UPDATE: CALCIUM UPTAKE AND RELEASE BY SKELETAL MUSCLE MITOCHONDRIA AND SARCOPLASMIC RETICULUM

by

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Introduction

As muscle goes into rigor mortis it contracts. If unrestrained it contracts isotonically, that is, it shortens. If restrained it contracts isometrically, that is, it develops tension. For muscle to shorten or develop tension in the living animal or post-mortem, the free Ca^{2+} concentration of the sarcoplasm must increase. This applies equally well to thaw-shortening, cold-shortening and rigor-shortening. These different kinds of post-mortem shortening are illustrated in Figure 1. Thaw shortening is the very rapid shortening that occurs when muscle frozen pre-rigor is thawed (Chambers and Hale, 1932; Perry, 1950). Cold-shortening is the shortening that commences immediately when muscle is exposed to temperatures below about 14°C within a few hours after slaughter (Locker and Hagyard, 1963). Rigor-shortening is the shortening that occurs during the rapid phase of rigor onset in muscles which do not cold-shorten either because they are not capable of cold-shortening or because they are kept at temperatures above about 14°C (Bendall, 1951; Marsh, 1954; Newbold, 1966). Rigor-shortening also occurs following thaw or cold-shortening (Bendall, 1973*b*, 1975; Newbold, 1966). In some muscles, when they are under load, a relaxation phase is apparent after thaw- or cold-shortening has reached its peak and before rigor-shortening commences (Bendall, 1960, 1973*a*, 1973*b*, 1975, 1978; Newbold, 1966). This relaxation most likely reflects the recapture of some of the Ca^{2+} released during the first shortening phase.

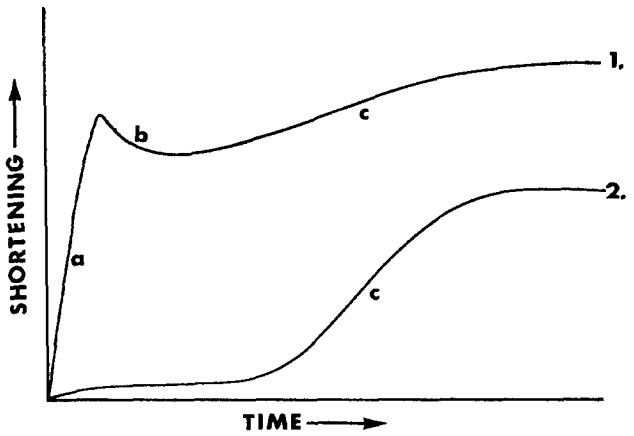
Where does the additional sarcoplasmic Ca^{2+} required for contraction come from and where does it go during relaxation? The two most likely regulators of the sarcoplasmic Ca^{2+} concentration are the mitochondria and the sarcoplasmic reticulum (SR), both of which systems are known to accumulate and release calcium under certain conditions. The mechanisms of calcium uptake and release by these organelles were discussed at the Reciprocal Meat Conference two years ago (Greaser, 1977).

Muscles differ in the amounts of mitochondria and SR they contain, those of the fast, white type containing more SR and fewer mitochondria than those of the slow, red type. In addition, white muscle SR takes up Ca^{2+} faster and in greater amounts than does red muscle SR.

Thaw-shortening occurs in both red and white muscles but, in general, only red muscles cold-shorten. (Chicken pectoralis major muscle (Smith *et al.*, 1969) and pig longissimus dorsi muscle (Bendall, 1975) are exceptions in that they are white muscles which cold-shorten). To explain why red muscles cold-shorten while white ones do not Buege and Marsh (1975) have presented evidence which suggests that the release of Ca^{2+} from mitochondria is responsible for the shortening.

While comparing the properties of red and white muscles it is worth noting that white muscle is ca-

FIGURE 1.



POST MORTEM SHORTENING
1. IN MUSCLE UNDER LOAD DURING THAW RIGOR OR UNDER COLD-SHORTENING CONDITIONS
a. THAW-SHORTENING OR COLD SHORTENING
b. RELAXATION PHASE
c. RIGOR-SHORTENING
2. IN MUSCLE AT TEMPERATURES ABOVE ABOUT 14°C
c. RIGOR-SHORTENING

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pable of greater glycolytic activity than red muscle. Thus, under the anaerobic conditions that prevail post-mortem, white muscle metabolizes faster than red muscle.

The work described in this report relates to three questions:—

1. Which of the cell organelles, the mitochondria (as suggested by Buege and Marsh (1975)) or the SR (as suggested by Newbold, 1966; Marsh, 1966; Cassens and Newbold, 1967; Davey and Gilbert, 1974), or both controls the sarcoplasmic Ca^{2+} concentration post-mortem?
2. What causes the release and uptake of Ca^{2+} in muscle post-mortem?
3. Why do some muscles cold-shorten while others do not?

First, some studies of the effect of inorganic orthophosphate (P_i) concentration on Ca^{2+} uptake and release by SR are considered. These studies were undertaken because P_i is known to increase the CA^{2+} accumulating ability of the SR greatly (Lorand and Molnar, 1962; Hasselbach, 1964) and has been observed to cause the relaxation of contracted, glycerinated muscle fibres (Newbold and Rose, 1957). Martonosi and Feretos (1964) considered that P_i was not likely to play a role in Ca^{2+} uptake and release by the SR under physiological conditions, the effective concentration of P_i being too high. However, the P_i concentration increases rapidly in muscle post-mortem, reaching a level of about 40 μmoles/g muscle when rigor is complete. (This concentration is more than 50 mM, assuming muscle contains 75% water and the P_i is evenly distributed in this.) It could therefore play an important role in the control of the sarcoplasmic Ca^{2+} concentration post mortem.

