# Changes in Oxalate-Stimulated Calcium Accumulation in Particulate Fractions from Post-Mortem Muscle

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This study was conducted to determine the changes that occur in the calcium accumulating ability of various subcellular fractions of post-mortem porcine muscle. The ability to accumulate calcium dropped markedly with increasing time post-mortem, with a 40% decrease occurring in the heavy sarcoplasmic reticulum fraction by 3 hours. At 24 hours post-mortem, activities of all fractions had fallen to 10% or less of initial values. However, electron microscopic observations of the vesicular fractions demonstrated no differences. The possible relationship of this inactivation to pH change and to rigor mortis is discussed.

Since the first description of a relaxing factor in muscle (Marsh, 1951, 1952), a considerable amount of information about its composition and mode of action has been obtained. This particulate relaxing factor consisted of microsomal material from muscle homogenates which could be separated by differential centrifugation (Bendall, 1958; Kumagai *et al.*, 1955; Portzehl, 1957) and was thought to be fragments of the sacroplasmic reticulum (Ebashi and Lipmann, 1962; Muscatello *et al.*, 1961; Nagai *et al.*, 1960). The discovery that these reticular fragments could actively remove $Ca^{+2}$ from solution led to the postulation that relaxation was caused by reducing the $Ca^{+2}$ concentration in the region of the myofibrils to a concentration below the critical level necessary for contraction (Ebashi, 1961; Hasselbach and Makinose, 1961; Weber *et al.*, 1963). This theory is consistent with observations that intracellularly injected $Ca^{+2}$ elicits contraction (Caldwell and Walster, 1963; Heilbrunn and Wiercinski, 1947; Niedergerke, 1955), and that isolated myofibrils, natural actomyosin, and glycerinated muscle fibers contract and relax as a function of the $Ca^{+2}$ concentration of the medium (Ebashi, 1961; Weber, 1959; Weber and Herz, 1962; Weber and Winicur, 1961).

Since most work concerning the relaxing factor has been concerned with its function in living muscle, little information is known about changes in its activity post-mortem. Relaxing factor material obtained from muscle immediately after death has been shown to lose its activity during storage at 0° C. and neutral pH (7.0 to 7.4), with losses of 0 to 50% reported during the first day after isolation (Ebashi and Lipmann, 1962; Lee *et al.*, 1965; Muscatello *et al.*, 1962; Seraydarian and Mommaerts, 1965). Normally, muscle pH drops to below 6.0 within a few hours after death and the muscle temperature remains fairly high during this period when the muscle is left in the carcass. Both pH and temperature possibly exert a detrimental effect on the relaxing factor. Also, the relaxing factor has been shown to affect myofibrillar ATPase activity markedly (Baird and Perry, 1960; Marsh, 1951, 1952). These observations suggest that there may be an appreciable loss of functional integrity of the relaxing factor system when left in situ and that this loss might have an effect on the rate of development of post-mortem changes, particularly the breakdown of ATP, the rate of anaerobic glycolysis, and the development of rigor mortis.

The present investigation was conducted to determine the stability of the relaxing factor in muscle post-mortem and to attempt to relate the calcium accumulating activity of the relaxing factor to post-mortem changes.

## METHODS AND MATERIALS

Muscle samples from the longissimus dorsi of six pigs were obtained within 5 minutes after exsanguination and at 1, 2, 3, and 24 hours post-mortem. Isolation procedures for the various sedimented fractions were essentially those of Martonosi and Feretos (1964). Samples were homogenized with 4 volumes of ice cold 0.1*M* KCl and 5m*M* histidine (pH 7.2) for 2 minutes in a Waring Blendor in 10- to 15-second bursts. Myofibrils (including nuclei, connective tissue, and undisrupted fiber segments) were sedimented by centrifugation at 1000 × G for 20 minutes. Mitochondria were removed from the supernate by centrifugation at 8000 × G for 20 minutes. The resulting supernate was then subjected to centrifugation at 30,000 × G for 1 hour and its supernate to 60,000 × G for 1 hour. The precipitate of the latter fractions will be referred to as heavy sarcoplasmic reticulum and light sarcoplasmic reticulum, respectively. The sedimented material was resuspended in 0.1*M* KCl–5m*M* histidine, and protein content was determined using the biuret procedure (Gornall *et al.*, 1949) with bovine serum albumin as a standard.

