

Autoradiographic Studies of Intracellular Calcium in Frog Skeletal Muscle

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ABSTRACT Autoradiographs consisting of a 1000 Å thick tissue section and a 1400 Å thick emulsion film have been prepared from frog toe muscles labeled with Ca^{45} . The muscles had been fixed with an oxalate-containing osmium solution at rest at room temperature, at rest at 4°C, during relaxation following K^+ depolarization or after prolonged depolarization. From 6 to 39 per cent of K^+ contracture tension was produced during fixation. The grains in the autoradiographs were always concentrated in the center 0.2 to 0.3 μ of the I band and the region of the overlapping of the thick and thin filaments. The greater the tension produced during fixation, the greater was the concentration in the A band and the smaller the concentration in the I band. Autoradiographs of two muscles fixed by freeze-substitution resembled those of muscles which produced little tension during osmium fixation. Muscles which shortened during fixation produced fewer grains. In the narrow (<2.0 μ) sarcomeres of the shortened muscles, grain density decreased with decreasing sarcomere width. A theoretical analysis of the significance of these grain distributions is proposed and discussed.

A considerable body of evidence has been accumulated to suggest that calcium plays a critical role in excitation-contraction coupling in muscle. Small amounts of calcium are necessary for maximum contraction and maximum adenosine-triphosphatase activity in muscle models (1, 31). Associated with contraction of intact muscle fibers there is an accelerated exchange of calcium probably involving both membrane and intracellular calcium (2, 3). Hill has shown on theoretical grounds that insufficient time exists between membrane depolarization and muscle activation for a substance to diffuse from the cell surface to the cell center (4). Therefore, the "activator" which directly initiates the contraction must either be transmitted by a process faster than diffusion or must be stored inside the cell at a site near the contractile proteins. Huxley and Taylor have shown that the membrane excitation may be con-

ducted into the cell interior along the triad portion of the endoplasmic reticulum (5). If calcium activates the contractile filaments, it should be stored near them in the resting cell, possibly in the endoplasmic reticulum, and should be intimately associated with the contractile proteins in the contracted state. To determine whether this is so, an autoradiographic study was made of the intracellular location of calcium in skeletal muscle.

METHOD

A. Preparation and Fixation of the Muscles

The extensor longus digiti IV muscle of the frog (*Rana pipiens*) was isolated and suspended in a continuous flow chamber, where it was bathed with Ringer's solution (NaCl 85 mM, KCl, 2.5 mM, CaCl₂ 1.0 mM, NaHCO₃ 30 mM) through which 95 per cent O₂-5 per cent CO₂ had been bubbled. One end of the muscle was fixed and the other end was either attached to a Statham strain gauge for continuous recording of tension or to a weight sufficient to maintain the resting length of the muscle at the desired level.

After a 1 hour soak in normal Ringer's solution, the bathing solution was changed to Ca⁴⁵ Ringer's (15 to 70 μc/ml), and the bathing with radioactive solution was continued for 8 hours. In some of the experiments during the last 30 minutes of the soak the temperature of the solution was lowered to 4°C, and all subsequent solutions were used at 4°C. No change in resting tension was observed as a result of the decrease in temperature. This reduction in temperature had been used to try to decrease the amount of activation of the muscle that was produced by the fixative. At the conclusion of the exposure to the isotope, the muscles were washed with non-radioactive Ringer's solution for 10 minutes to remove extracellular Ca⁴⁵ and then were fixed with a solution of osmium tetroxide or by freeze-substitution. The osmium fixation schedule was 15 minutes in 0.01 per cent osmium, 15 minutes in 0.1 per cent osmium, and 45 minutes in 1 per cent osmium. In addition to osmium, each liter of fixation solution contained 93 mmoles of choline chloride, 3.7 mmoles of KCl, 2 mmoles of oxalic acid, 8 mmoles of sodium acetate, and 5.8 mmoles of sodium veronal. The solution was approximately isotonic with Ringer's solution. Choline was used instead of sodium to decrease the likelihood of action potentials during fixation. The oxalic acid was added with the hope that it would precipitate any free or loosely bound cellular calcium at or near its physiological location. After fixation the muscles were dehydrated over 135 minutes in a graded ethanol series and finally embedded in butyl methacrylate.

Two muscles were fixed by freeze-substitution. Before the exposure to Ca⁴⁵, these muscles were fastened at both ends to a stainless steel rod. At the conclusion of 8 hours in Ca⁴⁵ Ringer's solution and 10 minutes in non-radioactive Ringer's solution, the muscles were held with their long axis horizontal and were manually plunged into liquid freon, which had been cooled to -155°C by liquid nitrogen. After 1 minute in the liquid freon, the muscles and their holders were quickly transferred to a large volume of ethanol containing 1 per cent osmium. This solution had been pre-cooled to -75°C by solid CO₂ and acetone. The muscles were kept in this solution

at this temperature for 3 days and then switched to cold ethanol containing no osmium. Three days at -75°C is ample time to dehydrate a tissue as thin as toe muscle (34). The muscles were brought up to room temperature over 3 to 4 hours and then embedded in butyl methacrylate. Aliquots of the ethanol solutions were saved for measurement of radioactivity to determine how much Ca^{45} had been lost by the muscles during the prolonged soak in ethanol.

In an attempt to fix a muscle with no activation, another group of muscles was depolarized before and during the exposure to Ca^{45} and the fixative solution. The muscles were first incubated for 15 minutes in a solution which contained 1 mmole CaCl_2 , 30 mmoles NaHCO_3 , 37 mmoles NaCl , 50 mmoles KCl , and 45 mmoles of sucrose per liter and through which 95 per cent O_2 had been bubbled. The sucrose was added to prevent the swelling that would have occurred from the net influx of KCl until $[\text{K}^+]_o \cdot [\text{Cl}^-]_o$ equaled $[\text{K}^+]_i \cdot [\text{Cl}^-]_i$ (35). The muscle was then bathed for 8 hours in a Ca^{45} solution of the same composition followed by 10 minutes in an identical non-radioactive solution. Previous experiments had shown that the effect of such a period of depolarization is reversible and that contractility can be essentially completely restored. Fixation was effected with solutions containing graded concentrations of osmium plus 50 mmoles KCl , 46 mmoles NaCl , 2 mmoles oxalic acid, 8 mmoles sodium acetate, and 5.8 mmoles sodium veronal.

The resting length of the different muscles studied was varied from approximately 100 per cent to 130 per cent of *in vivo* length. All but two muscles were firmly suspended at each end to prevent a significant shortening during fixation. These two were permitted to shorten during fixation, but were then tied to prevent further shortening during dehydration.

B. *Measurement of Calcium and Ca^{45}*

The radioactivity lost by the muscle during fixation and dehydration was measured with a Picker manual windowless gas flow counter. A section of fixed muscle from each experiment was weighed on a Sartorius semimicro balance (precision ± 0.02 mg), dissolved in warm concentrated nitric acid (A.C.S. purity), and evaporated to dryness. The section of tissue generally weighed 0.3 to 0.4 mg, so that an error of less than 10 per cent could have been introduced in the weighing. The residue was ashed at 560°C for 12 hours and then taken up in 0.1 N HCl (A.C.S. purity). All glassware used in the calcium determination was washed with an EDTA solution, 0.1 N HCl , and finally with deionized water. All water had been passed through a Barnstead bantam demineralizer to a final conductance of 0.1 part per million (expressed as NaCl) before it was used.