Secondly, some studies of the factors affecting the uptake and release of Ca^{2+} by muscle mitochondria in the presence of adenosine triphosphate (ATP) are described. These studies were prompted by the following considerations: Energy dependent uptake of Ca^{2+} by mitochondria can be supported by respiration or by ATP hydrolysis. Most previous studies have been made on liver or heart mitochondria and have related to respiration-linked rather than ATP-linked uptake. In post-mortem muscle, respiration quickly ceases through lack of oxygen but ATP is present until the full development of rigor mortis.

Experimental Procedures

Sarcoplasmic reticulum was isolated from rabbit psoas muscle (a white muscle which does not cold-shorten), rabbit soleus and semimembranosus proprius muscles combined (red muscle) and ox sternomandibularis muscle (a muscle which cold-shortens) essentially by the method of Martonosi, Donley and Halpin (1968). Mitochondria were prepared only from the ox muscle. These subcellular fractions were obtained by differential centrifugation of a muscle homogenate in 0.1M KCl, 5 mM histidine, 1 mM dithiotheitol, pH 7.3, the mitochondrial fraction being that which centrifuged down at 1000 - 8000 x g and the SR fraction that which centrifuged down at 8000 - 28000 x g. Except where noted these fractions were used on the day of preparation.

Calcium uptake and release were measured using ^{45}Ca and filters with an average pore size of 0.22 μm by the Millipore filtration technique (Martonosi and Feretos, 1964). The medium contained 0.1M KCl, 20 mM histidine, 5mM $MgCl_2$ and, except where noted, 5 mM ATP. With SR it usually contained, in addition, 5mM azide, 2.5 mM phosphoenolpyruvate, 8 units pyruvate kinase/ml and varying amounts of P_i. With mitochondria, the concentrations of succinate and P_i, when present, were 5 mM except where noted. The pH of the medium was 6.4 or 7.2, usually 6.4 with SR and 7.2 with mitochondria. The membrane concentration was usually about 100 μg/ml reaction mixture and the $^{45}CaCl_2$ concentration 25 μM except with rabbit red muscle preparations when it was 5μM. For measurement of the total amount of the Ca^{2+} accumulated by mitochondria the medium contained 500 μM $^{45}CaCl_2$. The incubations were carried out at room temperature (23°C) unless noted otherwise.

Effect of P_i on Ca^{2+} accumulation by SR

Table 1 shows that, in the presence of P_i, SR prep-

TABLE 1

AVERAGE AMOUNTS OF CALCIUM ACCUMULATED BY SARCOPLASMIC RETICULUM AND MITOCHONDRIA IN THE PRESENCE AND ABSENCE OF P_i AND PRESENCE OF ATP.

Source of Membrane	Amount accumulated (nmoles/mg protein) No P_i	+P_i *
Sarcoplasmic reticulum -		
White rabbit muscle	240	>3000
Red rabbit muscle	40	>2000
Ox sternomandibularis	80	>3000
Mitochondria -		
Ox sternomandibularis	2000	2000

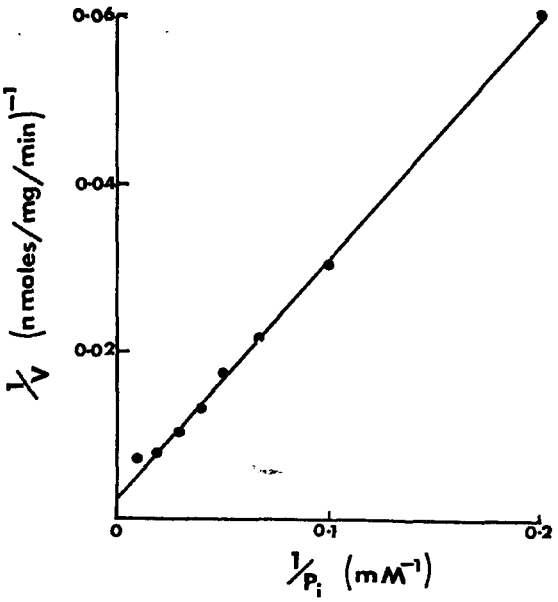
* 5-10 mM

arations from each of the three different sources took up more than 30 times the amount of Ca^{2+} they took up in the absence of P_i.

The effect of P_i concentration on the rate of Ca^{2+} accumulation is shown in Figure 2. This particular set of curves was obtained with SR from ox muscle. Similar sets of curves to that shown have also been obtained with SR from rabbit white muscle and rabbit red muscle (Newbold and Tume, 1979). Figure 2 shows clearly that in the presence of P_i the initially high rate of Ca^{2+} accumulation falls until a constant rate is reached and that the constant rate is greater and is reached sooner the higher the P_i concentration.

