EFFECTS OF CAFFEINE ON THE INTRACELLULAR DISTRIBUTION
OF CALCIUM IN FROG SARTORIUS MUSCLE

by

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ABSTRACT

The influence of caffeine on the intracellular distribution of calcium in the frog sartorius muscle was studied by differential centrifugation in an attempt to identify the locus of action of this alkaloid. The problem was approached in two ways. In the first, the locus of action was sought by relating the kinetic functions of $^{45}\text{Ca}$ washout curves of muscles to changes in the distribution of $^{45}\text{Ca}$ in the isolated fractions from the same muscles. It was not possible to make any correlation of the $^{45}\text{Ca}$ washout curves to the activity in the fractions; the relative distribution of this nuclide remained essentially unchanged at 1-, 2-, and 3-hour intervals along the curve. The washout curves appear to be the net effect of a complex interaction of the calcium in pools containing both readily exchangeable calcium and calcium which has a slow exchange or turnover rate. The second approach centered upon the examination of the effect of caffeine on the intracellular distribution of $^{45}\text{Ca}$ and of calcium among the cellular fractions. Caffeine treatment resulted in a distinct increase in the calcium content of the mitochondrial fraction and a decrease in the calcium of the microsomal fraction. Electron micrographic studies revealed significant morphological changes in the whole muscle and in the isolated mitochondrial fraction after the muscle had been exposed to caffeine in a concentration producing irreversible contracture or rigor (10 mM). The increase in calcium content of the mitochondrial fraction after caffeine treatment may be due to an actual accumulation of calcium by the mitochondria or may be the consequence of the appearance of granular vesicles in the fraction.
I. INTRODUCTION

Numerous reviews have been written about excitation and contraction in muscle and the intimate role that calcium plays in the process (Sandow, 1965; Bianchi, 1968). Nevertheless, very little is actually known about the distribution and the movement of intracellular calcium or about the function of subcellular organelles, such as mitochondria and elements of the sarcoplasmic reticulum, in determining the distribution and in regulating the movement of calcium.

Reports have appeared on the intracellular distribution of $^{45}$Ca in chicken breast muscle (Cosmos, 1964) and in rat heart and skeletal muscle (Bondani and Karler, 1965; Patriarca and Carafoli, 1968), but data on the absolute amount of calcium in the various cell fractions are lacking. Winegrad (1970) has approached the problem of the distribution of calcium in resting and stimulated muscle by employing autoradiography and has described the general disposition of $^{45}$Ca within the sarcomere. However, it is difficult to correlate autoradiographic distribution of $^{45}$Ca in a sarcomere to discrete subcellular structures. There have been a number of reports on the calcium distribution in conjunction with several different ions in tissues other than muscle (Griswold and Pace, 1956; Thiers and Vallee, 1957; Cassidy et al., 1969). In general, the fractions containing mitochondria and microsomes were found to have a significant concentration of calcium regardless of the cell type from which they were derived.

Many investigators have shown that mitochondria and the membrane fragments of the sarcoplasmic reticulum are able to accumulate calcium in vitro;
these studies have been extensively reviewed (Weber, 1966; Lehninger et al., 1967). The relevance of the in-vitro findings to the role of the subcellular organelles in the intact cell is one of the major unresolved problems in muscle physiology. According to the findings of a number of workers (e.g., Portzehl et al., 1964), the level of free intracellular calcium in the resting muscle is very low. Upon stimulation of muscle, the level of free calcium rises transiently in association with contraction (Jöbsis and O'Connor, 1966; Ridgway and Ashley, 1967). The nature and cellular locus of the mechanism controlling the free calcium level remain to be elucidated.

In his monograph, Bianchi (1968) relates the effects of caffeine on contractility to its ability to affect the state of intracellular calcium. In low concentrations, caffeine may mobilize bound or stored calcium which then acts additively with calcium mobilized by an action potential; this could account for the resulting potentiation of twitch tension produced by caffeine. In higher concentrations, caffeine mobilizes enough calcium to activate the contractile apparatus and cause a spontaneous contraction, and in still higher concentrations (\( > 5 \) mM) an irreversible contracture occurs.

In the past, investigations that have dealt with the effects of caffeine on calcium metabolism in muscle have been of two general types. The first of these focuses upon \(^{45}\)Ca efflux (washout) curves from muscle. Bianchi (1961) demonstrated that caffeine increases the rate of \(^{45}\)Ca efflux from frog sartorius muscle preparations. The second concentrates upon the ability of cell
fractions to accumulate or release calcium. Nayler and Hasker (1966) investigated the effects of caffeine on cellular constituents of toad cardiac muscle. Caffeine was shown to interfere with both the uptake and release of calcium by mitochondria. Additionally, microsomal (reticular) fractions isolated from the cardiac muscle bathed in a calcium-free Tyrode solution were found to contain significantly more calcium than did fractions isolated from a similarly treated muscle bathed in the same solution containing 5 mM caffeine. On the other hand, Caravalho (1968), who studied both actively and passively bound calcium in the sarcoplasmic reticulum isolated from rabbit skeletal muscle, was unable to demonstrate a release of calcium after exposure of the reticulum to caffeine. However, under certain experimental conditions, a small fraction of the total calcium of the reticulum was released in response to caffeine (Caravalho and Leo, 1967). In addition to the above-cited findings, Bondani and Karler (1970) were not able to detect any change in the ability of reticulum isolated from rat skeletal muscle to bind calcium during exposure to caffeine.