Total calcium of the solution of the ash and total calcium of the nitric acid were measured with a Beckman DU spectrophotometer with a photomultiplier and an oxygen-hydrogen flame (3). The intensity at $556 \text{ m}\mu$ was used as a measure of calcium. A correction was made for the small amount of calcium introduced by the nitric acid. The radioactivity of the solution of the ash was measured in a gas flow counter. The values of calcium and Ca^{45} per milligram tissue were corrected for the loss of weight during fixation by multiplying them by the ratio of weights of five control muscles before and after this treatment.

C. *Preparation of the Autoradiographs*

Sections which produced gold or gold to silver interference colors (20) when spread with xylol vapor were cut, floated on 10 per cent acetone, and picked up on glass coverslips. The coverslips, which had been previously washed in chromic acid and distilled water, were fastened to glass slides. Within 2 days the coverslips were covered with a layer of Ilford L₄ emulsion in the following way. The entire procedure was performed in total darkness except for 30 to 60 seconds when an Ilford brown light was on 6 feet away. Bulk emulsion which had been packaged with ice and flown to the United States by a low flying plane to minimize background was melted at 48°C on a previously warmed hot plate. The emulsion was diluted 1:7 with quartz-distilled water warmed to 48°C. The diluted emulsion was carefully and slowly stirred. Each slide was quickly dipped into and out of the emulsion and then kept in a vertical position on absorbent paper until dry. It is important that the slide be at about 25°C because at higher temperatures the emulsion dries too rapidly to permit the draining necessary to produce very thin films. At lower temperatures the viscosity of the emulsion limits the degree to which the emulsion can drain off the slide. During the entire procedure a heated water bath maintained a high relative humidity in the dark room to slow the drying of the emulsion films.

The thickness of a series of films prepared in this way was measured by interference microscopy. They consist of one layer of fairly densely packed grains (1400 Å mean diameter). During the drying, the films would occasionally crack. Although increasing the humidity in the dark room by the water bath decreased the incidence, it did not eliminate it. Fortunately, this cracking only occurred during the initial drying of the film and not during the drying following the developing procedure.

The slides were stored with all the autoradiographs facing in the same direction so that the thickness of a glass slide separated each autoradiograph from the adjacent ones. Drierite in a small, porous paper sack was added, and the slides were kept at 4°C for 2 to 12 weeks before developing. The slides were developed with 1/9 Kodak D19b developer for 20 minutes at 25°C, rinsed in distilled water, soaked in 1 per cent acetic acid for 1 minute, rinsed again in distilled water, fixed in plain hypo for 40 minutes, and washed in water for 90 minutes. Care was taken to maintain all solutions at the same temperature during processing.

Autoradiographs of tissues incubated in non-radioactive Ringer's were prepared as controls with each experiment. Any experiment in which the control had a grain density of greater than 0.5 grain per 100 μ^2 was discarded with two exceptions (see Results). Generally control grain density was about 0.3 grain per 100 μ^2 . This equalled less than 10 per cent of the grain density of all isometric experiments.

After the glass coverslips containing the tissue and film had been developed, they were turned over with the emulsion side down, sealed to the glass slides with paraffin around the edges, and visualized with a Zeiss phase contrast objective $\times 100$, N.A. 1.32. Photomicrographs were made with Kodak plus X panchromatic film.

THEORETICAL

Autoradiography involves the conversion of silver bromide grains to silver grains as a result of interaction of the grain with a beta emission and subse-

quently with a developing agent. After the emulsion is developed, the location of the grains with respect to the underlying tissue structure is determined. From an analysis of the number of silver grains and their locations, the amount and location of the isotope can be inferred. An accurate analysis requires knowledge of the total calcium content of the tissue and the degree of resolution of the system.

Doniach and Pelc (12) have quantitatively analyzed some of the important factors involved in the spread of grains around a radioactive source. Hill (13) has applied this to autoradiography of tritium in skeletal muscle. The primary consideration of these authors was the exponential decline in radioactivity with distance from a point source.

High resolution autoradiography with calcium, however, presents additional problems, which can be overcome by using very thin specimens and a very thin layer of emulsion. Since the Ca^{45} beta emission has an energy of about 0.25 Mev its range in the nuclear emulsion will be over 30 μ . It will reduce more grains per centimeter path as it moves farther from its source since the rate of energy loss of an electron per unit path length increases exponentially as the energy decreases. These two factors considerably reduce the resolution of autoradiographs. The need for multiple ionizations within a AgBr grain to make it capable of being developed and the effects of electron scattering facilitate resolution, however, to the point where localization of Ca^{45} within a sarcomere is possible (14, 15).

The specific number of ionizations which must occur along the path of an electron passing through a single grain varies with different grains and determines emulsion sensitivity. It may be as high as 15. Since ionizations occur with a certain frequency per unit path length an electron must have a certain minimum path length within the grain to produce the required ionizations. Pelc (15) has estimated that the path of a 0.15 Mev electron must be at least 1000 A for one of the more sensitive grains. It would have to be longer for the 0.25 Mev electron emitted by Ca^{45} . For even the most sensitive of the small grains, therefore, the path of the Ca^{45} beta particles must be a large fraction of the grain diameter for the grain to be developed.

In a classic experiment, Rutherford (16) showed that a certain number of alpha particles passing through a thin layer are scattered through large angles. Electrons are scattered in a similar way (43). The percentage of scattered electrons varies inversely with the square of the energy of the electron and inversely with $\sin^4 \frac{\theta}{2}$ where θ is the angle the electron path makes with the plane of the layer at the electron's point of entrance.

When very thin layers of emulsion and tissue are in direct contact, the paths of most electrons which do not enter the emulsion within the immediate vicinity of the isotope will form relatively small angles with the plane of the emulsion. Deviation through only a relatively small angle is necessary to

scatter the electron back in the direction of the tissue section. If the electron is scattered out of the grain before its path within the grain has reached the minimum length and therefore before a sufficient number of ionizations has occurred, the grain will not be capable of being developed unless hit by a second electron. The probability of this type of scatter occurring with those electrons which enter the emulsion at a significant distance from the radioactive source is fairly high. Electron scattering thus greatly improves resolution. Caro (17) and Hill (18) have observed better resolution in autoradiographic studies than can be predicted without consideration of the effect of scatter. Dudley (19) has shown that a diffuse beam of 0.25 Mev electrons produces only about one-third of the grains per unit area that a beam focused perpendicular to the surface of the film does.

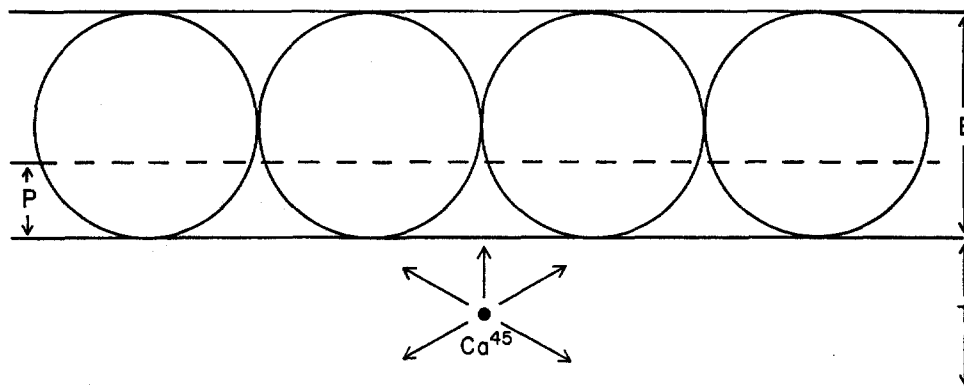


FIGURE 1. Schematic diagram of the model used for analysis of the grain distribution within the autoradiographs. E is emulsion thickness; T is tissue thickness; and P is equal to the minimum distance an electron must pass through a grain to make it capable of being developed. The Ca^{45} source is a line running perpendicular to the plane of the paper and located in the middle of the tissue thickness.