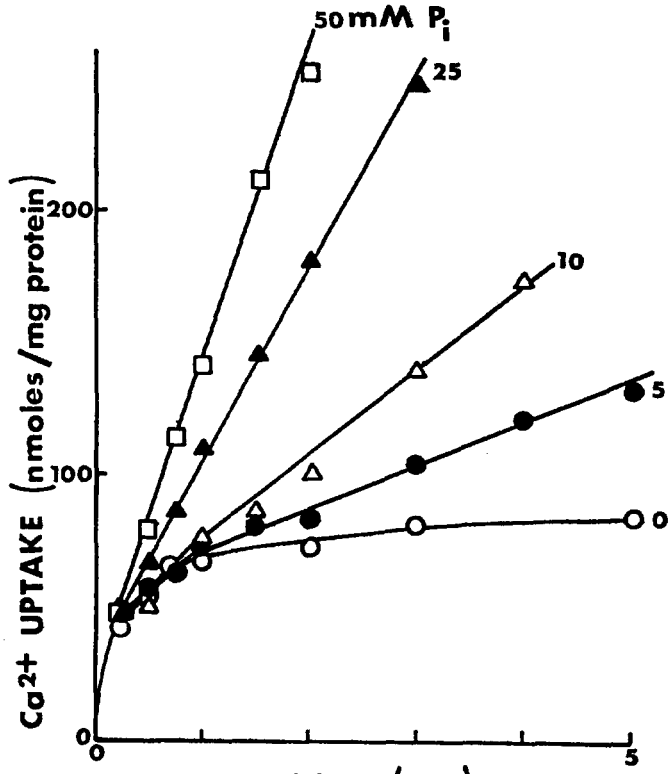
When the constant rate of Ca^{2+} accumulation at each P_i concentration is plotted against the P_i concentration in a double reciprocal plot, a straight line is obtained (Figure 3). From the slope and intercept of this line the maximum possible rate of Ca^{2+} accumulation and the P_i concentration required to give half the maximal rate can be calculated. Values obtained in this way are shown in Table 2.

FIGURE 3.



DOUBLE RECIPROCAL PLOT OF CONSTANT RATE AGAINST P_i CONCENTRATION

FIGURE 2.



EFFECT OF $[P_i]$ ON Ca^{2+} UPTAKE BY OX MUSCLE SR

The maximum rate for rabbit white muscle SR is 25 times that for rabbit red muscle SR and two and a half times that for ox muscle SR. On the other hand, the P_i concentration required for half-maximal rate is about 20-25 mM for SR preparations from rabbit muscle, red or white (Newbold and Tume, 1979), and 3-4 times greater, (about 80 mM) for SR from ox muscle. When rabbit white muscle SR is stored at 1°C for a few days (that is, when it is 'aged') the P_i concentration required for half-maximal rate of Ca^{2+} accumulation more than doubles in four days of aging although the maximum velocity remains the same (Newbold and Tume, 1979.) This possibly reflects an increase in the 'leakiness' of the vesicles during aging and suggests that freshly-prepared SR preparations from ox muscles are 'leakier' than the corresponding preparations from rabbit muscles, red or white.

TABLE 2

AVERAGE RATES OF CALCIUM UPTAKE* BY SR AND P_i CONCENTRATIONS FOR HALF MAXIMUM VELOCITY

Source of SR	Constant rate in the presence of - 5mM P_i	25mM P_i	50mM P_i (nmoles/mg protein/min)	Maximum velocity (nmoles/mg protein/min)	$[P_i]$ for half maximum velocity (mM)
Rabbit white muscle	220	570	820	1000	25
Rabbit red muscle	11	25	34	39	18
Ox sternomandibularis	22	120	170	400	80

* At pH 6.4 and 23°C.

For comparative purposes, Table 2 also gives average values for the constant rates at three different P_i concentrations and shows that increasing the P_i concentration from 5 mM to 50 mM increases the rate of Ca^{2+} accumulation by more than 8 times for ox muscle SR compared with 3-4 times for rabbit muscle SR.

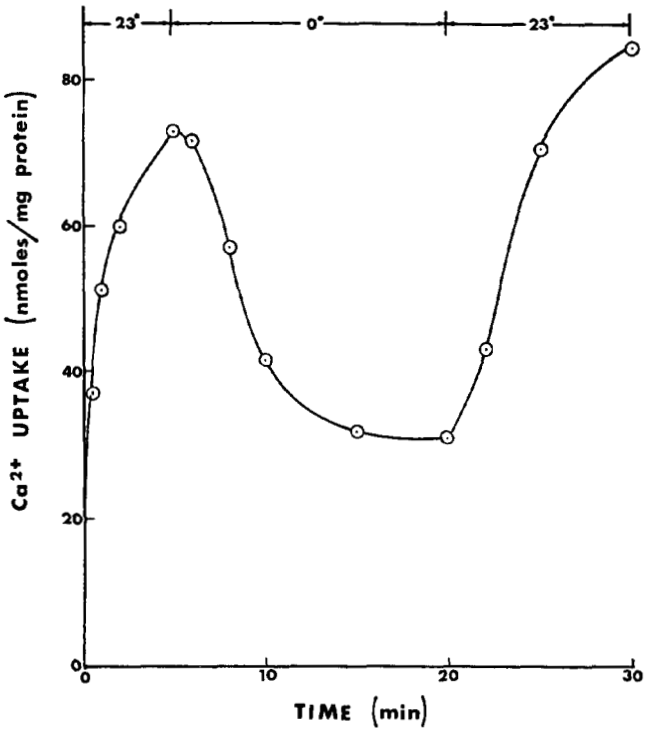
Effect of P_i on Ca^{2+} release from SR