The purpose of the present study was to attempt to find a cellular locus of action of caffeine. The experimental approach was two fold. In the first, $^{45}$Ca washout curves from intact muscle were investigated in order to try to correlate the first-order functions of the curves with the $^{45}$Ca activity in isolated subcellular fractions. If the washout curves could be described in terms of selective changes in the $^{45}$Ca activity of the isolated fractions, then it would be
feasible to relate the caffeine response to a particular fraction; hence, a crude isolation of the locus of action would be effected. The second approach measured the effect of caffeine on the intracellular distribution not only of $^{45}$Ca but also of calcium. The cell fractions examined were the nuclei, the mitochondria, the microsomes, and the soluble fraction.
II. METHODS AND MATERIALS

A. General procedures

All experiments were performed on the sartorius muscle of the frog (Rana pipiens) at room temperature, 23°-25°C. Animals weighing approximately 30 grams were obtained from dealers in Wisconsin and Arizona. After pithing the animal, both muscles were excised, attached to a stainless steel support rod by means of silk ties at the pelvic and tendon ends of the muscle, and loaded with a 3-gram weight in such a way as to allow for isotonic contraction. The barrel of a 5-ml glass syringe served as the muscle chamber. The bathing solution in the chambers and the reservoirs supplying the chambers was gassed with a 95% O₂ and 5% CO₂ mixture throughout each experiment unless noted otherwise. In experiments in which the bathing solution was gassed, a Barkan type of Ringer solution similar to one described by Boyle and Conway (1941) was employed. This solution had the following composition: 100 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 17.5 mM NaHCO₃, 0.1 mM NaH₂PO₄, 0.7 mM Na₂HPO₄, and 3.9 mM glucose. For the ⁴⁵Ca efflux studies, a non-gassed tris-Ringer solution was utilized and was composed of 113 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, and 10 mM tris buffer. The pH of all the solutions was maintained between 7.2 and 7.4. ⁴⁵Ca was obtained as a CaCl₂ salt solution (10-15 mc/mg calcium) from the New England Nuclear Corp. Triton X-100, 2,5-diphenyloxazole (PPO), and 1,4-bis-(2-(5-phenyloxazolyl))-benzene (POPOP) were obtained from the Packard Instrument Company, Inc. Lanthanum oxide, atomic absorption grade, was obtained from Alfa Inorganics and anhydrous caffeine alkaloid from Sigma Chemical Company.
B. Preparation and analyses

**Fractionation of the muscle.** The muscles were blotted lightly with Whatman No. 42 filter paper, weighed, and immediately homogenized with an all-glass, 5-ml homogenizer, either a Corning Glass Works type or Kontes Duall, in 2 ml of ice-cold 0.25 M sucrose and 0.05 M tris-buffer solution, pH 7.9 at 0°C. Some of the Corning-type tissue grinders did not homogenize the tissue adequately, but the Kontes Duall type always yielded satisfactory preparations. A 0.2-ml aliquot was removed from the homogenate and processed with the fractions as a check on the recovery of either calcium, $^{45}$Ca, or protein. The remaining homogenate was transferred to a 0.78 x 4.84 cm cellulose nitrate tube, placed into a Beckman 40 rotor with a nylon adapter, and centrifuged in a Spinco model L preparative ultracentrifuge according to the scheme presented in figure 1. This fractionation scheme was chosen because, compared with more conventional schemes, it yielded better recoveries of mitochondria, as determined visually with the use of Janus green B vital stain. After spinning for 5 minutes at 1,100 x g, the supernatant was removed by a needle and syringe and transferred to another tube. The crude nuclear pellet, referred to in the text as fraction I, was kept for analysis. The supernatant was centrifuged for 10 minutes at 9,000 x g. The pellet obtained, the crude mitochondrial fraction (fraction II), was held for later analysis and the supernatant transferred, as described above, to a third tube and centrifuged again. The supernatant was spun for 30 minutes at 145,000 x g. The crude microsomal
pellet and the supernatant, termed fractions III and IV, respectively, were then analyzed along with the other two fractions and the 0.2-ml aliquot of the original homogenate. The particulate fractions were resuspended in glass-distilled water before the various analyses were made.