A model based on electron scattering and the exponential decline of radioactivity with distance has been formulated to predict the distribution of grains around a Ca^{45} source (Fig. 1). Tissue sections 1000 Å thick are directly in contact with a dense layer of grains 1400 Å in diameter. All the radioactivity is localized in parallel lines running in a plane 500 Å from the emulsion. It is assumed that an electron must pass through at least one-third of the grain diameter to make the grain developable. All the electrons with perpendicular incidence scattered through an angle greater than the incident angle and one-half of the other electrons deflected through angles greater than the incident angles will be scattered back to the tissue specimens. If this deviation of path occurs before the electron path equals one-third of the grain diameter it is assumed that the grain must be hit again to be reduced. The pathway within the grain after the change in path direction from scattering and the fact that

an electron can completely traverse a grain without scatter but with a path equal to less than one-third the grain diameter have been neglected. These last two effects are opposing. Overlooking the second eliminates the differences that would result from the specific positioning of the source with respect to the grain or the grains nearest it. No difference exists whether the source is below the middle of a grain or the space between two grains. The predicted distribution of grains around a source as a function of horizontal distance from the source was found by graphical solution and is given in Fig. 2. The hump in the curve results from the fact that the effects of electron scattering only become important 2000 to 3000 A from the source. The grain count falls essentially to zero at 5500 A. The effect of a source of radioactivity is therefore limited to less than one sarcomere width.

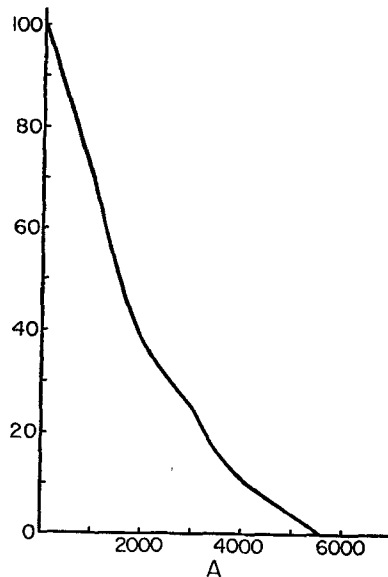


FIGURE 2. The theoretical decline in grain density from a line source of Ca^{45} as a function of horizontal distance from the source (see text).

If one assumes that about 75 per cent of the total surface of the tissue section is covered with AgBr grains and that every beta particle which traverses one-third of the thickness of a grain sensitizes it to the developer, then more than one grain should be produced for every three radioactive disintegrations. This calculation neglects latent image fading.

RESULTS

A. *The Effect of Osmium Fixation on Tension and Calcium and Ca^{45} Content of Toe Muscles*

Exposure of a toe muscle to a buffered solution containing osmium caused a rapid increase in tension for 30 to 60 seconds and a further slow increase over

the next 5 to 7 minutes. This occurs with or without oxalate present in the solution. If the muscle had been previously bathed in a solution containing isotopic calcium, an abrupt increase in the rate of loss of Ca^{45} from the muscle occurred simultaneously (Fig. 3). The size of each of these changes depended

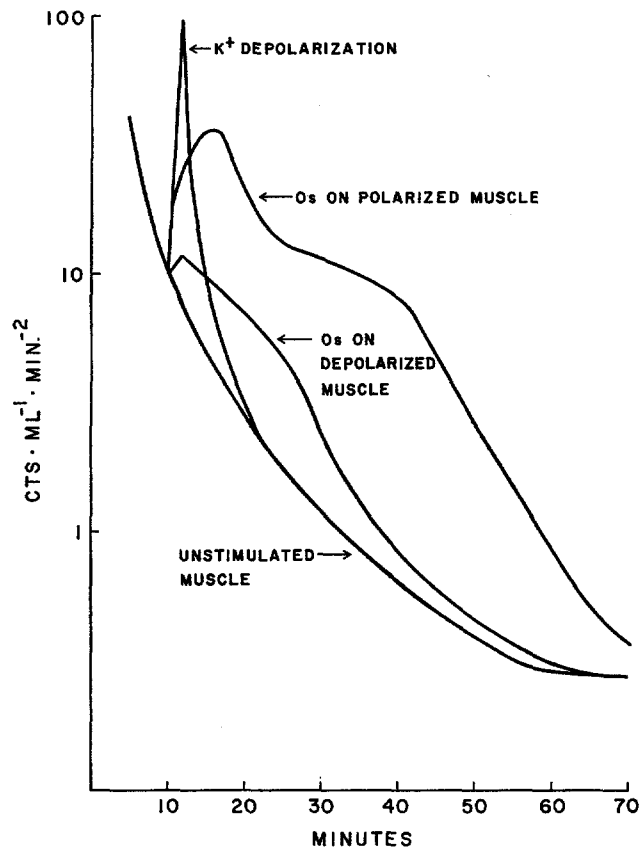


FIGURE 3. The loss of Ca^{45} from toe muscles previously loaded with the isotope. One of the muscles was depolarized with high K^+ before the exposure to Ca^{45} , and the depolarization was maintained with high K^+ for the rest of the experiment. The muscles were washed out in equal volumes of non-radioactive Ringer's (or non-radioactive Ringer's containing 50 mM KCl in the case of the depolarized muscle) for 10 minutes. They were then soaked in equal volumes of Ringer's solution, Ringer's plus 80 mM KCl , or osmium solution as indicated. The comparative radioactivity of 2 minute collections of the washout solutions has been normalized by arbitrarily setting the amount collected between 8 and 10 minutes for each muscle equal to 10.

in part on the circumstances under which the muscle was exposed to osmium. In muscles at *in vivo* resting length at 25°C , 4°C , or after prolonged K^+ depolarization 0.01 per cent osmium with added oxalate caused the development of 36 per cent, 19 per cent, and 9 per cent of K^+ contracture tension re-

spectively. Prolonged depolarization did not decrease a muscle's contractility, for within 15 minutes of return to normal Ringer's solution after 8 hours in a high K^+ solution, the tetanus tension returned to at least 90 per cent of the pre-depolarization value.