Measurement of $Ca^{+2}$ uptake was performed in a medium consisting of 0.1*M* KCl, 5m*M* histidine (pH 7.2), 5m*M* $MgCl_2$, 5m*M* $K_2C_2O_4$, 5m*M* ATP, 0.1m*M* $CaCl_2$ (containing 0.1 μc. of $^{45}Ca^{+2}$), and with protein concentration sufficiently limited so that the final free $Ca^{+2}$ concentration never dropped below $10^{-5}M$ during the assay. The approximate protein concentrations used were as follows: myofibrils, 0.5 mg. per ml.; mitochondria, 0.05 mg. per ml.; heavy sarcoplasmic reticulum, 0.03 mg. per ml.; and light sarcoplasmic reticulum, 0.05 mg. per ml. Following the addition of the resuspended fractions to the assay medium and incubation for 15 minutes at 22° to 24° C., the particulate material was removed by

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filtration through Millipore filters—type HA, 0.45-micron average pore diameter (Martonosi and Feretos, 1964). The $Ca^{+2}$ uptake was determined by difference by transferring aliquots of the filtrates to stainless steel planchets and measuring the radioactivity of these samples and appropriate standards with a Nuclear Chicago thin end window Geiger counter.

Samples of mitochondria, heavy sarcoplasmic reticulum, and light sarcoplasmic reticulum were obtained at 0 and 24 hours post-mortem for electron microscopy. Pellets from centrifugation were fixed in 6.25% biological grade glutaraldehyde in 0.1*M* potassium phosphate (pH 7.4) for 2 hours at 0° C. The samples were then rinsed in 0.1*M* potassium phosphate (pH 7.4) and 0.2*M* sucrose for 2 hours. The pellets were then fixed in 1% osmic acid and 0.1*M* potassium phosphate for 1 hour, dehydrated in a graded series of ethanol-water mixtures, and embedded in an Epon-araldite mixture. Sections were cut with a Sorvall Porter-Blum ultramicrotome, mounted on uncoated copper grids, stained for 50 to 60 minutes with a 5% uranyl acetate solution, and examined in a Siemens Elmskop I electron microscope at an accelerating voltage of 80 kv.

Following exsanguination and evisceration, the carcasses were stored in a 4° C. cooler beginning about 30 minutes post-mortem and held at this temperature through the remaining sample periods. Muscle pH was determined by use of a glass electrode placed directly on the freshly cut surface of the muscle (Briskey, 1964). All muscle fractions were maintained at 0° to 4° C. following homogenization. Distilled water which had been passed through a mixed-bed ion exchange column was used throughout the study. Dowex 50X-8 ($H^+$ form) was used to remove any contaminating $Ca^{+2}$ present in the ATP (Seidel and Gergely, 1963). Assays of $Ca^{-2}$ uptake for the samples taken at 0, 1, 2, and 3 hours post-mortem were conducted at the same time; those taken at 24 hours post-mortem were assayed a day later.

## RESULTS

Since the method of separating various cellular organelles by differential centrifugation rarely yields pure fractions, the total protein obtained in these fractions at the different times post-mortem was measured using the biuret procedure (Table I). If the heavier fractions became more fragile to the homogenization procedure at increasing times after death, a greater amount of protein might be obtained in the lighter fractions. Conversely, if the muscle fragments were disintegrated less easily during the latter post-mortem periods, the protein yields would decrease. The results indicate that protein content of the various fractions remained constant post-mortem for the most part with no statistically significant difference. Thus there did not appear to be an increase in protein content in the lighter fractions as a result of greater disintegration of the heavier fractions. The total protein yield of the most active $Ca^{+2}$ accumulating fraction (the heavy sarcoplasmic reticulum) was about 3 mg. per gram of muscle (Table I). This value is in agreement with reports on rabbit muscle from which yields of 1.5 to 3.0 mg. per gram have been reported (Ebashi and Yamanouchi, 1964; Inesi *et al.*, 1964; Seraydarian and Mommaerts, 1965; Weber *et al.*, 1964).