When SR vesicles are loaded with Ca^{2+} at 23°C in the absence of P_i and then rapidly cooled to 0°C they release up to 60% of their accumulated Ca^{2+} within about 8 minutes (Taniguchi and Nagai, 1970; Horgan *et al.*, 1973; Newbold and Tume, 1977). This cold-induced release of Ca^{2+} is completely reversible (Figure 4. See also Newbold *et al.*, 1973). It occurs with SR preparations from muscles that do not cold-shorten as well as with preparations from muscles that do, and so does not provide a simple explanation for cold-shortening.

The amount of Ca^{2+} released in the cold depends on the P_i concentration of the medium (Newbold and Tume, 1977). This is illustrated in Figure 5.

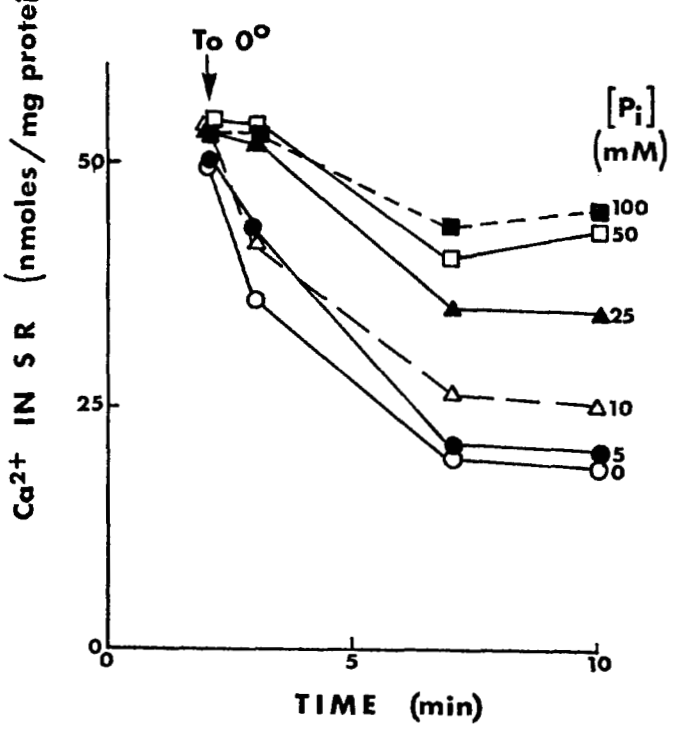
When the pH is not adjusted to be the same at 0°C as at 23°C a small part of the Ca^{2+} released on cooling is released because of the change in pH brought about by cooling (Newbold and Tume, 1977). The results cited below were obtained in experiments where the pH was kept constant.

FIGURE 4.



Ca^{2+} ACCUMULATION BY AND COLD-INDUCED RELEASE FROM OX SR

FIGURE 5.



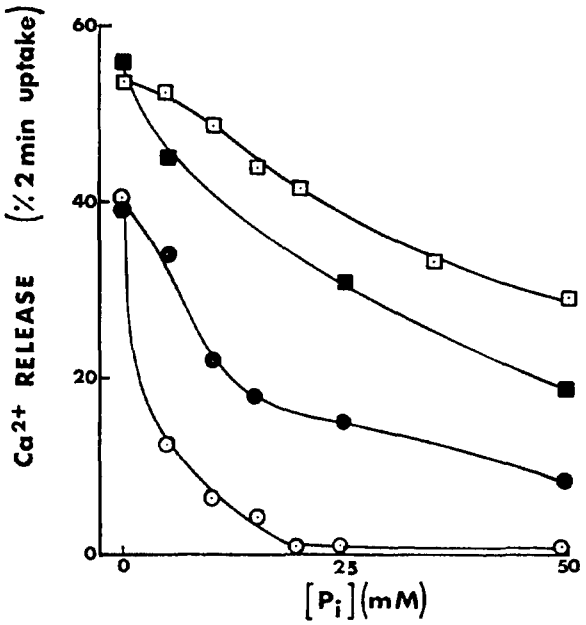
EFFECT OF P_i CONCENTRATION ON COLD-INDUCED RELEASE OF Ca^{2+} FROM OX MUSCLE S R

With rabbit muscle (red or white) SR preparations about 40% and with ox muscle SR preparations about 50% of the Ca^{2+} accumulated in the absence of P_i was released in the cold. No release accumulated from rabbit white muscle SR when the P_i concentration was 20 mM or more, whereas with ox muscle SR almost 30% of the accumulated Ca^{2+} was still released when the P_i concentration was 50 mM (Figure 6).

These results raise the possibility that rabbit white muscle does not cold-shorten because it metabolizes so quickly post-mortem that, by the time it is exposed to cold-shortening conditions, it contains enough P_i to prevent the cold-induced release of calcium.

From Figure 6, the effect of P_i concentration on the cold-induced release of Ca^{2+} from an aged SR preparation from rabbit white muscle is seen to resemble its effect on fresh SR preparations from ox muscle, in that 20% of the accumulated Ca^{2+} is still released when the P_i concentration is 50 mM. This, like the

FIGURE 6.