TABLE I
LOSS OF CALCIUM AND Ca^{45} DURING MUSCLE FIXATION

Procedure	Tension	Ca ⁴⁵ lost	Residual	Residual Ca ⁴⁵	Initial Ca *	Residual	Residual Ca
			Ca ⁴⁵	Initial Ca ⁴⁵		Ca	Initial Ca
	<i>mg</i>	<i>ml/gm</i> ‡	<i>ml/gm</i> ‡		<i>μmole/gm</i>	<i>μmole/gm</i>	
Os fixation at 25°C	350	0.21	0.58	0.73	1.93±0.12	1.15	0.60
	420	0.20	0.68	0.77		1.04	0.54
	430	0.18	0.63	0.78		1.20	0.62
Os fixation at 4°C	110	0.16	0.72	0.82	2.04±0.08	1.29	0.63
	140	0.22	0.76	0.78		1.25	0.61
Os fixation relaxing muscle	300	0.23	0.51	0.69	1.93±0.12	1.04	0.54
Os fixation of depolarized muscle	70	0.08	0.85	0.91	1.95±0.14	1.23	0.63
	120	0.10	0.88	0.90		1.37	0.71
Isotonic		0.55	0.14	0.20	1.93±0.12	0.68	0.35
			0.67	0.22	0.25	0.53	0.27
Frozen		0.04	0.87	0.96	1.93±0.12	1.76	0.91

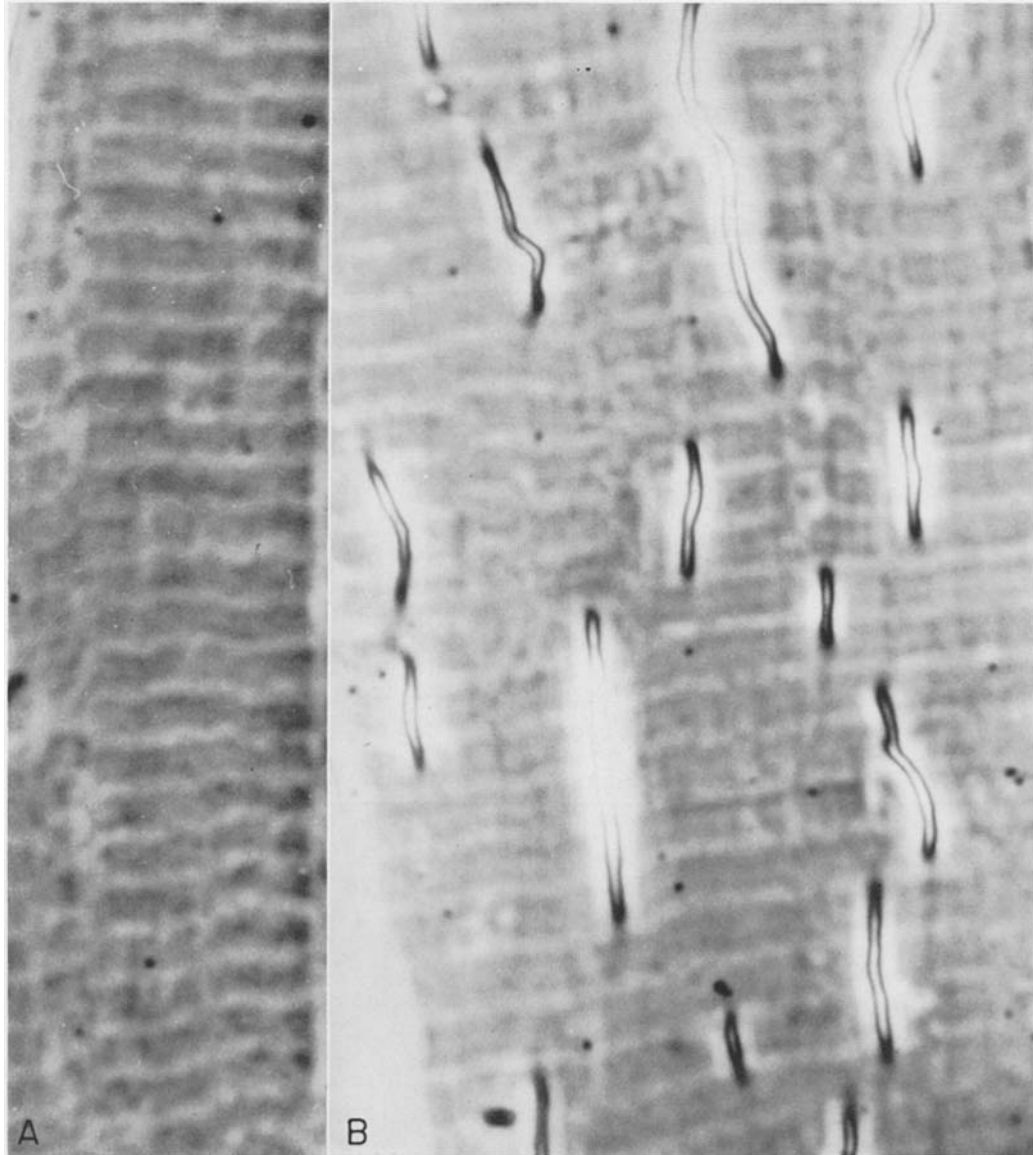
The tension given for the relaxing muscle is that estimated to have been present when the muscle was exposed to osmium.

* Determined on a separate series of fifteen unfixed muscles soaked in the same prefixation solution.

‡ Expressed in terms of radioactivity of the Ca^{45} soak solution. These values can be converted to minimum losses of Ca^{++} by multiplying by 1 $\mu\text{mole/ml}$.

The change in rate of Ca^{45} loss from the muscle during osmium fixation also depended upon the circumstances of the fixation (Fig. 3). Table I gives the amounts of Ca^{45} and of calcium lost during fixation for each muscle studied. Greater Ca^{45} loss was associated with greater tension development. Very little Ca^{45} was lost during dehydration of the tissue in ethanol, and essentially none was lost to the methacrylate embedding medium or the 10 per cent acetone onto which the muscle sections were floated after they were cut. The loss of total calcium during fixation in most cases exceeded the minimum loss of Ca^{45} by 15 to 20 per cent. This, presumably, was due to the loss of rapidly exchangeable calcium, which had lost most of its Ca^{45} during the 10 minute wash in non-radioactive solution before fixation.

The loss of calcium and Ca^{45} from the two muscles which shortened during fixation was much greater than from the rest.



FIGURES 4 *a* and 4 *b*. Autoradiographs of two muscles fixed in 0.01 per cent osmium at room temperature. A bands are dark. In Fig. 4 *b* an H zone is visible in the middle of the A band. In Fig. 4 *b* the vesicle-like structures running perpendicular to the striation pattern are artifacts produced by cracks in the emulsion film. Sarcomere width in both autoradiographs is 2.3μ . The small black spots present almost exclusively in the A band are the silver grains produced by the electrons emitted from the Ca^{45} in the tissue sections.

B. Ca^{45} Distribution in Muscles Fixed in Osmium without Shortening

Autoradiographs from two of the three resting, polarized muscles fixed in osmium at room temperature without shortening are shown in Figs. 4 *a* and 4 *b*. The distribution of over 2000 grains counted in autoradiographs of several different sections of the three muscles is shown in Fig. 5. To localize these grains, the sarcomere was arbitrarily divided into eight regions. The

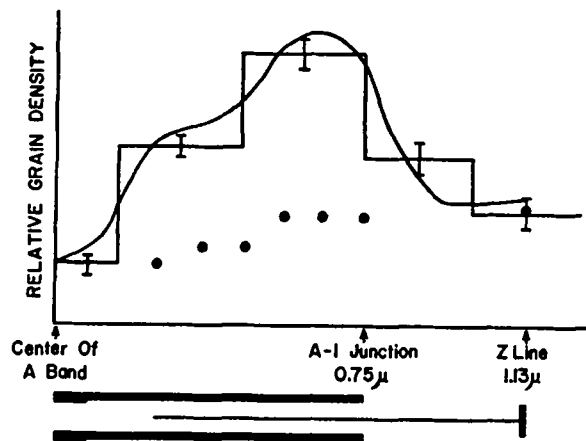


FIGURE 5. The distribution of grains in autoradiographs of three muscles fixed in 0.01 per cent osmium at room temperature is expressed with respect to the sarcomere structure and the inferred position of the thick and thin filaments. The distribution of at least 700 grains in autoradiographs of each muscle was determined separately. The three separate distributions have been averaged and expressed in one graph with standard errors of the mean. The thicker part of the thick filaments corresponds to the part where there are no cross-bridges. The half-sarcomere has been divided into five regions, and the relative grain density of each region is described by the block graph. The smooth curve represents the theoretical grain distributions that would result if the muscle Ca^{45} were distributed in parallel lines within the sarcomere at locations shown by the solid points. The relative intensities of these line sources are indicated by the position of the points with respect to the ordinate.

center of each grain was determined and the number of grain centers in each region counted. The density of grains has been expressed relative to the configuration of thick and thin filaments inferred from the striation pattern (7, 8). The length of the filaments and the degree of overlap are those which are presumed to exist after the shortening produced during dehydration of the tissue (9). A muscle held at both ends during fixation and dehydration shortens only a few per cent. As much as 13 per cent shortening can occur in the muscles unrestrained during dehydration (9).