Calcium uptakes by the various subcellular fractions at the different times post-mortem were measured in a medium of 0.1*M* KCl, 5m*M* histidine, 5m*M* $MgCl_2$, 5m*M* $K_2C_2O_4$, 5m*M* ATP, and 0.1m*M* $CaCl_2$ (Table I). The most active fraction was the heavy sarcoplasmic reticulum, which accumulated 2.27 μmoles of $Ca^{+2}$ per mg. of protein. This value is comparable with rat and rabbit preparations which have been shown by other workers to be capable of accumulating 1.5 to 3.0 μmoles of $Ca^{+2}$ per mg. of protein in oxalate-stimulated systems (Carsten and Mommaerts, 1964; Hasselbach and Seraydarian, 1966; Martonosi and Feretos, 1964).

Calcium uptake values for mitochondria and light sarcoplasmic reticulum fractions were less than half those of the heavy sarcoplasmic reticulum fraction. The activity of the mitochondria fraction from pig muscle is similar to that found in a comparable preparation from rat muscle (Martonosi and Feretos, 1964). Although mitochondria from tissues other than muscle are known to be capable of removing $Ca^{+2}$ from solution (Carafoli *et al.*, 1965; Rossi and Lehninger, 1964), other workers have shown that purification of the mitochondrial fraction by zonal centrifugation gave a preparation that had no relaxing activity (Schuel *et al.*, 1965). Also, substances that completely suppress $Ca^{+2}$ accumulation by mitochondria from other tissues decrease the $Ca^{+2}$ uptake by the muscle mitochondrial fraction by less than 10% (Weber *et al.*, 1966). Preliminary work using the mitochondria in-

**Table I. Protein Content and Calcium Uptake of Subcellular Muscle Fractions**

| Fraction | Mg. Protein/G. Muscle [Mean(6) ± S.E.] Time Post-Mortem, Hours | | | | | μmoles $Ca^{+2}$/Mg. Protein [Mean(6) ± S.E.] Time Post-Mortem, Hours | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 24 | 0 | 1 | 2 | 3 | 24 |
| Myofibrils (1000 × G) | 192 ±7 | 197 ±10 | 183 ±11 | 181 ±6 | 184 ±7 | 0.03 ±0.01 | 0.03 ±0.01 | 0.02 ±0.01 | 0.02 ±0.01 | 0.01 ±0.01 |
| Mitochondria (8000 × G) | 2.83 ±0.19 | 2.61 ±0.15 | 2.57 ±0.24 | 2.77 ±0.15 | 2.70 ±0.31 | 1.12 ±0.21 | 1.02 ±0.15 | 0.92 ±0.20 | 0.59 ±0.13 | 0.12 ±0.03 |
| Heavy sarcoplasmic reticulum (30,000 × G) | 3.11 ±0.15 | 3.22 ±0.16 | 3.23 ±0.35 | 2.90 ±0.49 | 2.01 ±0.27 | 2.27 ±0.18 | 1.91 ±0.29 | 1.80 ±0.24 | 1.38 ±0.13 | 0.10 ±0.02 |
| Light sarcoplasmic reticulum (60,000 × G) | 1.33 ±0.14 | 1.25 ±0.09 | 1.06 ±0.07 | 0.88 ±0.08 | 0.96 ±0.15 | 0.81 ±0.14 | 0.58 ±0.07 | 0.65 ±0.12 | 0.36 ±0.11 | 0.08 ±0.02 |