EFFECT OF P_i CONCENTRATION ON COLD-INDUCED Ca^{2+} RELEASE FROM S R
○ RABBIT WHITE MUSCLE S R
● RABBIT RED MUSCLE S R
□ OX MUSCLE S R
■ 'AGED' RABBIT WHITE MUSCLE S R

high P_i concentration it requires for half-maximal velocity, again suggests that freshly prepared SR from ox muscle is 'leakier' than freshly prepared SR from rabbit muscle.

This leakiness is unlikely to have developed during preparation since, although ox muscle loses its Ca^{2+} uptake activity faster than rabbit white muscle SR during aging, it still retains about 50% of its Ca^{2+} uptake activity on the day after preparation by which time rabbit red muscle SR is virtually inactive.

Ca^{2+} accumulation by mitochondria

As has been noted earlier, only Ca^{2+} accumulation by mitochondria in the presence of ATP will be considered here because ATP is present in muscle during post-mortem shortening. Figure 7 (also Table 1) shows that ox muscle mitochondria can accumulate, on average, about 2000 nmoles Ca^{2+}/mg protein when 5 mM ATP is present, whether or not 5 mM P_i or 5 mM succinate (a respiratory substrate) or both are also present. This lack of effect of P_i on the calcium accumulating capacity of mitochondria is in marked contrast to the very large effect it has on the calcium accumulating capacity of SR.

As for the effect of P_i on the rate of Ca^{2+} uptake, under the conditions used in the experiments summarized in Figure 7 (500 μM $^{45}CaCl_2$, pH 6.4), the rate in the absence of both P_i and succinate was slower than that in the presence of one or both of these substances.

Figure 8 shows the effect of P_i concentration on the rate of uptake at 23°C. Comparing the results in this Figure with those in Figure 2 it is clear that P_i concentration affects the rates of Ca^{2+} accumulation by mitochondria and SR in different ways. With mitochondria, increasing the P_i concentration from 5 mM to 50 mM slows the rate at which Ca^{2+} is accumulated whereas, with SR, the rate in the presence of 50 mM P_i is 8 times the rate in the presence of 5 mM P_i (Table 2).

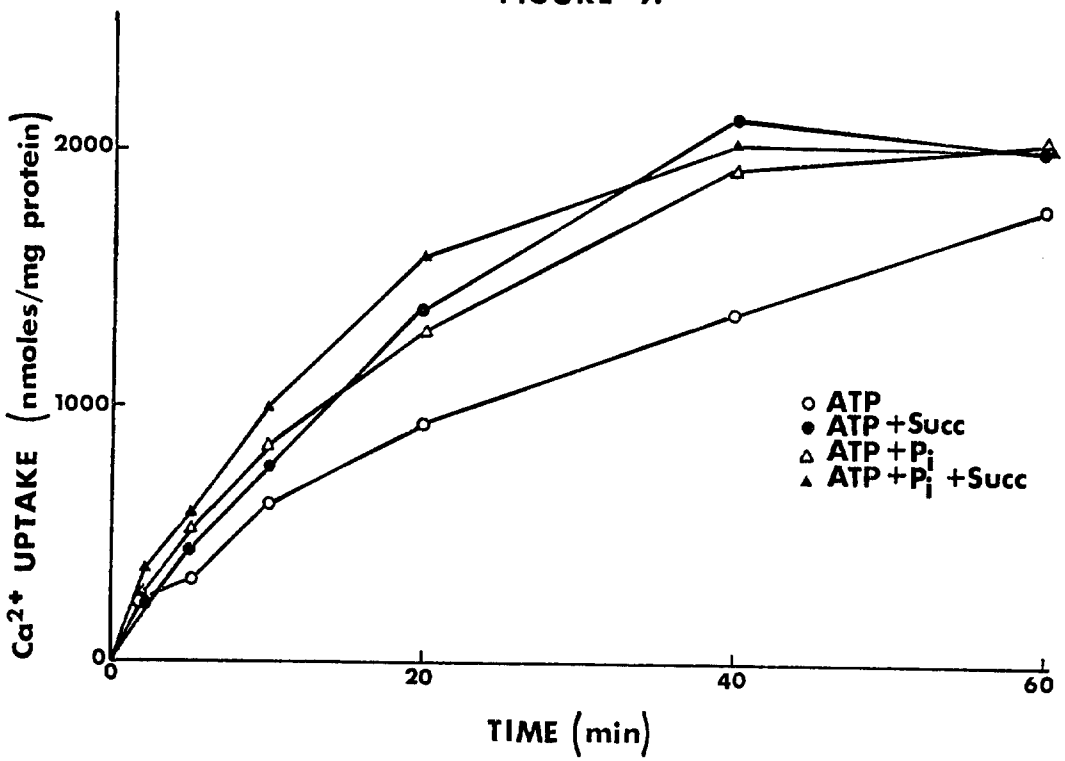
In Figure 9 the effects of P_i concentration on the accumulation of Ca^{2+} by mitochondria and SR at 0°C are compared. The main points of interest are the rapid increase in the rate of Ca^{2+} accumulation by SR as the P_i concentration increases and the large amount of Ca^{2+} that can be accumulated by SR at 0°.