The grains are localized to the region of the overlap of the thick and thin

filaments. Relative grain density is highest nearest the A-I junction. According to the theoretical model, six parallel line sources of Ca^{45} located every 1000 Å in the overlap zone and one line source in the center of the I band would produce a similar distribution of grains. This theoretical distribution is not the only one which approximates the observed distribution. The best fit of the experimental data, however, does result only from theoretical distributions similar to the one given in Fig. 5. It is not possible to distinguish, for in-

TABLE II
THE DISTRIBUTION OF GRAINS AND CALCIUM IN AUTORADIOGRAPHS

Procedure	Tension	Overlap	Grains at overlap	Ca at overlap		Ca/myosin at overlap†	Grains in center of I band	Ca in center of I band	
	P_0^*			$\mu\text{mole/gm}$	$\mu\text{mole/gm}$				
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>			<i>per cent</i>	<i>per cent</i>	
Os fixation at 25°C	32-39	70-83	87-93	1.0§	0.7	5	7-13	0.1§	0.1
Os fixation at 4°C	10-13	42-46	69-76	0.9	0.6	7	19-23	0.3	0.2
Os fixation of depolarized muscle	6-11	60-68	50-54	0.7	0.5	4	35-41	0.5	0.3
Isotonic¶ (1.6-1.7 μ sarcomeres)		100	63-72	0.4	0.1	1	28-37	0.2	0.1
Os fixation relaxing muscle	27	64	63	0.6	0.4	3	25	0.3	0.2

Values given are the range for all the experiments (listed in Table I) performed with the given procedure.

* P_0 equals peak tension developed during depolarization with 80 mM KCl Ringer's solution.

† Assuming 0.18 $\mu\text{mole/gm}$ of myosin (11) and assuming that the Ca^{45} did not label the inexchangeable Ca.

§ Assuming that the Ca^{45} distribution reflects the distribution of all the residual calcium.

|| Assuming that the Ca^{45} distribution reflects the distribution of the total residual calcium minus 0.4 $\mu\text{mole/gm}$ inexchangeable calcium.

¶ The calcium concentration in the overlap zone and the center of the I band is inferred by assuming that the calcium concentration within the 1.6 to 1.7 μ sarcomeres equals the average of the whole muscle. Therefore, these are minimum values.

stance, between one large source in the center of the I band and two smaller sources near each other in the same region. Ninety per cent of the muscle Ca^{45} would be present in the A band. The distance of 1000 Å has been chosen somewhat arbitrarily for the sake of mathematical simplicity. About one grain has been produced for every seven radioactive disintegrations.

This indicates that about 0.1 μmole of exchangeable calcium per gram muscle is present in the center of the I band and about 0.7 $\mu\text{mole/gm}$ in the A band if the residual Ca^{45} has equally labeled all the residual calcium except for the 0.4 $\mu\text{mole/gm}$ which does not exchange with isotope in the bathing

medium (Table II) (21, unpublished results). If myosin is uniformly distributed within the region of the thick filament containing cross-bridges the $0.7 \mu\text{mole}$ corresponds to about five calciums per myosin present in the overlap zone.

Two muscles suspended at 130 per cent of resting *in vivo* length and fixed with 0.01 per cent osmium at 4°C developed only 10 and 13 per cent of K^+ contracture tension (Table II) during fixation. The distribution of grains in

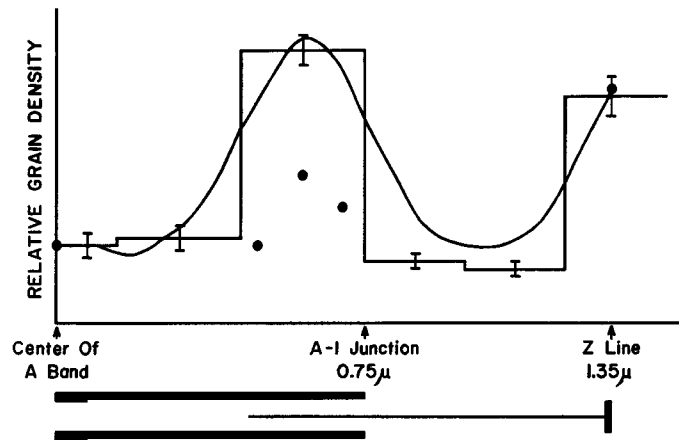


FIGURE 6. The distribution of grains in autoradiographs of a muscle fixed in 0.01 per cent osmium at 4°C (see legend for Fig. 5). The brackets show the variation expected from random fluctuation of radioactive disintegration. About 700 grains were counted.

autoradiographs of several sections of one of these muscles is shown in Fig. 6. The second muscle had a similar distribution. The grains are localized in two regions, the center of the I band and the region of the overlapping filaments. The widths of the overlap and of the concentration of grains within the A band are narrower than in the muscle fixed at room temperature.

According to the proposed theoretical model, this grain distribution would result from eight parallel line sources of Ca^{45} per sarcomere, a large one in the center of the I band, three smaller ones separated by a 1000 \AA in each region of overlapping filaments, and a small one in the center of the A band. The center of the I band would contain $0.2 \mu\text{mole}$ calcium/gm and the overlap zone of the A band $0.6 \mu\text{mole}$ calcium/gm or 7 moles of calcium per 1 mole of myosin.

In an attempt to fix a muscle without disturbing the resting location of the Ca^{45} , two muscles were depolarized before exposure to Ca^{45} and during the entire period of exposure to Ca^{45} . These muscles were suspended at approximately *in vivo* resting length. They developed only 6 and 11 per cent of K^+ contracture tension and lost only a small amount of Ca^{45} during fixation (Table I). The grain density of autoradiographs of these muscles (Figs. 7 and

8) is greatest in the center of the I band. A second concentration is present at the overlapping filaments.

Although the width of the zone of overlapping filaments is quite similar to that in the muscles fixed without prior depolarization, the width of the grain localization within the A band is much narrower in the muscles depolarized before fixation.