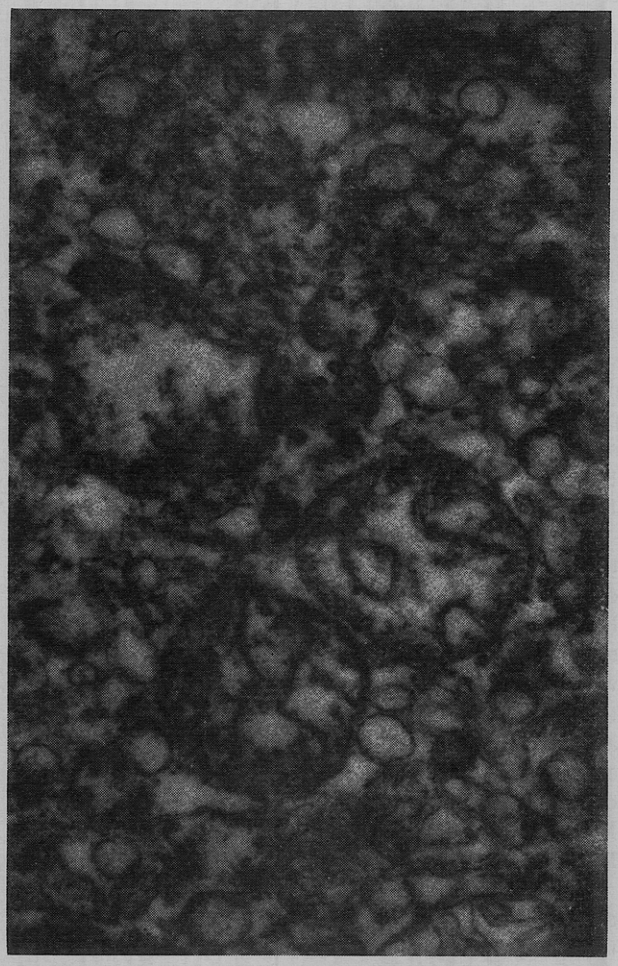

**Figure 1. 0-hour mitochondrial fraction**

Fixed in glutaraldehyde and post-fixed in osmic acid. Section stained with uranyl acetate. × 30,000

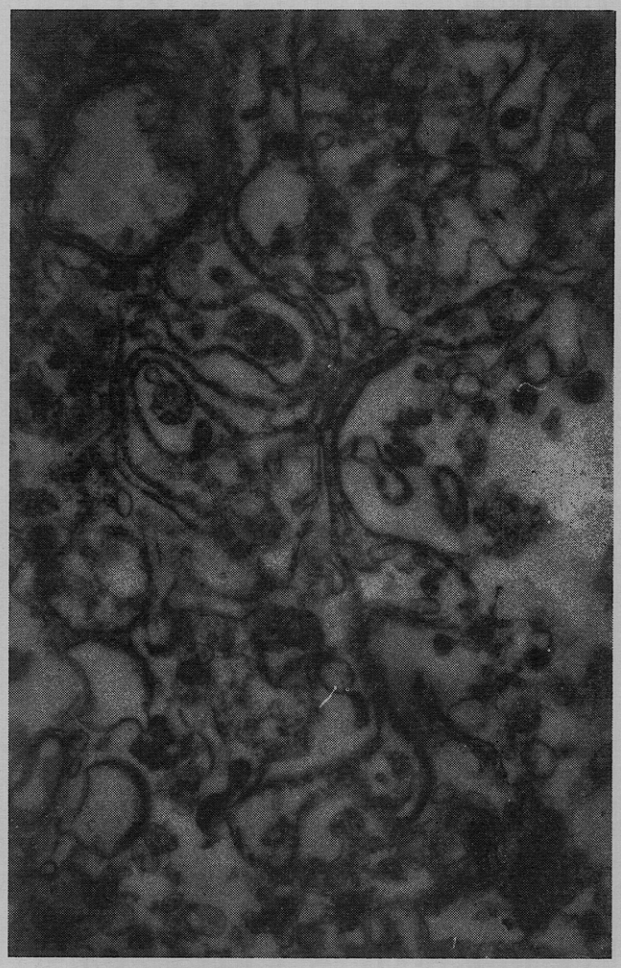

**Figure 2. 24-hour mitochondrial fraction**

Fixed in glutaraldehyde and post-fixed in osmic acid. Section stained with uranyl acetate. × 30,000