*45Ca experiments.* The muscles were labeled by injecting $^{45}$Ca-tris-Ringer solution into the ventral lymph sac of the animal ($16 \mu c/gram$). The animals were sacrificed one hour after the injection, the optimum time for maximally loading the muscles. For the $^{45}$Ca-distribution studies, muscles were either placed into the chambers for washout studies in tris-Ringer solution before fractionation or they were immediately fractionated and analyzed for $^{45}$Ca. In the caffeine experiments, the paired sartorii were washed for a period of one hour in Barkan-Ringer solution. At the end of the one-hour washout period, one muscle was exposed to a 10 mM caffeine-Ringer solution for 7 minutes; the other muscle was used as the control. In all the washout studies, 5-ml samples were collected every 10 minutes and the chambers refilled with 5 ml of fresh solution. For determining the radioactivity in the samples, a 2-ml aliquot of each collection was removed and placed into liquid scintillation vials containing 18 ml of the following "cocktail": 0.55% PPO, 0.01% POPOP, 66% toluene, and 33% Triton X-100. In preparing the isolated cell fractions for analysis, piperidine was added to the resuspended fractions to yield a final concentration of 40-50%. The fractions were then placed into a 60°C oven and left there for 1-2 hours. The piperidine procedure served to digest the
resuspended fractions. After removal from the oven, the fractions were further diluted with glass-distilled water to yield a final concentration of piperidine of approximately 25%. A 2-ml aliquot was removed and added to the scintillation "cocktail." All samples were counted on a Packard Tri-Carb liquid scintillation counter. In order to describe the effects of caffeine on the efflux of $^{45}\text{Ca}$, rate coefficient curves were prepared (Bianchi and Shanes, 1960) to describe the time course of the average percent change in activity per minute.

**Calcium determinations.** The resuspended fractions and a 0.2-ml aliquot of homogenate were placed into platinum crucibles for ashing. Sucrose-tris buffer solution and glass-distilled water were run as blanks to check for their calcium contamination. Only the sucrose-tris buffer was found to contain measurable amounts of calcium. Since essentially all of the original 2 ml of sucrose-tris buffer solution used in the fractionation procedure eventually appeared in the final supernatant, this was the only fraction corrected for contamination. The materials in the crucibles were ashed for 12-16 hours at 600°C in a muffle furnace. The ash was dissolved in either 1 or 2 ml of 1 N HCl and then diluted with a stock solution of lanthanum and strontium. The final concentrations in the samples were 0.20 mM HCl, 25 mM La, and 100 μM Sr; the samples were analyzed for calcium on an Instrumentation Laboratory model 153 atomic absorption spectrophotometer.

**Protein determinations.** The protein content of each fraction and of the 0.2 ml aliquot of homogenate was determined by the Lowry method (Lowry
et al., 1951) with human albumin as the standard.

**Electron micrographs.** The muscles and crude mitochondrial pellets from both control and caffeine-treated preparations were initially fixed in ice-cold 3% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4) and remained in this solution in a refrigerator for 24 hours. The specimens were then washed several times with the buffer solution and post-fixed for 1-2 hours in a 1% osmium tetroxide-Na cacodylate (0.1 M) solution. Dehydration was carried out with a series of alcohol washes and the specimens embedded in Epon. Sections were examined with a Zeiss or Hitachi electron microscope.
III. RESULTS

A. $^{45}\text{Ca}$ efflux studies

In the studies involving radiocalcium, muscles were labeled by injecting a $^{45}\text{Ca}$-Ringer solution into the ventral lymph sac of the animal rather than by the typical method of soaking the muscles for several hours in a $^{45}\text{Ca}$-Ringer solution. The $^{45}\text{Ca}$ was injected for several reasons. First, labeling occurs relatively rapidly; secondly, it eliminates a possible artifactitious distribution of calcium and $^{45}\text{Ca}$ which might arise from soaking the muscle for several hours in Ringer solution (Cosmos and Harris, 1961); and, finally, it is possible that a more uniform loading of individual fibers and subcellular organelles is achieved with the in-vivo technic. As can be seen in figure 2, a typical multi-function washout curve was generated even though a different loading procedure was employed (Harris, 1957; Shanes and Bianchi, 1959). However, in contrast to the more generally described two-component washout curve (Shanes and Bianchi, 1959), the curve presented in figure 2 appears to consist of three components. The unique component shown in figure 2 is the first fast one with a half-decay time ($T/2$) of 5 minutes; washout curves of muscles labeled by the soaking method do not appear to possess such a fast function. Unlike the in-vivo labeled muscles, those prepared in vitro are routinely rinsed with non-radioactive Ringer solution prior to the start of a study; therefore, the very fast component may be absent because of the initial rinse. The second fast component ($T/2 = 27$ minutes) and the slow component ($T/2 = 292$ minutes) are functions similar to those described by others (see Curtis, 1970). Shanes
and Bianchi (1960) suggested that the $^{45}$Ca constituting the former component is derived from extracellular fluid. They (1959) also proposed that the $^{45}$Ca constituting the slow component originates in the myoplasm. The latter component is of particular interest because most studies involving caffeine are conducted on this portion of the washout curve.