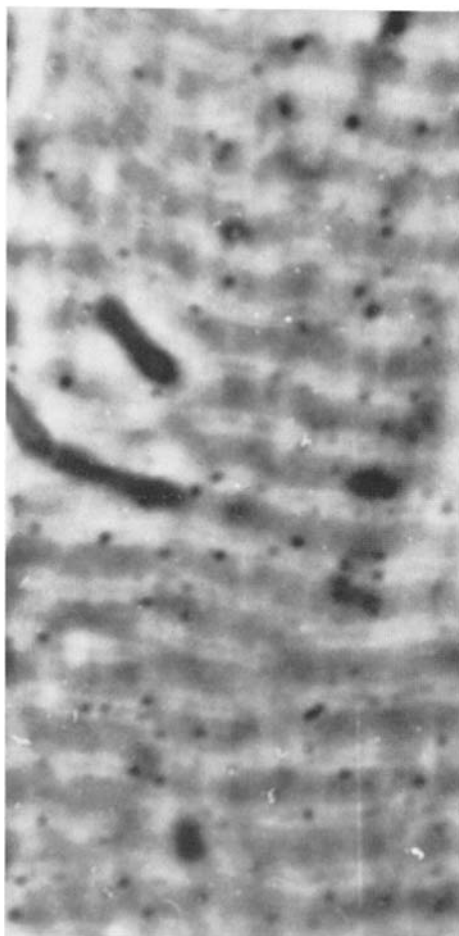


FIGURE 7. Autoradiograph of a muscle depolarized in high K^+ solution before and during the exposure to Ca^{45} and during fixation. A bands are dark. Sarcomere width 2.5μ . The grains are concentrated in the center of the I band and along the edge of the A band.

According to the theoretical model such a distribution could be produced by a very large source in the center of the I band, two smaller ones in the overlap region, and three very small ones in the center of the A band. Such a distribution would correspond to $0.3 \mu\text{mole Ca/gm}$ in the center 0.2 to 0.3μ of the I band, $0.5 \mu\text{mole Ca/gm}$ or 4 Ca/myosin in the overlapping region, and $0.1 \mu\text{mole Ca/gm}$ in the center of the A band.

After the 10 minute washout in non-radioactive Ringer's solution, one muscle was depolarized in Ringer's solution to which 80 mmoles per liter of KCl had been added. The muscle temperature had been lowered to 4°C during the last 30 minutes of the soak in Ca^{45} Ringer's solution and the muscle was maintained at this temperature until the end of the osmium fixation. This was done to prolong the duration of the contracture produced by the high K^+ .

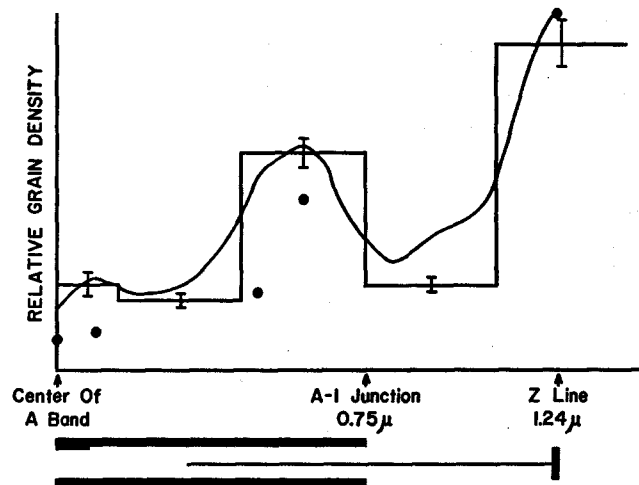


FIGURE 8. The distribution of grains in autoradiographs of two muscles depolarized in high K^+ solution before and during exposure to Ca^{45} and during fixation. About 750 grains were counted for each muscle. The two muscles have been analyzed separately and the distributions averaged as in Fig. 5.

Fifteen seconds after the introduction of the high K^+ solution, the 0.01 per cent osmium fixation solution replaced the K^+ Ringer's. The aim of the experiment had been to fix the muscle at the peak of K^+ contracture. By 15 seconds, however, the tension had already declined to about 32 per cent of its peak value. Following the introduction of the osmium, the rate of decline of tension slowed considerably, and after 3 to 4 minutes the tension remained constant at approximately 27 per cent of the highest tension it had reached. The grain distribution in autoradiographs of this muscle is shown in Fig. 9. The grains are localized to the center of the I band and the overlap region. Comparison with the grain distributions in the muscle fixed after complete relaxation following K^+ depolarization (Fig. 8) and in the muscles activated by osmium (Fig. 5) shows that the grain density at the two localizations in the partially relaxed muscle is intermediate between those in the "contracted" and the "fully relaxed" muscles. It corresponds to $0.4 \mu\text{mol/gm}$ of exchangeable calcium in the overlap region and to $0.2 \mu\text{mol/gm}$ in the I band.

C. *Autoradiographs of Muscles Permitted to Shorten during Osmium Fixation*

Two muscles were treated exactly as the muscles fixed with 0.01 per cent osmium at room temperature except that they were suspended in such a way as to permit considerable shortening. The paired muscles, one for an isometric study and one for an isotonic study, were suspended at the same resting length, soaked in the same Ca^{45} solution, and sectioned at the same thickness. They were covered on the same day, with the same batch of emulsion, stored together, and developed in the same solutions at the same time. These precautions were taken in order to strengthen the validity of a comparison of the grain counts of the two sets of muscles.

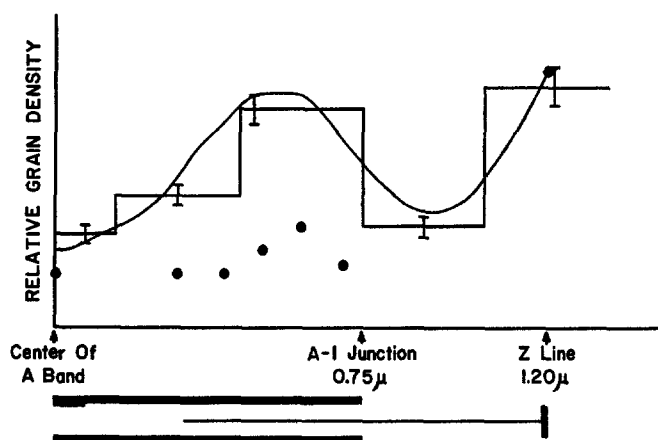


FIGURE 9. The distribution of grains in autoradiographs of a muscle fixed with osmium while relaxing from a K^+ contracture. About 850 grains were counted. The brackets indicate variation from random fluctuation of radioactive disintegration.

In the autoradiographs of the muscles which contracted isometrically during fixation, the sarcomere spacing was uniformly approximately 2.3μ . In the muscles which had contracted isotonicly, several sarcomere patterns were present: (a) sarcomeres 1.6 to 2.0μ in length with 1.3 to 1.5μ A bands; (b), sarcomeres 1.5 to 1.6μ in length with A bands only 0.7 – 1.0μ wide; (c) sarcomeres 1.3 to 1.5μ wide with contraction bands; and (d) a repeating pattern of light and dark bands with the total width of the two bands equal to 0.8 to 1.0μ . The second pattern probably refers to that stage just before the appearance of the C_M contraction band (8). The fourth pattern may be the result of extreme shortening to the point where thick and both thin filaments completely span the distances between Z lines and possibly are folded at their ends (22).

Table III shows the grain counts associated with one set of paired osmium-

TABLE III
THE CONCENTRATION OF GRAINS IN AUTORADIOGRAPHS
OF MUSCLES FIXED IN OSMIUM AT 25°C

Contraction	Sarcomere width	A band width	Grains/100 μ^2
	μ	μ	
Isometric	2.3	1.5	5.62±0.17
Isotonic	1.6-2.0	1.3-1.5	1.55±0.09
	1.3-1.6		0.90±0.07
	0.8-1.0		0.66±0.12

induced isometric and isotonic contractions. The grain count of the muscle which contracted isometrically is over 3.5 times that of any region of the isotonicly contracted muscle. In the isotonicly contracted muscle, the grain density was greater in regions where the sarcomere width was larger.