Ca^{2+} release from mitochondria

Using ox muscle mitochondria pre-loaded with Ca^{2+} to 10-30% of their capacity in the presence of ATP, the effects of a number of different treatments on Ca^{2+} release have been examined. These loaded preparations are very stable and can be stirred in air at 23°C for several hours without releasing Ca^{2+}.

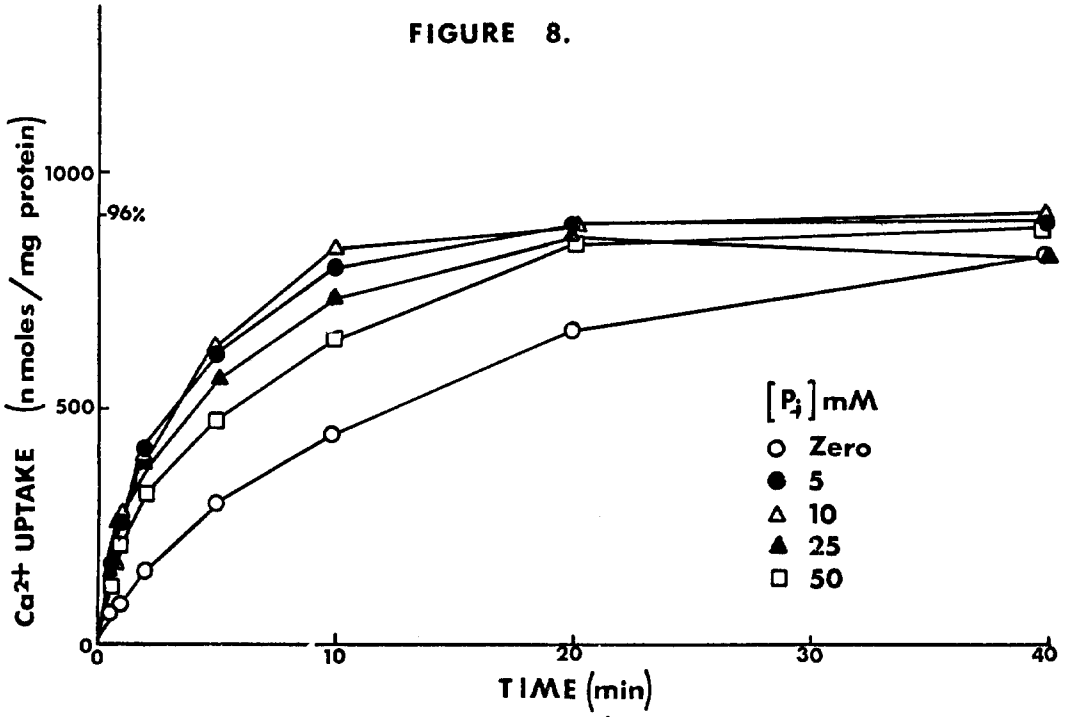
1. *Cooling.* For reasons that are not clear the results obtained when loaded mitochondria have been rapidly cooled to 0°C have been inconsistent. Sometimes loaded mitochondria have been gently stirred at 0°C for 21 hours without any apparent release of Ca^{2+} at any time, sometimes they have released a small amount of Ca^{2+} very slowly over the first 1-3 hours, sometimes they have released a small amount of Ca^{2+} in the first 10-20 minutes and then have released no more or have recaptured the Ca^{2+} previously lost. In spite of these inconsistencies, it is clearly apparent that if there is any cold-induced loss of Ca^{2+} from loaded mitochondria it is very much smaller than the large, rapid loss that takes place from SR on cooling. This conclusion is supported by the finding of Drahota *et al.* (1965) that rat liver mitochondria do not lose more than 1-2% of the Ca^{2+} taken up at 30°C in the presence of ATP and P_i when they are cooled to 0°C. The small release observed in our experiments may possibly have been due to the presence of SR in the preparation.

FIGURE 7.



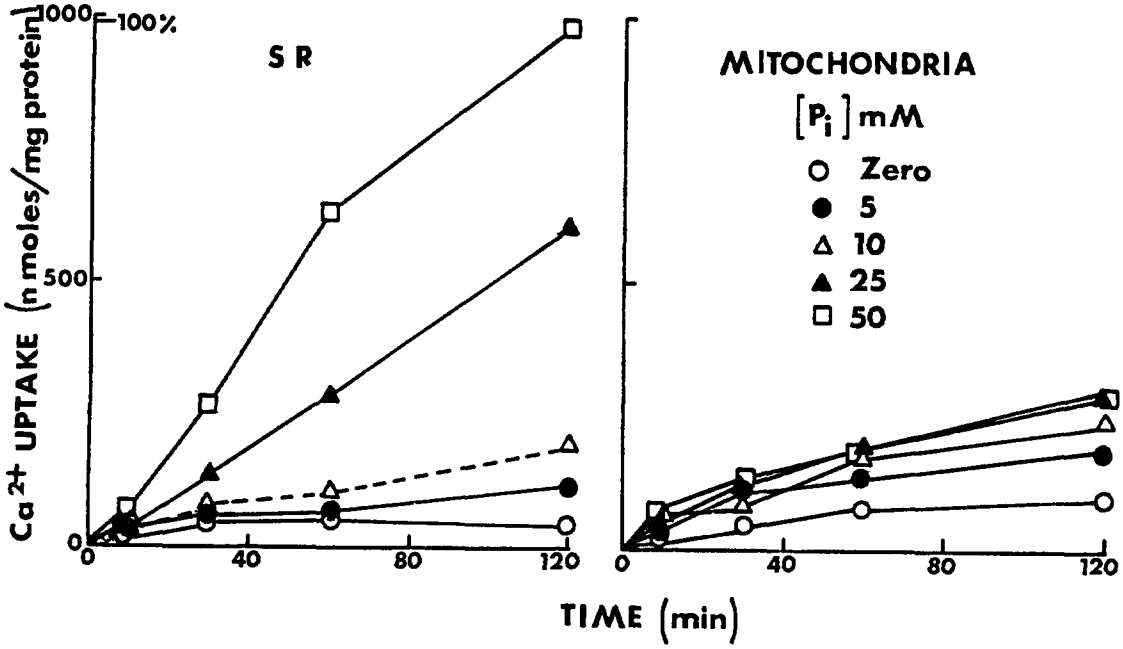
Ca^{2+} UPTAKE BY MITOCHONDRIA IN PRESENCE OF ATP, pH 6·4