Figure 3 illustrates the effect of 10 mM caffeine on the efflux of $^{45}$Ca from the frog sartorius. The drug produced a substantial and sustained increase in the exchange of calcium, reflected as an increased rate of $^{45}$Ca efflux, during the slow component of the washout curve. The response to caffeine is comparable to that reported by other investigators using in-vitro labeled muscles (Bianchi, 1961). If the $^{45}$Ca washout functions of the whole muscle could be correlated with the $^{45}$Ca washout of various cell fractions, then a crude isolation of the locus of action of caffeine could be achieved. For this purpose, muscles were homogenized at selected time intervals along the washout curve and the homogenates were subjected to the differential centrifugation scheme presented in figure 1. If a washout fraction is extracellular in origin, as has been postulated, there should be a change in the pattern of the distribution of $^{45}$Ca in the isolated fractions with time. The final supernatant, fraction IV, probably contains the $^{45}$Ca of the extracellular space and therefore should account for a progressively smaller proportion of the total radiocalcium remaining in the muscle. Similarly, if $^{45}$Ca emerging from the muscle during the slow component is derived from the myoplasm, it is
possible that the $^{45}\text{Ca}$ is localized in discrete calcium pools associated with specific subcellular structures. Curtis (1970) proposed that the slow component of the washout curve, such as the one seen in the present study, represents the $^{45}\text{Ca}$ from the longitudinal reticulum. In the fractionation scheme employed, shown in figure 1, a large proportion of the longitudinal reticulum is assumed to appear as the membrane fragments in fraction III, the microsomal fraction. If such a discrete pool does serve as the primary source for this nuclide during the slow component of the washout curve, then the $^{45}\text{Ca}$ distribution should confirm the existence of such a source.

Figure 4 presents the results of the fractionation experiments. It is evident that the percent distribution of $^{45}\text{Ca}$ activity in the fractions at different periods of time along the curve does not change markedly. As a consequence, all of the fractions, even at three hours, still contribute substantially to the total activity remaining in the muscle. From these data, it is impossible to relate any of the fractions to any of the functions plotted in figure 2. One point is clear: the source of the radioactivity contributing to the development of the efflux curve from the sartorius muscle is complex. In contrast to the proposition of some investigators (c.f. Gilbert and Fenn, 1957), Harris maintains that there may be no justification for interpreting calcium movements in terms of a small number of separate first-order processes. The results presented in figure 4 would seem to support Harris’s view. Because it was not possible to correlate the washout of $^{45}\text{Ca}$ from cellular fractions with the
washout from muscle, and because caffeine is thought to produce changes in
the intracellular state of calcium (in addition to increasing calcium efflux),
another approach to the isolation of the locus of action of caffeine was under­
taken. For this purpose, the intracellular distribution of calcium in muscle
and the effect of caffeine on the distribution were investigated.

B. Calcium and protein distribution studies

Table 1 summarizes the results of experiments on freshly dissected
frog sartorius muscles. The various fractions of fresh muscles were ana­
lyzed for their calcium, $^{45}\text{Ca}$, and protein content. The determinations were
carried out during the months of August, September, and October. The rou­
tine determination of recoveries was included in the study for several reasons:
first, it provided a check on experimental technic; secondly, it allowed the
pooling of comparable experiments; and finally, it substantiated the differ­
ences in calcium, $^{45}\text{Ca}$, and protein distributions among the various frac­
tions.

The value for total muscle calcium in the present study (242 nmoles/100
mg muscle) is higher than some previously reported values (e.g., Gilbert
and Fenn, 1957; Cosmos, 1958); this discrepancy may be attributable to the
difference in calcium-measurement technic (atomic absorption versus titra­
tion, colorimetric, or emission flame photometry), or to a difference in
frogs. However, in the present data there is neither appreciable difference
in total tissue calcium between summer (table 1) and winter (table 2) frogs
nor between frogs from different suppliers.

An analysis of the intracellular distribution of protein and calcium indicates that fractions II and III together account for only 8% of the total tissue protein, but they account for nearly 40% of the total calcium. Figure 5 illustrates the relatively high concentration of calcium in these two fractions. In the experiments to determine the effects of 10 mM caffeine on the content and distribution of calcium, $^{45}$Ca, and protein in the sartorius, the muscles were washed for one hour before they were homogenized. The one-hour wash was initiated in the $^{45}$Ca experiments in order to minimize differences in the starting level of radioactivity of paired muscles; indeed, the total muscle $^{45}$Ca activity presented in table 2 verifies the similar starting levels of radioactivity. To assure uniformity in the preparations, a one-hour wash was also used in the calcium and protein experiments.