Within an area of the isotonicly contracted muscles where the sarcomere width was between 1.6 and 1.7 μ and the A band 1.3 μ , about 600 grains were localized with respect to the striation pattern. The result is shown in Fig. 10. The grains are sharply localized to the I band and the portion of the A band closest to the A-I junction. The density is larger in the center than in the lateral regions of the I band. From the sarcomere dimensions one would infer that during the contraction both thin filaments reached the center of the thick filaments and presumably about 0.2 μ of each thin filament either passed the center of the A band (23) or folded up in that region. During fixation and dehydration a certain undetermined amount of shrinkage of the thin filament occurred (9). In Fig. 10 the solid thin filament represents the smallest length that is likely after dehydration, while the dashed line extension indicates the

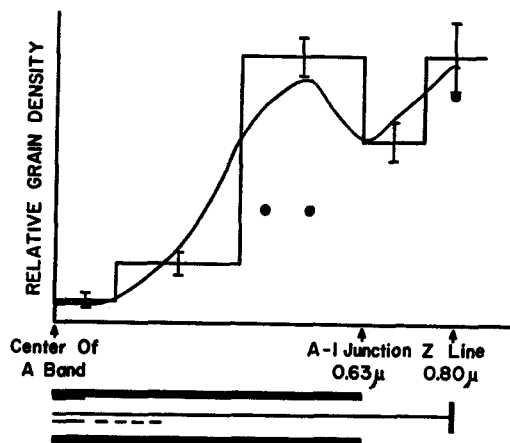


FIGURE 10. The grain localization in regions of autoradiographs of muscles permitted to shorten during fixation. About 600 grains were counted. (See Fig. 5 and text.) Only the areas where the sarcomere width was between 1.6 and 1.7 μ were considered. The two muscles have been analyzed separately and the distribution averaged as in Fig. 5.

largest. According to the theoretical model, this distribution of grains could be produced by Ca^{45} localized at the center of the I band and borders of the A band.

D. Autoradiographs of Muscles Fixed by Freeze-Substitution

Two Ca^{45} -labeled muscles were quick-frozen at a temperature of -155°C and subsequently exposed to 1 per cent osmium in ethanol for 3 days at -75°C . It was not possible to determine whether the muscle developed any tension before it was frozen, but less than 5 per cent of the muscle Ca^{45} was

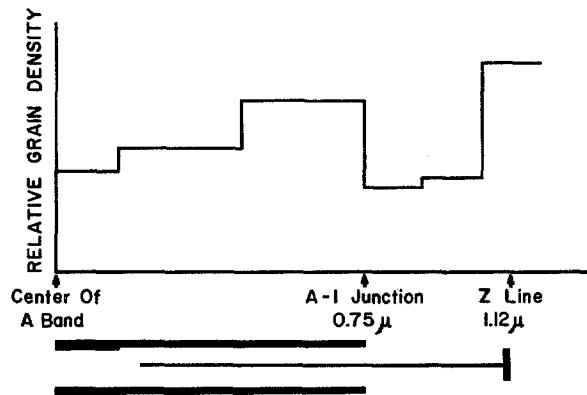


FIGURE 11. The grain localization in autoradiographs of a muscle fixed by freeze-substitution. About 1000 grains were counted.

lost during the entire fixation and dehydration procedure. It was obvious from the color of the tissue block and the amount of contrast in the tissue sections that these muscles had taken up considerably less osmium than the others. To visualize the striations clearly it was necessary to stain the tissue sections after the autoradiographs had been developed. Crystal violet was used according to the technique of Hill (39), and in general stained the A bands and the Z lines.

The distribution of grains in autoradiographs of several sections of one of the muscles is shown in Fig. 11. The grain density is highest in the center of the I band and in the region of the overlapping filaments but the grain yield in both of the muscles is almost twice that found in the other experiments. The sections from both muscles were covered with the same emulsion. The grain count in autoradiographs of an unlabeled quick-frozen muscle prepared as controls was much higher than in other controls although the distribution was random. The high background indicated by the control autoradiographs was sufficient to account for the unusually high grain yield in sections of Ca^{45} -labeled muscles. The most reasonable explanation for these observations is a

high background in the particular batch of emulsion used for these experiments. This emulsion was not used for any other experiments, as a policy of discarding once opened emulsion has been employed to help keep the background low. If a correction for high background is made, the localization of grains to the center of the I band and the zone of overlapping filaments becomes more striking.

In view of the large background, no attempt has been made to analyze quantitatively the distribution of calcium within the muscle. Nevertheless, the similarity in grain distribution in the muscles fixed by freeze-substitution and by osmium after prior depolarization, the two techniques most likely to retain the isotope in its normal resting position, is apparent. In each the grain density is highest in the center of the I band and second highest in the area of overlapping filaments. In each the density in the A band is greatest near the A-I junction.

DISCUSSION

By the use of very thin tissue sections and emulsion films directly in contact with each other, it has been possible to localize isotopic calcium within the sarcomere of skeletal muscle. An analysis of the distribution of grains based on the distance from the radioactive source and the effects of electron scattering has been used to infer the amount and location of the Ca^{45} in the tissue. Resolution appears to be somewhat better than the proposed theory predicts. Since the minimum path of an electron for grain reduction is uncertain, the value used in the model may be low, causing an underestimation of resolution. In favor of this consideration is the fact that the observed yield of one grain per seven radioactive disintegrations is less than that predicted by the model. Latent image fading, however, has not been considered in the theoretical formulation.

The purpose of these experiments was to describe the location of calcium in resting and actively contracting muscles and to determine whether a displacement capable of coupling contraction with excitation occurs. The interpretation of the autoradiographic data within this context requires that certain features of the experiment be clarified: (*a*) What component of cell calcium has been labeled with Ca^{45} ; (*b*) how closely does osmium-induced contraction simulate the events of physiological contraction; and (*c*) how closely does the calcium distribution of a muscle in prolonged depolarization or quickly frozen represent the location in the truly resting state.

From kinetic studies with frog sartorius, Shanes and Bianchi (6) and Gilbert and Fenn (21) have divided the muscle calcium into several components, extracellular fluid, connective tissue-bound, rapidly exchanging cellular (presumably bound to the cell surface), slowly exchanging cellular (presumably intracellular), and inexchangeable. The half-times of exchange for the

two cellular components, 10 minutes and 300 to 500 minutes, are similar to those found in the toe muscle. Since the Ca^{45} -loaded toe muscle is much thinner than the sartorius, and is exposed to non-radioactive Ringer's for 10 minutes before fixation, it is likely that most if not all the Ca^{45} studied in these experiments corresponds to the slowly exchanging cellular calcium.

Hasselbach (25) has presented evidence to suggest that most of the muscle calcium is bound to actin, myosin, and the granular fraction of muscle. He and Barany (26) have shown that myosin- and granule-bound calcium is exchangeable and that the actin-bound calcium is relatively inexchangeable. It is likely, therefore, that the autoradiographs have been produced by Ca^{45} which was bound to myosin and to the granules before fixation and has been retained as bound or insoluble calcium.