FIGURE 8.



EFFECT OF P_i ON RATE OF Ca^{2+} UPTAKE BY MITOCHONDRIA pH 7·2

FIGURE 9.



EFFECT OF P_i ON Ca^{2+} ACCUMULATION BY SR AND MITOCHONDRIA AT 0°C

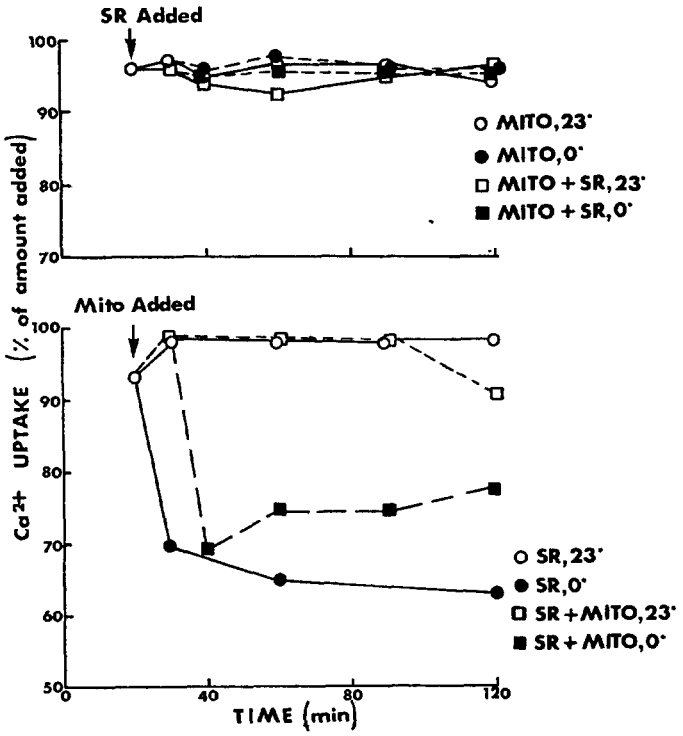
In a few experiments, mixtures of mitochondria and SR vesicles were used, one of the membrane systems loaded with Ca^{2+} and the other not loaded. For example, mitochondria were loaded with Ca^{2+} (600 nmoles/mg protein) in the presence of ATP and 5 mM P_i for 20 minutes, by which time 95% or more of the Ca^{2+} had been taken up. An amount of sarcoplasmic reticulum equal in protein content to the amount of mitochondrial protein was then added and, after a further 10 minutes, the reaction mixture was rapidly cooled at 0°C. Similarly, mitochondria were added to Ca^{2+}-loaded SR and the mixture was cooled. The results of such an experiment are shown in Figure 10, from which it can be seen that there was no cold-induced loss of Ca^{2+} from the mitochondria in the presence or absence of SR. It can also be seen that the cold-induced release of Ca^{2+} from SR was not affected by the presence of mitochondria although, in time, there was some uptake of the released Ca^{2+}, presumably by the mitochondria.

2. *Freezing and thawing.* Preliminary results indicate that mitochondria or SR loaded with 1500 - 3000 nmoles Ca^{2+}/mg protein in the presence of P_i lose one third to one half of their accumulated Ca^{2+} during freezing and thawing, more with mitochondria than with SR. This Ca^{2+} is not subsequently taken up again. When loaded to only a limited extent (250 nmoles Ca^{2+}/mg protein) in the presence of 5 mM P_i mitochondria and SR both release calcium during freezing and thawing. The released CA^{2+} is taken up again rapidly and completely by SR, slowly and less completely by mitochondria.

3. *Anaerobiosis.* Buege and Marsh (1975) have suggested that, under the anaerobic conditions prevailing in muscle post-mortem, Ca^{2+} is released from the mitochondria and that, at low temperatures, the SR is not able to take up this Ca^{2+} as fast as it is released. This implies that Ca^{2+} is released from the mitochondria for several hours, for cold-shortening can occur when muscle is cooled several hours after slaughter (Locker and Hagyard, 1963). Several reports have appeared of the release of Ca^{2+} from mitochondria under anaerobic conditions (Lehninger *et al.*, 1969; Thomas *et al.*, 1969; Cheah and Cheah, 1976) but these have all related to release from mitochondria in ATP-free media.

Using a Clark oxygen electrode it was shown that, with ox muscle mitochondria in a medium containing succinate and P_i (pH 7.2) but no ATP, Ca^{2+} was lost slowly when conditions became anaerobic, 75% of the Ca^{2+} taken up previously being released in about 5 hours. On the other hand, in a medium con-

FIGURE 10.



EFFECT OF COOLING Ca^{2+}-LOADED SR IN THE PRESENCE OF MITOCHONDRIA AND VICE VERSA (pH 6·4)

taining ATP and P_i (pH 7.2) there was no Ca^{2+} release in 5 hours, either at 23°C or when the temperature was reduced to 2-3°C after conditions had become anaerobic. This finding does not fit the Buege-Marsh hypothesis.

4. *Mitochondrial inhibitors.* Neither azide, an inhibitor of respiration, nor dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, has any effect on the uptake of Ca^{2+} by SR (Fanburg, 1964; Fanburg and Gergely, 1965) or on the cold-induced release of Ca^{2+} from SR. They do, however, almost completely inhibit Ca^{2+} accumulation by mitochondria (Vasington and Murphy, 1962). Hence, in most of our studies of SR 5 mM azide has been included in the media to inhibit the activity of any mitochondria present in the SR preparations.

The addition of DNP to Ca^{2+}-loaded mitochondria causes the release of Ca^{2+} (Drahota *et al.*, 1965). The effect of adding DNP (0.4 mM) to ox muscle mitochondria loaded with Ca^{2+} is shown in Figure 11. At 23°C virtually all the Ca^{2+} was released in less than an hour. At 0°C it was released more slowly. The results obtained when DNP was added to a mixture containing equal amounts (mg protein) of SR and Ca^{2+}-loaded mitochondria are also shown in Figure 11. In the presence of SR, Ca^{2+} was released more slowly than in the absence of SR, both at 23°C and 0°C, presumably because some of the released Ca^{2+} was taken up by the SR. Similar results to the above were obtained using azide (5 mM) instead of DNP.

5. *Apyrase.* When, after loading mitochondria or SR with Ca^{2+} in the presence of ATP, the enzyme apyrase (5 units/ml) was added to break the ATP down to AMP and P_i, Ca^{2+} was released from both membrane preparations, about half of the previously accumulated Ca^{2+} being released in 25 minutes.

Conclusions

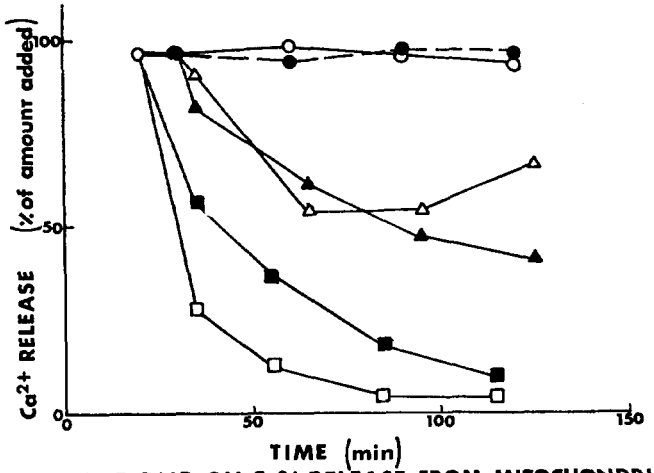
On the basis of these results it is suggested that:

1. Thaw-shortening which, as has been noted earlier, occurs in both red and white muscles, is due to the release of Ca^{2+} from SR and mitochondria.

2. The relaxation after the first shortening phase in thaw-rigor is due to an increase in the Ca^{2+} uptake activity of the SR as a result of increasing temperature and increasing P_i concentration.

3. Cold-shortening is probably due to the release of Ca^{2+} from the SR, for cold induces a substantial and reversible loss of Ca^{2+} from SR but little or no loss from mitochondria.

FIGURE 11.



EFFECT OF DNP ON Ca^{2+} RELEASE FROM MITOCHONDRIA AND MITOCHONDRIA-SR MIXTURE

4. The relaxation following cold-shortening is brought about by the increase in the Ca^{2+}-accumulating ability of the SR with increasing P_i concentration.

5. Rigor-shortening, which occurs while ATP is being lost, is due to the loss of ATP and the release of Ca^{2+} from both SR and mitochondria. Loss of ATP would lead to loss of activity of the Ca^{2+} pump of the SR and to reduction of the Ca^{2+}-binding capacity of the mitochondria (Drahota *et al.*, 1965).

6. In muscles that do not cold-shorten the cold-induced release of Ca^{2+} from the SR is completely inhibited by P_i, and sufficiently high concentrations of P_i for complete inhibition are reached soon after slaughter.

It is realized that the cold-induced release of Ca^{2+} from SR does not explain Buege and Marsh's (1975) observation that cold-shortening requires anaerobic conditions and does not occur in muscle stored in oxygen, but in view of the evidence that, in the presence of ATP, mitochondria do not release Ca^{2+} under anaerobic conditions, this observation cannot be explained in terms of mitochondria, either. Thus the problem of cold-shortening remains an intriguing one.

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