Tables 2 and 3 summarize the results of the various intracellular determinations. The experiments were done during the months of November through February. The most salient feature of the data in table 2 is the significant change in the absolute amounts of calcium in fractions II and III after the muscle was exposed to 10 mM caffeine for 7 minutes. In fraction II, the total calcium content increased approximately 50%, while the protein content decreased 50%. Fraction III lost approximately 30% of its calcium; however, its protein content did not change radically. Caffeine appears to have caused a quantitative calcium shift from the microsomal fraction (III) to the
mitochondrial fraction (II). The percent distribution of calcium, $^{45}$Ca, and protein among the various fractions before and after caffeine exposure is provided in table 3.

Relative specific activity values (figure 6) were obtained by calculating the ratio of the percent distribution of $^{45}$Ca to the percent distribution of total calcium. (The values calculated this way are essentially identical to the values obtained if one determines the true specific activity in terms of dpm/m mole of calcium for each fraction and then adjusts the values to the specific activity of the whole muscle.) Fractions I and IV have considerably greater relative specific activities than do fractions II and III, the mitochondrial and microsomal fractions, respectively. A greater relative specific activity in fractions I and IV connotes a more rapid exchange or turnover of calcium than that in the other two fractions. From the data on $^{45}$Ca distribution at various times along the washout curve there was no evidence of different calcium turnover rates in the fractions. Caffeine did not alter the relative specific activity of any of the fractions; therefore, the increase in calcium in fraction II after caffeine treatment was apparently derived from fraction III.

The problem of simple redistribution is always present in any homogenization and fractionation procedure and has been discussed by others (Griswold and Pace, 1956; Cassidy et al., 1969); however, in this study redistribution does not seem to be a significant factor after a muscle is homogenized and fractionated. If simple redistribution occurs, large amounts of
calcium would be released into the homogenizing solution and then would be found with the bulk of the cell material (fractions I and IV); relatively lesser amounts would be found in the remaining smaller fractions. The results of such a redistribution would be equal specific activities of the fractions. The relative specific activity values in figure 6 do not support the occurrence of redistribution. In addition, the values for the total calcium and protein content of fractions II and III (table 2, figure 5) make it clear that there is a concentration of calcium in these two fractions. After exposure to caffeine, a noticeable shift in the intracellular distribution of the ion occurs but is unaccompanied by a corresponding shift in protein. Because caffeine produces measurable changes in the intracellular distribution of calcium, the argument against simple redistribution after homogenization is further strengthened.

C. Morphological changes after caffeine treatment

Caffeine treatment results in a gross change in the appearance of the fraction II pellet. The caffeine pellet is much smaller in size, and a layer of material normally visible as a translucent "halo" around the central core of the pellet is absent. Several density gradient experiments were conducted on the fraction II pellet obtained from control and caffeine-treated muscles. After isolating the pellets by the procedure described in the Methods, the supernatants were removed with a needle and syringe and the pellets resuspended in 1 ml of 0.25 M solution of sucrose. The resuspensions were layered on a discontinuous gradient consisting of 0.5 ml of 1.0 M and 0.5 ml
of 1.5 M sucrose solution. The tubes were inserted into adapters, placed into a Beckman SW39L rotor, and spun for 12 hours at 25,000 x g. In the control preparation, a pellet was produced at the bottom of the centrifuge tube and a band of material was formed at the junction of the 1.0 M and 1.5 M sucrose layers. In contrast, no bottom pellet was recovered from the caffeine-treated preparation; also the band formed at the junction of the sucrose gradient was much wider than the one produced in the control tube and, in fact, appeared to be two separate bands. Thus there was some change in the physical characteristics of the mitochondrial fraction after the muscle had been exposed to caffeine.

Because of the change in the physical character of the fraction II pellet, it was examined with an electron microscope. In addition, because of the pronounced change effected by caffeine in the functional state of a muscle, the structure of the intact muscle after exposure to caffeine was also examined under the electron microscope (figures 7-9). Electron micrographs of the control mitochondrial fraction (fraction II) are shown in figure 10 and illustrate the heterogeneity of the elements present. Contained in the fraction are numerous small fragments of membranes and clumps of what appear to be myofilaments, in addition to mitochondria. After caffeine treatment, fraction II, illustrated in figure 11 contains many granular vesicles and an occasional spiral-shaped element, as well as its normal constituents. The vesicular and spiral elements seem to have their origin in
morphologically similar structures present in the intact muscle fiber; they apparently originate from the sarcoplasmic reticulum, as shown in figures 8 and 9. Another feature of the caffeine-treated muscle is damaged mitochondria which also occur in the fraction II pellet from the treated muscle. Conway and Sakai (1960) reported that caffeine-induced contracture severely damaged muscle fibers; however, they did not describe the nature of the damage. The extensive structural alterations observed in the present study may well account for the irreversible character of the caffeine contracture.
IV. DISCUSSION

The extensive documentation of the effect of caffeine on the movement of skeletal muscle calcium suggests that its effect on contractility is the consequence of an action on the intracellular state of calcium; that is, it is believed to cause an increase in the concentration of ionized calcium in the sarcoplasm. In the experiments described here, attempts were made to identify the locus of the subcellular action of caffeine on calcium; in other words, to localize a subcellular pool of calcium affected by the action of caffeine.

