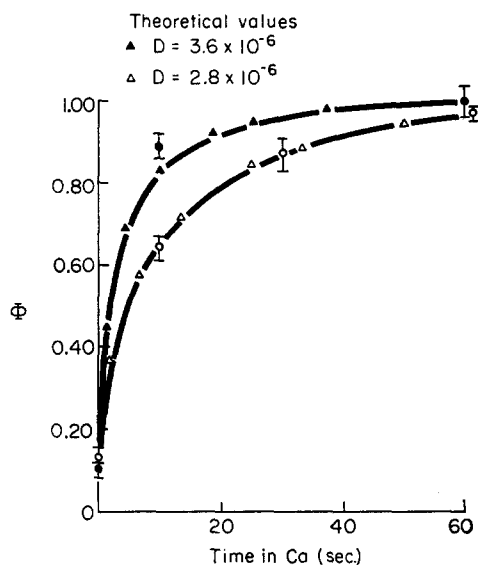


FIGURE 10. Time course of recovery on restoration of Ca after washout for 15 minutes in Ca-free solution. The closed symbols represent 1.8 mM Ca and the open are 450  $\mu$ M Ca. The D values shown give the best fit, but a change of up to 10 per cent does not change it appreciably (see text). Three muscles were used and the standard error is indicated by a bar.

TABLE II  
RECOVERY OF MEMBRANE POTENTIAL AFTER  
WASHOUT IN Ca-FREE SOLUTION

	Control	After 20 min. in Ca-free	After restoration of Ca (min.)		
			½-1½*	1½-2½*	2½-3½*
Average potential $\pm$ SE	78 $\pm$ 2.0	61 $\pm$ 2.5	72 $\pm$ 3.0	73 $\pm$ 3.0	74 $\pm$ 3.0
No. of penetrations	(119)	(70)	(43)	(49)	(39)

\* The values are the averages of all successful penetrations during this interval.

quire a 5 to 10 second period of impalement to be certain the potential is steady. Recording on film requires only 1 to 2 seconds and in this period of time the smaller "slow" fibers (Gray, 1958) are less likely to be badly damaged.

The repolarization is essentially complete in about one minute at 18°C. after the reintroduction of calcium to the bathing solution. This is much faster than the 60 minutes reported by Pauschinger *et al.* (1964) for the whole sartorius after this muscle had been soaked in Ca-free solution under somewhat different conditions.

## DISCUSSION

Because of the temperature sensitivity of the recovery after a washout in calcium-free solution and the low value of  $D$  calculated from its time course, these results do not seem to support Frank's (1958) contention that the calcium involved is completely extracellular. There are several explanations for these effects, however, which may resolve the discrepancies.

The temperature dependence of recovery may be due to the partial depolarization which occurs when the muscle is exposed to calcium-free solutions. Though repolarization after depolarization with  $K$  is not slowed by cooling (Grieve, 1960), repolarization following depolarization by  $Ca$  removal might behave differently. Moreover, if the muscle was soaked in a calcium-free solution for only 4 minutes instead of 15 minutes, the recovery in the cold was more rapid than after the 15 min wash, but was still slower than could be accounted for by physical factors alone (Milligan, unpublished results). A 4 minute wash in calcium-free solution produces only a slight depolarization, and the fibers will depolarize another 10 to 15 mv if kept in the calcium-free solution for 20 minutes (Curtis, 1963). Thus, it cannot be stated with certainty that the temperature sensitivity of recovery is entirely due to repolarization, but it seems likely that a portion of it is.

To determine whether  $D$ , the diffusion coefficient, is really smaller than would be expected it is necessary to decide what its normal value should be under the conditions of the experiment. The value of  $11.91 \times 10^{-6}$  of Harned and Owen (1958) and the value of  $7.78 \times 10^{-6}$  of Wang (1953) were obtained in dilute solutions with only  $CaCl_2$  present. Vinograd and McBain (1941) showed that under certain conditions the diffusion coefficient of divalent cations in aqueous solutions was decreased in the presence of monovalent cations.

Further, the calcium ions can diffuse only in the aqueous portion of the muscle bundle and so any diffusion coefficient selected must be corrected for the increased mean free path of diffusion. Harris and Burn (1949) treated the fibers as uniform, closely packed, circular cylinders and calculated a correction factor of  $\pi^2/4$ , based on the square of the relative increase in distance for diffusion. The actual correction factor should probably be less than this, depending on the closeness of packing of the muscle fibers.

There is some evidence that the endoplasmic reticulum system may be directly connected to the extracellular space. Page (1962) found that the mannitol space was much larger than the inulin space in cat papillary muscle and Johnson and Simonds (1962) found a similar discrepancy between chemical and histological determinations of extracellular space in rabbit ventricle. Both groups suggested that this was due to direct connections with the endoplasmic reticulum. Recently Huxley (1964) has shown that a fairly large molecule, ferritin, can penetrate into the transverse tubules of frog sartorius muscle

fibers and Endo (1964) has shown that a fluorescent dye can enter the same region in as little as 2 minutes. This structure is only a small fraction of the endoplasmic reticulum, but calcium ions diffusing from it would be expected to have their diffusion path increased by an unknown amount and, therefore, to have a reduced diffusion coefficient as determined above.

Another condition that can produce a smaller apparent value for  $D$  is binding of calcium to a site at a rate much faster than the rate of diffusion, such that the amount bound is always in equilibrium with the external solution, and thus diffusion is the rate limiting process. In the simplest case where the amount bound is directly proportional to the concentration it can be shown for the one-dimensional case that  $D$  should be divided by  $(1 + A)$ , where  $A$  is the proportionality constant for the binding (Crank, 1956, Chapter VIII). It is apparent then, that the larger the value of  $A$ , the smaller will be the apparent value of  $D$ .

While there is no direct evidence that this "washout" calcium is bound to some portion of the muscle membrane in this fashion, the effects of  $\text{NO}_3$  and cold potassium solutions upon the relation between relative area and external Ca concentration could be readily explained if it were so. Bianchi and Shanes (1960) attributed the slowing in the washout of  $\text{Ca}^{45}$  from muscle in the presence of  $\text{NO}_3$  to tighter binding of Ca to the muscle membrane and increased binding of Ca in muscle at low temperatures has been postulated by Apter and Koketsu (1960) and indirectly shown by Lorković (1963). A model, based on Langmuir chemisorption and assuming that contracture area is proportional to chemisorbed calcium will explain the relation between external calcium and contracture area (Milligan, 1964). This relation can also be explained using Eisenman's (1962) general theory for ion exchange reactions as applied to glass membranes.

These results do not directly identify the mechanism of the abolishment of contracture ability by a wash in calcium-free solution. If the mechanism is primarily due to calcium removal *per se* it is strange that the recovery should be so temperature sensitive. If depolarization, related to the calcium removal, is the mechanism, why is it so insensitive to temperature when the repolarization is quite sensitive? Brecht *et al.* (1963) found that the time course of depolarization was different from that of the loss of contracture ability and suggested from this that the two processes were not directly related. The failure of a muscle fiber to respond to an electrical stimulus in the absence of calcium has been attributed by Jenden and Reger (1963) to an elevation of the membrane potential at which the cell becomes mechanically refractory. This change in threshold may also be partly responsible for the loss of contracture ability, but this implies that threshold is some function of the external calcium concentration. Lüttgau (1963) has reported an increase in potassium con-



tracture threshold in 5 mM calcium, but Curtis (1963) has reported no change in threshold on reducing external calcium to 100  $\mu$ M.

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#### BIBLIOGRAPHY

- APPLETON, H. D., WEST, M., MANDEL, M., and SALA, A. M., 1959, *Clin. Chem.* **5**, 36.  
APTER, J. T., and KOKETSU, K., 1960, *J. Cell. and Comp. Physiol.*, **56**, 123.  
BIANCHI, C. P., and SHANES, A. M., 1960, *J. Cell. and Comp. Physiol.*, **56**, 67.  
BOYLE, P. J., and CONWAY, E. J., 1941, *J. Physiol.*, **100**, 1.  
BRECHT, K., KUTSCHA, W., and PAUSCHINGER, P., 1963, *Arch. ges. Physiol.*, **277**, 178.  
BROWN, A. S., 1934, *J. Am. Chem. Soc.*, **56**, 646.  
BÜLBRING, E., HOLMAN, M., and LÜLLMAN, H., 1956, *J. Physiol.*, **133**, 101.  
CRANK, J., 1956, *The Mathematics of Diffusion*, Oxford University Press, London.  
CURTIS, B. A., 1963, *J. Physiol.*, **166**, 75.  
EISENMAN, G., 1962, *Biophysic. J.*, **2**, 259.  
ENDO, M., 1964, *Nature*, **202**, 1115.  
FRANK, G. B., 1958, *Nature*, **182**, 1800.  
FRANK, G. B., 1960, *J. Physiol.*, **151**, 518.  
GRAY, E. G., 1958, *J. Anat., London*, **92**, 559.  
GRIEVE, D. W., 1960, *J. Physiol.*, **152**, 25P.  
HARNED, H. S., and OWEN, B. B., 1958, *The Physical Chemistry of Electrolytic Solution*, New York, Reinhold Publishing Corp.  
HARRIS, E. J., and BURN, G. P., 1949, *Tr. Faraday Soc.*, **45**, 508.  
HILL, A. V., 1949, *Proc. Roy. Soc. London, [Series B]*, **136**, 399.  
HILL, A. V., and MACPHERSON, L., 1954, *Proc. Roy. Soc. London, [Series B]*, **143**, 81.  
HÖBER, R., 1945, *Physical Chemistry of Cells and Tissues*, Philadelphia, The Blakiston Company.  
HODGKIN, A. L., and HOROWICZ, P., 1960a, *J. Physiol.*, **153**, 386.  
HODGKIN, A. L., and HOROWICZ, P., 1960b, *J. Physiol.*, **153**, 404.  
HUTTER, O. F., and NOBLE, D., 1960, *J. Physiol.*, **151**, 89.  
HUXLEY, H. E., 1964, *Nature*, **202**, 1067.  
JENDEN, D. J., and REGER, J. F., 1963, *J. Physiol.*, **169**, 889.  
JOHNSON, J. A., and SIMONDS, M. A., 1962, *Am. J. Physiol.*, **202**, 589.  
KAHN, A. J., and SANDOW, A., 1950, *Science*, **112**, 647.  
KIESSLING, A., 1960, *Arch. ges. Physiol.*, **271**, 124.  
LORKOVIĆ, H., 1962, *Am. J. Physiol.*, **202**, 440.

- LORKOVIĆ, H., 1963, *Arch. Internat. Physiol. et de Biochim.*, **71**, 594.
- LÜTTGAU, H. C., 1963, *J. Physiol.*, **168**, 679.
- MILLIGAN, J. V., 1964, Ph.D. Dissertation, publication 65-135, Ann Arbor, University Microfilms Inc.
- PAGE, E., 1962, *J. Gen. Physiol.*, **46**, 201.
- PAUSCHINGER, P., LORKOVIĆ H., and BRECHT, K., 1964, *Arch. ges Physiol.*, **278**, 541.
- VINOGRAD, J. R., and MCBAIN, J. W., 1941, *J. Am. Chem. Soc.*, **63**, 2008.
- WANG, J. H., 1953, *J. Am. Chem. Soc.*, **75**, 1769.