hibitor sodium azide showed an average of less than 5% reduction in calcium accumulation by the mitochondria fraction from three preparations. Thus, most of the $Ca^{+2}$ removing ability of the mitochondrial fraction apparently was due to contamination by the larger fragments of the sarcoplasmic reticulum. The myofibrillar fraction had very little ability to accumulate $Ca^{+2}$ and this ability also may have been due to residual sarcoplasmic reticulum contamination which has been shown to be present in myofibrils (Weber and Herz, 1962; Briggs and Fuchs, 1963).

A marked decrease in activity of the three most active $Ca^{+2}$ accumulating fractions with increasing time post-mortem was noted. Myofibrillar values were so low that the validity of comparisons between different time periods is questionable. In the heavy sarcoplasmic reticulum fraction, an approximately 40% decrease in $Ca^{+2}$ accumulating ability occurred during the first 3 hours post-mortem. An even larger decrease in $Ca^{+2}$ binding of mitochondrial and light sarcoplasmic reticulum fractions was observed.

By 24 hours post-mortem, $Ca^{+2}$ accumulating activity had fallen to very low levels. Less than 5% of the initial activity remained in the heavy sarcoplasmic reticulum fraction. Mitochondrial and light sarcoplasmic reticulum values for $Ca^{+2}$ binding were about 10% of those obtained from these fractions immediately after death.

The pH values obtained by use of a glass electrode at the various post-mortem times (six tests) are:

| 0 hour | 1 hour | 2 hours | 3 hours | 24 hours |
|---|---|---|---|---|
| 6.6 | 6.3 | 6.0 | 5.8 | 5.5 |

By 3 hours after death, muscle pH had fallen to a level only slightly higher than its ultimate pH (24-hour value).

Electron micrographs of the mitochondrial fraction at 0 and 24 hours post-mortem are shown in Figures 1 and 2. Identifiable mitochondria in the 0-hour sample were swollen with few cristae remaining (Figure 1). The poor preservation was probably due in part to the hypotonicity of the homogenization medium.

There was a considerable contamination of the mitochondrial fraction by vesicular and tubular elements, myofibril pieces, and nuclei. The large numbers of sacs and vesicles could easily account for the $Ca^{+2}$ uptake by this fraction and supports the conclusions of other workers that muscle mitochondria themselves may not play a major quantitative role in the $Ca^{+2}$ uptake of this fraction under the conditions studied (Weber *et al.*, 1966; Schuel *et al.*, 1965).