In the initial experiments, the washout of $^{45}$Ca from the major cellular fractions was studied. The purpose was to determine the relationship between the $^{45}$Ca washout from different cellular fractions and the different components characteristic of the washout curve obtained from whole muscle. The data indicate that the percent distribution of $^{45}$Ca in the isolated fractions does not change appreciably with changes in the total muscle $^{45}$Ca (figures 2 and 4). Therefore, these results do not reveal any simple pools within the muscle serving as a source of $^{45}$Ca for the major functions in the washout curve. Because of the inability to correlate changes in the distribution of $^{45}$Ca with washout functions, it is also not possible to relate the effect of caffeine on the calcium efflux to any specific cell fraction.

As described above, it is not feasible to ascribe the muscle washout functions to the turnover of calcium in any of the cellular fractions. However, the various cellular fractions do appear to contain distinctly
different pools of calcium, exemplified by the lower relative specific activities of the mitochondrial and microsomal fractions as compared with the other two fractions (figure 6). The physiological significance of intracellular pools of calcium with different turnover rates is unknown.

The experiments designed to determine the influence of caffeine on the intracellular distribution of calcium yielded the first information concerning the relative distribution of calcium in skeletal muscle. As has already been pointed out, the crude mitochondrial and microsomal fractions (fractions II and III, respectively) contain more than one third of the total calcium in the muscle; in addition, on the basis of the protein content of the fractions, there is a pronounced concentration of calcium in fractions II and III. The latter feature would implicate the structures making up the fractions in the regulation of calcium ion levels within the muscle cell. Exposure of a muscle to caffeine resulted in a conspicuous exchange of calcium between fractions II and III; the amount of calcium gained by the former can be accounted for by the loss from the latter. As can be seen in figure 6, caffeine does not produce a change in the specific activity in any of the fractions despite the shift in calcium. The initial conclusion drawn on the basis of the observed movements of calcium and $^{45}$Ca was that caffeine had released calcium from the elements which make up the microsomal fraction, and the released calcium was then accumulated by the mitochondria. Nevertheless, such an interpretation of the observed shift in calcium may not be
correct. The interpretation is complicated because of the notable decrease in the size as well as in the protein content of the fraction II pellet. The electron micrographic analyses of the fraction II pellet and the whole muscle illustrated striking structural changes produced by caffeine treatment. Mitochondria were damaged, numerous granular vesicles appeared, and some bizarre spiral-shaped structures were formed. The structural changes probably resulted in an altered fractionation pattern of the subcellular elements.

The electron micrographic observations suggest two explanations for the changes in the intracellular distribution of calcium induced by caffeine. One is that the increase in calcium in fraction II is a consequence of the appearance of relatively large granular vesicles apparently derived from structures normally found in fraction III. The granular character of the caffeine-induced vesicles is similar to that of the lateral sacs and terminal cisternae of the sarcoplasmic reticulum. Normally, the sarcoplasmic reticulum is considered to be represented as small vesicles in fraction III. If the increase in calcium in fraction II is a consequence of a shift in a structural component from fraction III, then the concentration of calcium in this component must be very high. The observation of a large decrease in calcium from fraction III accompanied by a small decrease in protein would tend to support the above conclusion.

A second explanation for the increase in the calcium content of fraction
II is that the mitochondria have actually accumulated the calcium. Treatment does produce pronounced morphological changes in the mitochondria of both the whole muscle and fraction II; many mitochondria are swollen and their cristae are in different states of dissolution. The changes in the mitochondria are of special interest. The ability of these structures to accumulate substantial amounts of calcium in vitro is well documented (e.g., Lehninger et al., 1967). It remains to be demonstrated in vivo whether their ability to accumulate calcium is of physiological significance. When mitochondria accumulate calcium in vitro, they undergo perceptible morphological changes; most notable are swelling and marked changes in the cristae (Greenawalt et al., 1964). Similar morphological changes have been found in vivo in the mitochondria of severely exercised skeletal muscle (Gollnick and King, 1969) and in the mitochondria of nerve terminals after extensive periods of stimulation (Jones and Kwanbunbumpen, 1970). Because calcium is important in muscular contraction, in transmitter release, and presumably in the action of caffeine on muscle contractility, it is conceivable that the alterations in the mitochondria observed in the above-cited studies and in the present investigation are related to a change in their calcium content.

Experimentally it may be possible to resolve the problem of which structure in fraction II accounts for the caffeine-induced increase in calcium. The density gradient experiments described in the Results tend to
indicate that this approach may be a useful technic for subfractionation of fraction II, particularly in order to isolate the mitochondria from the other elements.
LITERATURE CITED


Caravalho, A. P. 1968 Calcium-binding properties of sarcoplasmic reticulum as influenced by ATP, caffeine, quinine, and local anesthetics. J. Gen. Physiol., 52: 622-642.