The osmium-induced contracture is accompanied by a rapid depolarization of about 60 mv (unpublished results). When the osmium is introduced very slowly, several small twitches may precede the development of a contracture. These twitches are about one-half the size of those elicited by electrical stimulation. In the experiments described in this paper, no twitches preceded the contracture. Muscle tension increases much more slowly than in a K^+ contracture, the time to half-maximum tension being about 10 to 15 times longer for an osmium contracture. An abrupt increase in the rate of loss of Ca^{45} from the muscle accompanies the increase in tension with osmium or potassium. The degree of increase is similar in the two contractures, and the difference in the time course of the changes in Ca^{45} loss as well as of tension development is consistent with the difference in the diffusion rates of osmium and potassium. The tension developed during an osmium contracture is only 36 per cent of K^+ contracture tension and does not decline over 75 minutes whereas the K^+ contracture is over within 15 to 20 seconds. From these comparisons, it is tempting to assume that osmium activates muscle by directly depolarizing the membrane and then interacts with the contractile proteins, fixing them in some stage of their tension-developing interactions.

Three different procedures have been used to fix muscles in a state of inactivation or partial activation, namely fixation at 4°C , fixation of an already depolarized muscle, and quick-freezing. The changes in tension and in Ca^{45} loss indicate that some degree of activation occurred with the first two. There are no data to evaluate the amount of activation produced by quick-freezing. Cain *et al.* (27) have shown that a muscle can be frozen without any ATP splitting even when creatine phosphokinase is inhibited, but earlier steps in the contractile sequence may have already occurred to some degree. A large part of the cellular calcium, nevertheless, may be in its resting position.

Studies with caffeine show that a depolarized muscle cell still has its calcium-activated contractile mechanism intact (28-30). It is not known, however, whether the intracellular calcium which supports the caffeine-

induced contracture in depolarized muscle is the same as that involved in normal contraction.

The amount of tension produced and the amount of calcium released during osmium fixation of an already depolarized muscle suggest that the changes in calcium location produced under these conditions are much smaller than with osmium fixation of a polarized muscle.

In all three situations used to simulate the inactive muscle the grains were localized in the center of the I band and the region of overlap of the contractile filaments. In the quick-frozen muscles and the K^+ -depolarized muscles,



FIGURE 12. Electron micrograph of frog toe muscle. Sarcomere width is 3.62μ . Triads are present at the level of the Z line. Very few mitochondria are present.

the two conditions in which the calcium localization is most likely to reflect that in the resting state, the greatest grain density was in the center 0.2 to 0.3 μ of the I band. According to the theoretical model proposed 38 per cent of the muscle Ca^{45} and, by inference, of the exchangeable calcium is localized in the center of the I band and the rest in the overlap zone. If all the exchangeable calcium is distributed in the same way as the Ca^{45} , about 0.3 μmole calcium per gram muscle is present in the center of the I band. If the Ca^{45} reflects total tissue calcium location, the amount is 0.5 μmole . In view of the loss of some calcium during fixation and the limitations of quantitative analysis of autoradiographic data, this value compares favorably with the 0.5 $\mu\text{mole/gm}$ found by Hasselbach to be bound to the granular fraction of rabbit psoas homogenate (25). Since the triads of the endoplasmic reticulum are located in the center of the I band in frog toe muscle (Fig. 12) and are part of the granular fraction of the muscle homogenate (32, 40), they are the most likely site of I band Ca^{45} . It should be noted, however, that the Ca^{45} is localized at the center 0.2 to 0.3 μ of the I band while the width of the triads in these muscles is about 0.5 μ . The center of the I band is also the location of a large concentration of ash (41). Mitochondria are relatively uncommon in frog toe muscle and are generally located in another part of the I band.

The validity of the small localization in the center of the A band described by the model cannot be evaluated without more precise information about the nature of the reaction of the AgBr grains to Ca^{45} disintegration.

A localization of grains in the overlap zone was found in the autoradiographs of all muscles. Within the overlap zone, the density is always greater nearest the I band. The greater the tension, the larger the percentage of the grains and, by inference, exchangeable calcium found there. The correlation between the absolute amount of calcium and the developed tension is less impressive. There are approximately 4 to 7 moles of exchangeable calcium per mole of myosin within the zone of overlapping filaments, if it is assumed that myosin is uniformly distributed throughout the portion of the thick filaments in which cross-bridges are located. Although the technique is too imprecise for the differences in this ratio in the different experiments to be meaningful, the ratio is similar to that found in maximally precipitated myofibrils by Weber (personal communication).

Hasselbach (25) has shown that the calcium binding of actomyosin may be different from the binding of actin and myosin alone. He has found that although the amounts of calcium bound by actomyosin and the equivalent quantities of the isolated proteins are very similar, 50 per cent of actomyosin-bound calcium is removable by EDTA but little of the calcium bound to isolated protein is. Parker and Gergely (42) have also observed that a large part of the myosin calcium could not be removed with EDTA, although it is exchangeable (26). These results are consistent with the autoradiographic

findings that the calcium binding characteristics of the overlapping thin and thick filaments are different from those of either filament alone.

The presence of a greater concentration of exchangeable calcium in the center of the I band in the less activated muscles and among the overlapping filaments in the more activated muscles suggests that a shift from the former region to the latter occurs during activation. This conclusion is supported by the nature of the grain distribution within the overlap zone. The data of Guld and Sten-Knudsen show that the time from stimulation to the development of tension increases as the I band widens up to sarcomere widths of 3.1μ . This observation is consistent with such a shift's being an integral part of the activation process (33).

The autoradiographs of muscles which have been permitted to shorten during fixation differ from those which have not in two important respects. The grain density in the isotonically contracted muscles is much less. Even within these muscles the grain density appears to be related to sarcomere width, the narrower the sarcomere below 2.0μ , the less the grain density. The second important difference is the grain distribution. In sarcomeres 1.6 to 1.7μ wide where the thin filaments have crossed in the middle of the A band (23) or possibly folded, most of the grains in the A band lie on the edge of the A-I junction zone. These two findings can be explained if the special binding properties of overlapping filaments are inhibited at sarcomere widths below 2.0μ by an interference from the thin filaments which have crossed or folded in the center of the A band.

Another factor may also be involved in producing a low grain density in isotonic contractions. A. F. Huxley (10) and Podolsky (36) have pointed out that in a sliding model of muscle contraction involving multiple cyclic interactions, the number of interactions at any instant will be dependent on the velocity of movement of the filaments past each other; the greater the velocity, the fewer the interactions. If calcium is bound at the site of this interaction then less calcium would be bound during an isotonic contraction than during an isometric one. This phenomenon could also explain the observation that the rate of Ca^{45} loss during an isotonic contracture is over twice that during an isometric contracture (24).

Hill (18) has used autoradiography to show that nucleotides in depolarized muscles fixed in osmium are bound in the overlap region and in the center of the I band. Although the relative amount of nucleotide in each of these regions was different from that found for calcium in comparably fixed muscles, it is possible that the calcium is bound to the nucleotide in these regions. The greater affinity of nucleotide for magnesium than for calcium (37) and the larger concentration of magnesium (38) do not eliminate this possibility. Hasselbach (25) however, has found that about $9.2 \mu\text{mole/gm}$ or 95 per cent of the nucleotide of muscle homogenate is lost to KCl washing while only

0.3 μ mole/gm or 15 per cent of the muscle calcium is lost in the same process. It would appear that the affinities of the myosin, actin, and microsomes for calcium at least before exposure to osmium are greater than that of the muscle nucleotides.

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