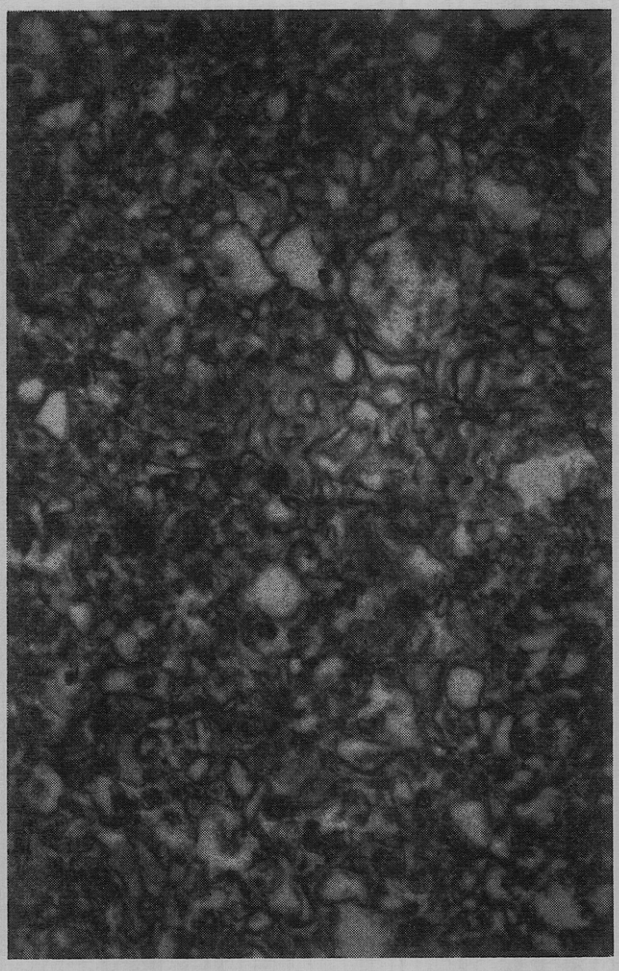

**Figure 3. 0-hour heavy sarcoplasmic reticulum fraction**

Fixed in glutaraldehyde and post-fixed in osmic acid. Section stained with uranyl acetate. × 30,000

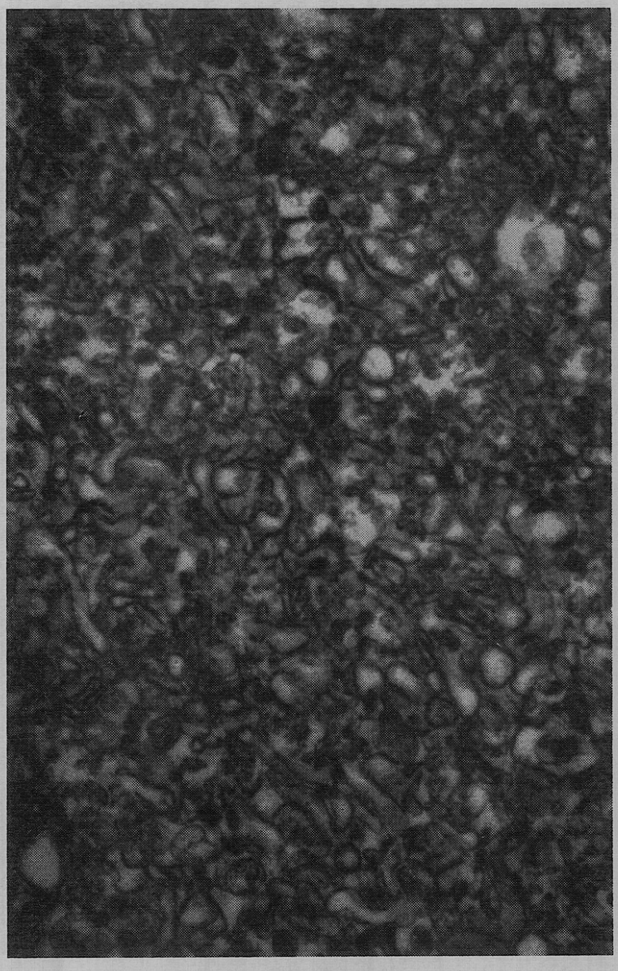

**Figure 4. 24-hour heavy sarcoplasmic reticulum fraction**

Fixed in glutaraldehyde and post-fixed in osmic acid. Section stained with uranyl acetate. × 30,000

The 24-hour mitochondria fraction appeared considerably different from that of the initial sample. Intact mitochondria were seldom present. An interesting feature of this fraction was the presence of a large number of extremely long membrane fragments which were of unknown origin. This fraction also showed extensive contamination with smaller vesicular materials.

The heavy sarcoplasmic reticulum fraction is shown in Figure 3. It consisted mainly of vesicles of various sizes and shapes and of some tubular elements. A few of the larger vesicles may have been of mitochondrial origin. The general appearance of this fraction was similar to preparations from rabbit muscle made by other workers (Nagai *et al.*, 1960; Sreter, 1964; Ebashi and Lipmann, 1962).

Figure 4 is a micrograph of the 24-hour heavy sarcoplasmic reticulum fraction. There appeared to be no morphological difference between the vesicles in this fraction and those obtained immediately post-mortem. There was also no difference in the proportion of membranous to nonmembranous material between the two sample time periods. This was an unexpected result, in light of the 10-fold decrease in $Ca^{+2}$ accumulating ability between 0 and 24 hours. Thus, the loss of $Ca^{+2}$ accumulating ability in vesicular fractions post-mortem apparently is due to an inactivation of the $Ca^{+2}$ pump and not to vesicular disintegration or extraneous protein contamination.

The light sarcoplasmic reticulum fractions at 0 and 24 hours post-mortem are shown in Figures 5 and 6. This fraction was extremely variable in nature, but usually contained some small vesicular elements, a large proportion of granular material which may have been of glycogen or ribosomal origin, and some filamentous material. Again there were no apparent consistent differences between samples at different time periods.

## DISCUSSION

The data presented demonstrates that $Ca^{+2}$ accumulating ability decreases rapidly in all the particulate fractions of muscle with increasing time post-mortem. The cause of this decrease is unknown. An obvious possibility is the effect of decreasing pH, since many enzymes are known to be denatured by changes in acidity. Other workers have shown that the relaxing factor is inactivated by pH values of less than 4.5 and greater than 9.0 (Marsh, 1952; Ebashi, 1958; Hasselbach, 1964a). The lowest muscle pH obtained was about 5.5 which does not fall very close

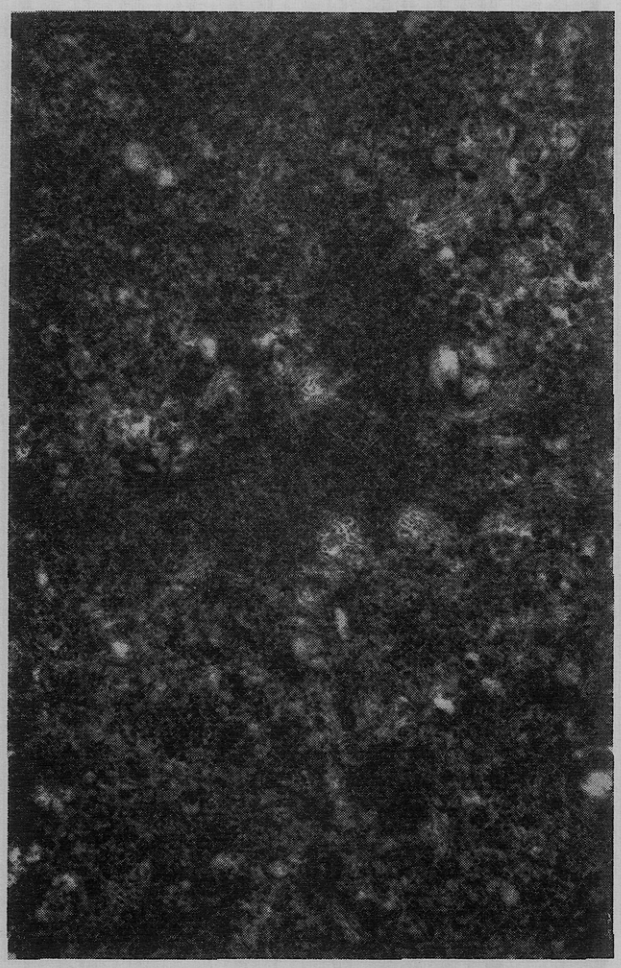

**Figure 5. 0-hour light sarcoplasmic reticulum fraction**

Fixed in glutaraldehyde and post-fixed in osmic acid Section stained with uranyl acetate. × 30,000

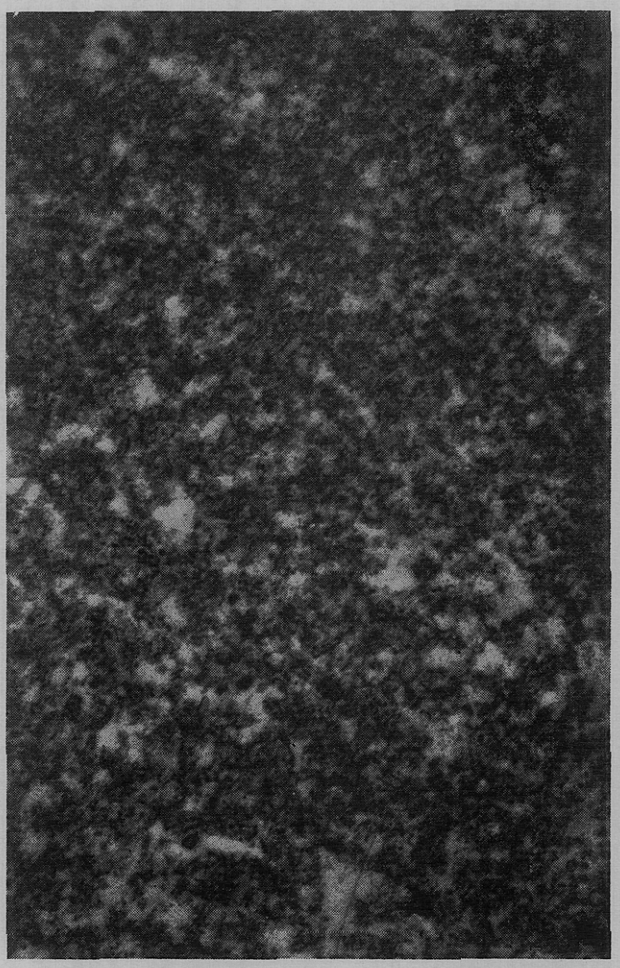

**Figure 6. 24-hour light sarcoplasmic reticulum fraction**

Fixed in glutaraldehyde and post-fixed in osmic acid. Section stained with uranyl acetate. × 30,000

to the region of complete inactivation. However, destruction of activity may proceed slowly in this higher pH range and be aided by the reasonably high muscle temperature which is maintained for several hours post-mortem (Briskey, 1964). The largest decrease in $Ca^{+2}$ accumulating ability during the first 3 hours post-mortem occurred during the final hour of this period when the average muscle pH was below 6.0. There also appeared to be a high correlation between decline of activity and rate of pH decline when individual animals were compared. Thus the increasing muscle acidity post-mortem may play an important role.

Whether the inactivation of the relaxing factor in post-mortem muscle is related to the process of rigor mortis is not known. Recent work with $^{45}Ca^{+2}$-labeled frog muscle indicated that there was an increased outflux of label into the bathing medium at the time of development of rigor mortis (Nauss and Davies, 1966). This outflux could have been due to relaxing factor inactivation or inability of the calcium pump to function in the absence of ATP.

Also, muscle is known to contract as it passes into rigor (Nauss and Davies, 1966), perhaps indicating a release of $Ca^{+2}$ by the relaxing factor. Other work on porcine muscle with post-mortem pH patterns similar to those in the present study suggests that loss of extensibility would just begin to occur at 3 hours post-mortem (onset of rigor), and that total loss of extensibility would occur at about 5 hours post-mortem (completion of rigor) (Briskey, 1964). Thus the present study does not give information on relaxing factor activity immediately before and after the completion of rigor. The fact that a greater than 10-fold decrease in activity of the most active fraction (heavy sarcoplasmic reticulum) occurred between 3 and 24 hours post-mortem suggests that this aspect should be further investigated.

One should question the validity of inferring from the oxalate-stimulated $Ca^{+2}$ accumulation by the isolated fractions to the conditions of the relaxing factor in situ. Whether the cellular organelles become more fragile to the homogenization process with increasing times post-mortem is a possibility which must be considered. However, electron microscopic examination of the particulate fractions at 0 and 24 hours post-mortem indicates little difference in either the vesicular composition or morphology. The evidence that relaxing factor activity is stable for longer periods of time following isolation

when assayed in the presence of oxalate than in its absence (Hasselbach, 1964b) might indicate that the values obtained in the present study are overestimates of the real integrity of the relaxing factor system in post-mortem muscle.

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