### TABLE 1
Intracellular distribution of $^{45}$Ca, calcium, and protein in freshly dissected frog sartorius muscle

<table>
<thead>
<tr>
<th>Fractions†</th>
<th>$^{45}$Ca</th>
<th>Calcium</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/100 mg muscle† (N = 7)</td>
<td>% distribution (N = 7)</td>
<td>nmoles/100 mg muscle† (N = 7)</td>
</tr>
<tr>
<td>I</td>
<td>98,058 ±19,100</td>
<td>32.6 ±3.3</td>
<td>57.2 ±1.8</td>
</tr>
<tr>
<td>II</td>
<td>32,822 ±4,503</td>
<td>11.3 ±0.8</td>
<td>25.4 ±0.9</td>
</tr>
<tr>
<td>III</td>
<td>74,066 ±12,112</td>
<td>24.8 ±1.1</td>
<td>69.7 ±3.4</td>
</tr>
<tr>
<td>IV</td>
<td>92,729 ±13,702</td>
<td>31.3 ±1.1</td>
<td>83.2 ±3.3</td>
</tr>
<tr>
<td>Total muscle</td>
<td>311,900 ±44,840</td>
<td>242.5 ±10.6</td>
<td>24.6 ±10.6</td>
</tr>
<tr>
<td>Recovery values</td>
<td>95.1 ±2.2</td>
<td>100.3 ±2.2</td>
<td></td>
</tr>
</tbody>
</table>

N = number of observations  
† wet weight of muscle  
‡ Values in table represent the mean ± S.E.M.
TABLE 2

Effects of caffeine on the intracellular distribution of $^{45}$Ca, calcium, and protein in one-hour washed muscles

<table>
<thead>
<tr>
<th>Fraction ‡</th>
<th>Control (N = 6)</th>
<th>Caffeine (N = 6)</th>
<th>Δ</th>
<th>Control (N = 8)</th>
<th>Caffeine (N = 8)</th>
<th>Δ</th>
<th>Control (N = 8)</th>
<th>Caffeine (N = 8)</th>
<th>Δ</th>
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<tbody>
<tr>
<td>Total muscle</td>
<td>28,482 ± 5,899</td>
<td>26,237 ± 5,533</td>
<td>-2,245</td>
<td>221.8 ± 3.5</td>
<td>222.0 ± 8.0</td>
<td>+0.1</td>
<td>25.1 ± 0.9</td>
<td>23.0 ± 0.8</td>
<td>-2.1</td>
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<tr>
<td>I</td>
<td>9,330 ± 1,535</td>
<td>9,449 ± 1,913</td>
<td>119</td>
<td>69.0** ± 3.5</td>
<td>71.4 ± 4.6</td>
<td>+2.4</td>
<td>16.6 ± 0.6</td>
<td>14.8 ± 0.4</td>
<td>-1.8</td>
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<tr>
<td>II</td>
<td>1,934 ± 294</td>
<td>2,636 ± 378</td>
<td>+702</td>
<td>29.0 ± 1.8</td>
<td>43.1 ± 3.8</td>
<td>+14.1*</td>
<td>0.4 ± 0.04</td>
<td>0.2 ± 0.01</td>
<td>-0.2*</td>
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<td>III</td>
<td>2,953 ± 446</td>
<td>2,238 ± 387</td>
<td>-715</td>
<td>53.6 ± 1.4</td>
<td>38.8 ± 1.5</td>
<td>-14.9*</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>-0.1</td>
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<tr>
<td>IV</td>
<td>14,708 ± 4,351</td>
<td>13,141 ± 3,174</td>
<td>-1,567</td>
<td>76.5 ± 4.5</td>
<td>74.9 ± 3.7</td>
<td>-1.6</td>
<td>4.9 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>-0.1</td>
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</table>

N = number of observations
‡ Values in table represent the mean ± S. E. M.
† wet weight of muscle

** N = 7
* P < 0.05
TABLE 3

Effects of caffeine on the % distribution of $^{45}$Ca, calcium, and protein recovered in the fractions from one-hour washed muscles

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$^{45}$Ca (% distribution)</th>
<th>Calcium (% distribution)</th>
<th>Protein (% distribution)</th>
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<tbody>
<tr>
<td></td>
<td>Control (N = 6)</td>
<td>Caffeine (N = 6)</td>
<td>Control (N = 7)</td>
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<tr>
<td>Recovery values</td>
<td>100.5 ±2.2</td>
<td>104.5 ±1.8</td>
<td>+3.8</td>
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<tr>
<td>I</td>
<td>33.6 ±1.6</td>
<td>34.6 ±1.0</td>
<td>+1.0</td>
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<tr>
<td>II</td>
<td>7.3 ±0.9</td>
<td>10.3 ±1.0</td>
<td>+3.0*</td>
</tr>
<tr>
<td>III</td>
<td>11.0 ±1.1</td>
<td>8.4 ±0.5</td>
<td>-2.6*</td>
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<tr>
<td>IV</td>
<td>48.1 ±2.8</td>
<td>46.7 ±1.8</td>
<td>-1.4</td>
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N = number of observations

* Values in table represent the mean ± S.E.M.

*P < 0.05
Figure 1. Differential centrifugation scheme of the fractionation studies.
HOMOGENATE

(muscle homogenized in 0.25 M sucrose-0.05 M tris-buffer solution and a 0.2 ml aliquot removed for determination of recovery value)

5' at 1,100 x g

SUPERNATANT

FRACTION I
(crude nuclear fraction)

10' at 9,000 x g

SUPERNATANT

FRACTION II
(crude mitochondrial fraction)

30' at 145,000 x g

FRACTION IV
(soluble fraction)

FRACTION III
(crude microsomal fraction)
Figure 2. $^{45}$Ca washout curve from frog sartorius muscle. Each point is the mean of 6 experiments. The graphically determined T/2 values correspond to the half-times of the individual first-order functions.
The graph shows the decay of $^{45}$Ca in muscle (as a percentage of initial activity) over time. The half-lives are indicated as $T/2 = 5'$, $T/2 = 27'$, and $T/2 = 292'$. The time axis is labeled as "TIME (MIN)" with values ranging from 0 to 240 minutes.
Figure 3. $^{45}$Ca efflux curve illustrating the effect of caffeine on the movement of $^{45}$Ca from the frog sartorius. The efflux of $^{45}$Ca is plotted as a rate coefficient which is determined as follows:

\[
\text{Rate coefficient} = \frac{\text{collected activity}}{\text{mean tissue activity} \times t} \times 100
\]

\( t \) = interval between collections (in minutes)
Figure 4. Distribution of $^{45}\text{Ca}$ in the isolated fractions at various times along the $^{45}\text{Ca}$ washout curve. Values represent the mean of $N$ experiments $\pm$ S.E.M. $N = 7$ at zero time; $N = 4$ at each of the remaining times.
Figure 5. Calcium concentration in the cellular fractions. The calcium/protein ratios are given with their 95% confidence limits. The ratios are calculated from the mean value of calcium and protein for each fraction given in tables 1 and 2.
Figure 6. Relative specific activity of the isolated fractions (% distribution of $^{45}$Ca/ % distribution of calcium). The calculated values are given along with their 95% confidence limits. Calculations were based on the data in table 3.
Figure 7. Longitudinal section of a control muscle. The terminal
cisternae (tc), mitochondria (m), transverse tubules (tt), and
the longitudinal tubules (lt) are labeled in the micrograph.
$\times 40,000$. 
Figure 8. Longitudinal section of a caffeine-treated muscle. x 40,000.

A. Swollen mitochondria (m) illustrating the marked changes in the cristae appearing after the muscle has been exposed to 10 mM caffeine for 7 minutes. B. Granular vesicles (gv) are formed throughout the muscle after caffeine treatment. Note the convoluted membranes of the sarcoplasmic reticulum (arrows).
Figure 9. Transverse sections through the frog sartorius. x 40,000.

A. Control muscle illustrating the longitudinal tubules (lt), mitochondria (m), and terminal cisternae (tc). B. Caffeine-treated muscle. Notice the spiral-shaped convolutions of the sarcoplasmic reticulum (arrow). C. Caffeine-treated muscle. Swollen mitochondria (m) similar to those in figure 8A.
Figure 10. Fraction II (crude mitochondrial pellet) obtained from a control muscle. Note the heterogeneity of the pellet. Mitochondria (m), small vesicles (v), and what appear to be clumps of myofilaments (mf) are evident throughout the field. $\times$ 30,000.
Figure 11. Fraction II pellet from a caffeine-treated (10 mM) muscle. x 30,000. A. In addition to mitochondria (m), numerous small vesicles are present along with spiral-shaped elements (arrow) which are occasionally seen in this pellet and are apparently derived from similar structures in the whole muscle; see figure 9B. B. The granular nature of the vesicles (arrows) is illustrated in this section.
**VITA**

<table>
<thead>
<tr>
<th><strong>Name</strong></th>
<th>Henry K. Borys</th>
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<tbody>
<tr>
<td><strong>Birthplace</strong></td>
<td>Chicago, Illinois</td>
</tr>
<tr>
<td><strong>Birthdate</strong></td>
<td>October 5, 1940</td>
</tr>
<tr>
<td><strong>Secondary Education</strong></td>
<td>East Leyden Community High School, Franklin Park, Illinois</td>
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<tr>
<td><strong>College and University</strong></td>
<td>Montana State University, Missoula, Montana; September 1958-June 1959</td>
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<tr>
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<td></td>
<td>Graduated cum laude</td>
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<tr>
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<td>National Science Foundation Summer Trainee, 1967</td>
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Publications:


