

# On the Mechanism of the Relaxing Effect of Fragmented Sarcoplasmic Reticulum

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**ABSTRACT** The vesicles of fragmented sarcoplasmic reticulum, *i.e.* the physiological relaxing factor, remove most of the exchangeable Ca bound to actomyosin and myofibrils. The extent to which they remove Ca and the extent to which they inhibit myofibrillar activity are closely correlated. Previous work has shown that those *in vitro* reactions of actomyosin with ATP which are equivalent to its contraction, *i.e.* superprecipitation and a high ATPase activity, require the formation of a Ca-actomyosin complex, and that actomyosin after the removal of most of its bound Ca is inhibited by physiological concentrations of ATP. The evidence now suggests that the factor achieves its relaxing effect through the dissociation of the Ca-actomyosin complex and that it has no other direct influence on the biological activity of actomyosin. Experiments, showing that under similar conditions the vesicles of the factor are capable of reducing the concentration of ionized Ca in the surrounding medium to about  $0.02 \mu\text{M}$  and less, suggest that the factor competes successfully with actomyosin for the available Ca through its mechanism of Ca accumulation.

## INTRODUCTION

A number of years ago Sandow (44) suggested that Ca might couple excitation to contraction in living skeletal muscle, a concept which meanwhile has acquired a considerable body of experimental evidence in its favor (5, 6, 15, 16, 35, 38, 39).

The mechanism by which Ca, introduced into the sarcoplasm, changes the state of relaxation into the state of contraction has not yet been established. At the moment two different possibilities are under consideration. On the one hand, Ca might inactivate a relaxing substance (7, 8, 34, 36). It has been postulated—on the basis of indirect evidence—that in the presence of ATP a soluble relaxing substance is released by the sarcoplasmic reticulum which maintains actomyosin in the relaxed state (8, 27, 33, 52).

On the other hand, one of us (46) proposed several years ago—also on the basis of indirect evidence—that Ca might combine with actomyosin. It was postulated that actomyosin hydrolyzed ATP at a maximal rate and superprecipitated under the influence of ATP only in the form of the Ca complex, whereas without Ca actomyosin had a very low ATPase activity and could not superprecipitate. Meanwhile it has been demonstrated directly (11, 48–51) that the state of actomyosin in presence of ATP (*i.e.* contracted or relaxed) can be regulated by the concentration of ionized Ca in the medium. We showed that, after the removal of most of the Ca bound to myofibrils, superprecipitation does not occur unless about 1 mole of Ca per mole of myosin has been added (50). Most of the Ca bound to myofibrils or actomyosin is in equilibrium with the ionized Ca of the medium (49): with a concentration of ionized Ca around 0.01  $\mu\text{M}$  very little Ca is bound in the presence of Mg and ATP, ATP does not induce superprecipitation, and is hydrolyzed at a very low rate. When the concentration of Ca is raised to a level of 2.0 to 10  $\mu\text{M}$ , about 1 to 2 moles Ca per mole myosin are bound, and superprecipitation and ATPase activity are maximal.

If in living muscle the transition from contraction to the resting state were caused by a shift in the equilibrium,  $\text{Ca-actomyosin} \rightleftharpoons \text{actomyosin} + \text{Ca}^{++}$  one might expect the concentration of ionized Ca in the cytoplasm to fall sharply during relaxation, from more than 5  $\mu\text{M}$  (necessary for contraction) to about 0.2  $\mu\text{M}$  required for the resting state. This could be achieved by the sarcoplasmic reticulum. The finding that the physiological relaxing factor, first described by Marsh (28) and Bendall (3), consists of fragmented sarcoplasmic reticulum (14, 32, 33, 40, 41) was followed by the discovery of Ebashi and Lipmann (12, 14) and of Hasselbach and Makinose (19, 20) that it is capable of removing Ca from the medium.

Therefore it seemed interesting to determine, first, whether the physiological relaxing factor can remove Ca from actomyosin to an extent sufficient to explain its relaxing effect on this basis and, second, whether the vesicles of the relaxing factor are capable through their mechanism of Ca accumulation of reducing the outside concentration of Ca to such low levels that the equilibrium between Ca and actomyosin is shifted towards dissociation. A preliminary account of some of these results has already been presented (50).

#### EXPERIMENTAL PROCEDURE

Treatment of glassware, choice of reagents, preparation of solutions, myofibrils, and actomyosin have been described elsewhere (48, 49, 51).

*The Relaxing Factor (RF)* This was prepared by a modification of Portzehl's procedure (42). Ground rabbit muscle was homogenized in a Waring blender for 40 sec., at 0°, in 3 volumes of 120 mM KCl–5 mM histidine of pH 6.5 (histidine buffer

adjusted to pH 6.5 at 25° has a pH of about 7.0 at 0°). After removal of the myofibrils and the bulk of the mitochondria by two centrifugations at 3,000 and 8,000 *g*, we precipitated the RF by centrifugation at 20,000 or 36,000 *g* for 1 hour. The bottom layer of the precipitate, which was brown-green in color and probably consisted of the remaining mitochondria, was rejected and the white fluffy top layer resuspended and washed twice by thirty- to fiftyfold dilution in 120 mM KCl–1.0 mM histidine, pH 6.5, followed by centrifugation at 89,000 *g*. The final precipitate was suspended in the same medium to contain from 1.2 to 2 mg N/ml. 1 mg nitrogen corresponded to about 9 mg dry weight of H<sub>2</sub>O–washed RF. Just prior to an experiment, the RF suspension was centrifuged for 7 min. at 8,000 *g* to remove any precipitate which had been present initially or which had formed during standing. Aggregates which are spun down during 2 min. centrifugation at 3,000 *g* are continuously being formed during storage. RF was used at most for 3 days after preparation. However, in high concentrations RF was active even after more than 1 week at 4° (see also, reference 1).

*The Binding of Ca by Myofibrils or Actomyosin* This was determined with Ca<sup>45</sup> as described elsewhere (49). The assay system (10 ml) contained 1.5 to 2.0 mg myofibrillar protein/ml, or 0.8 mg actomyosin/ml, RF as indicated, 10 mM imidazole pH 6.5, ionic strength ( $\Gamma/2$ ) of 0.12 adjusted with KCl, 0.01 to 0.02 mM Ca<sup>45</sup>Cl<sub>2</sub> (0.04 to 0.08  $\mu$ curie/ml), 1.0 to 4.0 mM Mg-ATP, and other additions as indicated. When present, the concentration of creatine phosphate (CP) was 10 mM and that of creatine phosphokinase (CPkinase) 0.1 mg/ml. The suspension was equilibrated at 24.6° for 10 min., with the exception of RF, Mg-ATP, and, if added, oxalate. (In the presence of Mg-ATP, Ca-oxalate was not precipitated by 60 min. centrifugation at 100,000 *g* (see also reference 19).) RF was added 3 to 5 min. prior to the assay which was started by the addition of Mg-ATP containing oxalate, if desired. The assay was terminated after 1 to 3 min. (as indicated) by precipitating the myofibrils or actomyosin by centrifugation at 3,000 *g* for 2 min. The radioactivities of supernatant and precipitate were measured as described elsewhere (49).

*Whereas the Calculation of the Isotope Bound to actomyosin is identical with that described previously (49), special considerations apply to the calculation of the isotope bound to myofibrils (examples of the calculation are given in Table III a) if RF is present. In that case, since most of the Ca is contained in the vesicles of the RF (compare Table VI) the isotope cannot be uniformly distributed throughout the solution and must be absent from the interior of the myofibrils. The vesicles are too large to penetrate the space between the filaments (33). The fraction of the radioactivity of the precipitate which is not bound to the myofibrils and which must be subtracted from the total radioactivity of the precipitate, therefore must be restricted to the extra myofibrillar volume. The extra myofibrillar volume is obtained by subtracting the wet weight of the myofibrils from the total weight of the precipitate, assuming a specific gravity of 1 for the weight of the remaining solvent (the specific gravity of a 0.13 M KCl solution is 1.005). We estimated the wet weight of myofibrils by weighing myofibril precipitates containing a known amount of myofibrillar protein after centrifugation at 100,000  $\times$  *g* for 5 min. under various conditions. The wet weights thus obtained (Table I) are too large by the amount of extra myofibrillar space in the pellet. However, this error is probably small and furthermore its elimination would*

increase the values for Ca removal by the relaxing factor from myofibrils, not decrease them. The values for the wet weights of myofibrils in the presence of 4 mM Mg-ATP were considerably increased if superprecipitation had been prevented as the result of removing Ca from the myofibrils by 2 mM ethylene glycol bis ( $\beta$ -amino-ethylether)-*N,N'*-tetraacetic acid<sup>1</sup> (EGTA) (48, 49). From these data we estimated by interpolation the myofibrillar wet weight in our precipitates according to the following expression:

$$w_{\text{ext}} = \left[ \frac{\text{vol}_{\text{ppt}} - \text{vol}_{\text{sppc}}}{\text{vol}_{\text{EGTA}} - \text{vol}_{\text{sppc}}} (w_{\text{EGTA}} - w_{\text{sppc}}) + w_{\text{sppc}} \right]$$

TABLE I  
WET WEIGHT OF MYOFIBRILS UNDER  
VARIOUS CONDITIONS AFTER 5 MIN.  
CENTRIFUGATION AT 100,000 G

Myofibril preparation	Medium	Myofibrils per gm myofibrillar protein
		<i>gm</i>
Cardiac	Solution 1*	8.0
Skeletal	Solution 1	8.6
Skeletal	Solution 1	8.0
Skeletal	Solution 1 + 4 mM Mg-ATP + 0.02 mM CaCl <sub>2</sub>	7.2
Skeletal	Solution 1 + 4 mM Mg-ATP† + 2 mM EGTA	11.3

\* Ionic strength, 0.12, 10 mM imidazole, pH 6.5.

† 24 mM CP and 0.1 mg/ml CPkinase present to prevent the reduction of ATP below 0.1 mM with consequent superprecipitation (48).

( $w_{\text{ext}}$ , extrapolated wet weight of the myofibrils in the assay;  $\text{vol}_{\text{ppt}}$ , volume of the precipitate as read in the calibrated tube after centrifugation at 3,000 *g* for 2 min.;  $\text{vol}_{\text{sppc}}$ , the volume of the same amount of myofibrils when fully superprecipitated;  $\text{vol}_{\text{EGTA}}$ , the volume of the myofibrils in the presence of ATP when superprecipitation had been fully inhibited by EGTA;  $w_{\text{EGTA}}$ , the wet weight of the myofibrils after centrifugation at 100,000 *g* for 5 min. in the presence of ATP when superprecipitation had been inhibited by EGTA (see Table I, row 5);  $w_{\text{sppc}}$ , the wet weight after maximal superprecipitation). The above considerations, and the value for the wet weight of myofibrils with inhibited superprecipitation were confirmed by the following experiment. RF alone was incubated with 0.02 mM Ca<sup>45</sup> in the usual

<sup>1</sup> The authors are indebted to Geigy Chemical Corporation for their generous gift of EGTA (trade name, Chel De). EGTA is similar in structure to ethylenediaminetetraacetic acid (EDTA), but binds Mg with an association constant 3 orders of magnitude smaller than EDTA, whereas the constant for Ca is identical with that of EDTA (43). Consequently, a system of EGTA + Ca-EGTA is an excellent Ca buffer also in presence of Mg.

assay system (see above) for 6 min. and myofibrils, prepared to contain very little exchangeable Ca (49), were added just prior to centrifugation at 3,000 *g*. The analysis for radioactivity (in four assays) showed that 0.66 to 0.71 gm precipitate contained from 82,000 to 93,000 CPM compared to 138,000 to 148,000 in the identical amounts of supernatant. Therefore, part of the volume of the precipitate (ppt), *i.e.* 0.254 to 0.264 gm,  $\left( \text{gm ppt} - \frac{\text{Total CPM in ppt}}{\text{CPM/gm supernatant}} \right)$  if no isotope were bound to the myofibrils, cannot have contained any isotope at all. Therefore, 0.25 to 0.26 gm presumably represented the wet weight of myofibrils containing 21 mg myofibrillar protein contained in each assay. This value, 12.5 gm per gm myofibrillar protein, compares fairly well with the value given in the 5th row in Table I. A control experiment (see below) established that RF bound 99.5 per cent of the isotope under similar conditions.

TABLE II  
CONTENT OF PCA-EXTRACTABLE AND OF EXCHANGEABLE  
Ca IN VARIOUS RF PREPARATIONS

	Ca, $\mu\text{moles/gm N}$ of RF	
	PCA-extractable	Exchangeable
Lowest value	105	33
Highest value	270	190
Average of 11 preparations	190	140

*The Calculation of the Exchangeable Ca Bound to Myofibrils or Actomyosin* For this it is necessary to determine whether a reduction in the amount of *isotope* bound to actomyosin or myofibrils following the addition of RF, might be caused by a dilution of the specific activity of the isotope as a result of the addition of more  $\text{Ca}^{40}$  introduced with the RF preparation. Perchloric acid (PCA) extracts of RF contained 100 to 200  $\mu\text{moles Ca}$  per gram nitrogen as determined by titration with EGTA (49). However, some of this Ca might be tightly bound and non-exchangeable and therefore not contribute to the dilution of the isotope. Since we found that in a saturated thymol solution all  $\text{Ca}^{46}$  was released from RF, we considered the fraction of Ca removed by thymol treatment from relaxing factor as the exchangeable Ca. (That this value for exchangeable Ca might still be too high, because the complete inactivation of RF by thymol (17) might result in the release of previously non-dissociable Ca, is not important since with lower values the calculated extent of Ca removal by RF would be increased.) This fraction was estimated in the following manner. A known volume of an extract of RF, heated in an aqueous solution of saturated thymol was incubated with myofibrils binding a known amount of Ca (as determined by isotope distribution) and containing a known amount of total Ca, with the concentration of ionized Ca above saturating level. If the concentration of  $\text{Ca}^{2+}$  is above saturation, an increase in its concentration by the thymol extract cannot change the amount of Ca bound to the myofibrils. The amount of exchangeable Ca added with the RF extract (free of

active RF after this treatment (17)) was calculated according to the expression  $a/(b + x) = c$  ( $a$ , exchangeable Ca bound to myofibrils;  $b$ , total exchangeable Ca before the addition of the extract;  $x$ , exchangeable Ca added with the extract;  $c$ , fraction of the total isotope bound to the myofibrils). The values thus obtained (Table II) were variable and usually lower than the total Ca titrated after PCA extraction. They were high enough to account for significant reductions in the amount of isotope bound to actomyosin, particularly if large amounts of RF were used (11). For the calculation of Ca bound to myofibrils or actomyosin in presence of RF, which requires the value for the concentration of total exchangeable Ca, the exchangeable Ca present in the RF was determined each time, in addition to the exchangeable Ca present in myofibrils and solutions (see reference 49).

*Discussion of Errors in the Calculation of Bound Exchangeable Ca* The error in the procedures leading to the determination of the total radioactivities of the various fractions was highest for the precipitates where values differed maximally by 15 per cent. We estimated the amount of Ca contaminating myofibrils and solutions in a manner described in great detail elsewhere (49). These estimations may be inaccurate by as much as 30 per cent. These inaccuracies are not fully reflected in the final results because the contaminating Ca represents only 30 to 50 per cent of the total Ca, the rest consisting of added Ca. Eliminating this fraction from the calculation decreases the calculated value for bound exchangeable Ca (compare in Table III *b* the values of  $R_1$  and  $R_2$ ) and also decreases the calculated value for the fraction of the bound exchangeable Ca removed by RF  $\left[ 1 - \frac{(L + N)(K_3/C)}{L(K/C)} \right]$  is smaller than  $1 - \frac{(L + M + N)(K_3/C)}{(L + M)(K/C)}$  as can be calculated from the data of Table III *b* ]. However, the existence of contaminating Ca in our assays is assured by the titration of dissociable Ca bound to myofibrils (37) (2.5 to 3  $\mu$ moles per gm which are fully removable by EGTA<sup>2-</sup> and Mg<sup>2+</sup> (49)) and the titration of Ca contained in ATP solutions (as much as 2  $\mu$ moles Ca per millimole of ATP were found).

If the values for exchangeable Ca introduced with the relaxing factor were increased to those of the total PCA-extractable Ca, the calculated values for Ca removed from the myofibrils would become smaller but not enough to significantly change the results

$$\left[ (L + M + N) \frac{C}{K_3} \text{ vs. } (L + M + O) \frac{C}{K_3} \right].$$

The interpolation of the values for the wet weight of myofibrils as described above presupposes that the wet weight of the myofibrils after centrifugation at  $100,000 \times g$  is linearly proportional to the variations in the precipitate volume after 2 min. centrifugation at  $3,000 \times g$ , which is not proved. However, a comparison of the values for  $K_1$  and  $K_3$  (Table III *a*) shows that the calculated removal of bound isotope by RF is not much altered if the interpolated wet weight of the myofibrils is substituted by their maximal wet weight resulting in a maximal figure for isotope bound to myofibrils

in the presence of RF. If it were assumed instead that the added isotope was not bound by the relaxing factor but was free in the solution, the calculated removal of exchangeable Ca from myofibrils would greatly increase.

The values for  $R_3/R_1$  (Table III *b*) represent the highest binding of Ca in the presence of RF (or the least removal of Ca by RF) as calculated with a combination of all those assumptions which minimize the removal of Ca from myofibrils as the cause for the reduction of radioactivity in the myofibril precipitate after RF addition. These values deviate most from the  $R_4/R_2$  values, which are presumably the more accurate ones, when the RF concentration is greatest; *i.e.*, when the Ca introduced with the RF becomes a large fraction of the total (see Table III *b*, Experiment 2). In that case the calculated removal of Ca becomes quite small. Since with low concentrations of RF, however, the  $R_3/R_1$  values also indicate a substantial removal of Ca (Experiment 1, and 150 sec. incubation in Experiment 3) and are comparable to the  $R_4/R_2$  values at high concentrations of RF, it seems justifiable to conclude that the assumptions and estimations underlying the  $R_4/R_2$  values are essentially correct.

*ATPase Activity of Myofibrils* This was measured in the same assay by which the binding of exchangeable Ca was determined as described elsewhere (49) by determining the inorganic phosphate released into the supernatant. Whereas the determination of the inorganic phosphate in the supernatant was precise, the estimation of the time interval during which it was liberated was less accurate. Since we had found that the liberation of inorganic phosphate into the supernatant ceased after the myofibrils had been fully precipitated by centrifugation at 3,000 *g* (12.1  $\mu$ moles P; after 5 sec., 12.5  $\mu$ moles after 120 sec. centrifugation for fully superprecipitated myofibrils), we calculated the rates on the basis of the time elapsed between ATP addition and full precipitation of the myofibrils. To obtain the precipitation times, we measured in control experiments the interval between the moment the centrifuge reached maximal speed and the time the volume of the precipitate had reached its final value (*i.e.* equaled the volume after 2 min. centrifugation) within 15 per cent. Since this interval varied with the degree of superprecipitation of the myofibrils, the precipitation times were estimated by interpolation from the following data: myofibrils precipitating to a final protein concentration in the precipitate of 130 mg/ml, 5 sec.; 85 mg/ml, 22 sec.; 50 mg/ml, 45 sec.; 36 mg/ml, 45 sec. However, the interval between ATP addition and full precipitation of the protein presents the maximum period for the liberation of phosphate. The rate at which phosphate was liberated into the supernatant presumably decreased appreciably before the protein was fully precipitated during the time when large aggregates were formed and part of the protein was concentrated in the bottom of the tubes because of a decrease in the concentration of ATP in the interior of aggregates. Fortunately this error is not as severe as one might fear because myofibrils with a high rate of ATP hydrolysis precipitate rapidly so that the precipitation time is small compared to the incubation period (when aggregation was prevented by rapid stirring), whereas the slowly precipitating myofibrils with inhibited ATPase were found to form much smaller aggregates with presumably a higher internal ATP concentration (steady state between ATP diffusion and breakdown). In one experiment, for instance, we

TABLE IIIa  
CALCULATION OF BOUND ISOTOPE

In total precipitate A	Radioactivity cpm per ml. supernatant B	In total assay C	Precipitate* total weight, gm D	at E	Weights of myofibrils <sup>§</sup> $w_{\text{MGT}}$ F	$w_{\text{MGT}}$ H	$w_{\text{ext}}$ I	Bound isotope Bound cpm K, K <sub>1</sub> , K <sub>2</sub> , K <sub>3</sub>	Bound isotope Total isotope
68,710	36,090	424,000	0.221	No RF (vol <sub>ppst</sub> 0.14 ml, vol <sub>MGT</sub> 0.48 ml) <sup>§</sup>	—	—	—	K = 61,360	0.145
46,560	66,870	682,800	0.494	RF, 0.016 mg N/ml (vol <sub>ppst</sub> 0.43 ml)	0.195	0.124	0.184	K <sub>1</sub> = 26,610 K <sub>2</sub> = 21,860 K <sub>3</sub> = 25,860	0.039 0.032 0.038
80,120	46,630	541,000	0.209	No RF (vol <sub>ppst</sub> 0.17 ml, vol <sub>MGT</sub> 0.65 ml) <sup>¶</sup>	—	—	—	K = 71,240	0.13
39,940	58,920	592,200	0.629	RF, 0.26 mg N/ml (vol <sub>ppst</sub> 0.65 ml)	0.214	0.136	0.214	K <sub>1</sub> = K <sub>2</sub> = 15,540	0.026
339,120	199,500	2,210,000	0.232	No RF (vol <sub>ppst</sub> 0.15 ml, vol <sub>MGT</sub> 0.52 ml) <sup>**</sup>	—	—	—	K = 296,120	0.134
213,390	228,700	2,480,000	0.263	RF (0.03 mg N/ml) 80 sec. incubation (vol <sub>ppst</sub> 0.21 ml)	0.191	0.121	0.133	K <sub>1</sub> = 196,990 K <sub>2</sub> = 183,590	0.079 0.074
167,480	225,260	2,170,000	0.324	150 sec. incubation (vol <sub>ppst</sub> 0.29 ml)	0.190	0.121	0.147	K <sub>1</sub> = 137,280 K <sub>2</sub> = 127,680	0.063 0.059

\* After the removal of the supernatant.

† Grams myofibrillar protein in precipitate.

§ Abbreviations explained in text.

||  $K [K = A - B(D - E)]$  is the calculated amount of radioactivity bound to Mf in the absence of RF;  $K_1 [K_1 = A - B(D - F)]$  is the maximal amount in the presence of RF that can be calculated,  $K_2 [K_2 = A - B(D - H)]$  the minimal amount,  $K_3 [K_3 = A - B(D - I)]$  the amount calculated using  $w_{\text{ext}}$  as defined in the text.  $K_3$  is the value used for Figs. 1 and 2 and Tables IV, V, and VIII.

¶ Taken from Fig. 1.

\*\* Taken from Fig. 2.



TABLE IIIb  
CALCULATION OF EXCHANGEABLE Ca BOUND TO MYOFIBRILS

Experiment No.	RF mg N/ml	Contaminating Ca, $\mu\text{moles}$ RF				FCA O	Total exchangeable Ca $\mu\text{moles}$	Bound exchangeable Ca	
		Added Ca L	Mf + solution M	Thymol N	$R_1 \rightarrow 4^*$ $\mu\text{moles}$			In presence of RF	In absence of RF
1	0	0.2	0.1	—	—	—	L = 0.2	$R_1 = 0.029$	
								$R_2 = 0.043$	
								$R_3 = 0.0097$	$R_3/R_1 = 0.34$
								$R_4 = 0.0123$	$R_4/R_2 = 0.28$
2	0	0.2	0.13	—	—	L + M = 0.33	$R_1 = 0.026$		
							$R_2 = 0.043$		
							$R_3 = 0.020$	$R_3/R_1 = 0.77$	
							$R_4 = 0.016$	$R_4/R_2 = 0.37$	
3	0	0.2	0.089	—	—	L + M = 0.289	$R_1 = 0.027$		
							$R_2 = 0.039$		
							$R_3 = 0.021$	$R_3/R_1 = 0.78$	
							$R_4 = 0.026$	$R_4/R_2 = 0.67$	
80 sec.	0.03	0.2	0.089	0.056	0.069	L + M + N = 0.345	$R_1 = 0.017$		
							$R_2 = 0.017$	$R_2/R_1 = 0.63$	
							$R_3 = 0.017$	$R_3/R_1 = 0.63$	
							$R_4 = 0.020$	$R_4/R_2 = 0.51$	
150 sec.	0.03	0.2	0.089	0.056	0.069	L + M + N = 0.345	$R_1 = 0.017$		
							$R_2 = 0.017$	$R_2/R_1 = 0.63$	
							$R_3 = 0.017$	$R_3/R_1 = 0.63$	
							$R_4 = 0.020$	$R_4/R_2 = 0.51$	

\*  $R_1 [R_1 = L \frac{K}{C}]$  is the minimal value of exchangeable Ca bound to Mf in the absence of RF that can be calculated,  $R_2 [R_2 = (L + M) \frac{K}{C}]$  the value that takes the contaminating Ca into account;  $R_3 [R_3 = (L + O) \frac{K_1}{C}]$  is the value of exchangeable Ca bound to Mf in the presence of RF calculated on the basis of  $K_1$  and of the maximal contamination of RF,  $R_4 [R_4 = (L + M + N) \frac{K_3}{C}]$  the value calculated on the basis of  $K_3$  and of the exchangeable Ca contaminating Mf and RF.  $R_2$  and  $R_4$  are used for Figs. 1 and 2 and Tables IV, V, and VIII.

found an apparent precipitation time of 38 sec. by dividing the inorganic phosphate, released into the supernatant after 2 min. centrifugation, by the steady rate of ATP hydrolysis (as measured by a conventional accurate enzyme assay (46, 48, 51)  $P_i/P_i/\text{min.}$ ) whereas the direct measurement of the precipitation time gave a value of 50 sec. These uncertainties affect particularly the steep part of the ATPase curves in Fig. 2 where the precipitation times change to a larger extent (particularly curve 4). Nevertheless, the general relation between the curve for Ca binding and that for ATPase (curve 1 *vs.* 3 and curve 2 *vs.* 5) remains, *i.e.* the ATPase curve falls much more steeply than the curve indicating bound Ca, even if the values are recalculated for a 50 per cent decrease in the precipitation times.

When the hydrolysis of ATP by myofibrils was determined in the presence of RF, the phosphate liberated by RF was determined in control experiments and subtracted from the total value. The range of ATP hydrolysis by RF was between 0.9 to 1.4  $\mu\text{moles ATP per min. per mg N}$  and was linear with RF concentrations from 0.013 to 0.1 mg N/ml. The presence of 1.0 mM oxalate decreased the rate only slightly (compare references 19, 20).

*Superprecipitation* We measured this as described previously (48, 51), but it is indicated here in milligrams of protein per milliliter of precipitate.

*The Removal of Ca from the Surrounding Medium by the Vesicles of RF* This was measured with  $\text{Ca}^{45}$ . The assay system was identical with that for Ca binding by myofibrils, with myofibrils omitted. After the addition of Mg-ATP, the RF suspension was, 10 to 25 min. later, centrifuged for 60 min. at 100,000 *g* at room temperature. The fraction of the total radioactivity remaining in the upper layers of the supernatant (0.5 to 1.0 ml) was determined, and the concentration of the total exchangeable Ca remaining in the supernatant calculated on the basis: total exchangeable Ca = added Ca + exchangeable Ca present in the RF preparation. The concentration of ionized Ca remaining in the supernatant was calculated with the association constants for Mg-ATP and Ca-ATP determined by Burton (10), and for  $\text{ATP}^{4-}$  by Martell and Schwarzenbach (30), and, if EDTA was present, with the various association constants for EDTA reported by Martell and Calvin (29). A calculation estimating the additional influence of 1 mM oxalate on the concentration of ionized Ca showed that it could be neglected. These calculated values for ionized Ca are approximations, since the reported association constants for complexes between ATP and divalent ions vary over a wide range. Burton's association constants are the highest reported so far. The concentration of protein remaining in the supernatant was determined by Lowry's procedure (26).

#### EXPERIMENTAL RESULTS

Relaxing factor which inhibited superprecipitation removed bound, exchangeable Ca from skeletal and cardiac (dog) myofibrils, as well as from actomyosin (Table IV). In the presence of 1.0 mM oxalate it was capable of reducing the amount of bound exchangeable Ca to the same extent as 2.0 mM EGTA. In the absence of oxalate, it usually removed less Ca

from myofibrils, but the reduction in bound Ca was still extensive enough to account for an inhibition of superprecipitation by Ca deficiency (49).

In decreasing concentrations the relaxing factor removed less Ca and inhibited myofibrillar activity less: more ATP was hydrolyzed and superprecipitation was increased (Fig. 1). Fig. 1 gives the impression that the relaxing factor inhibited the hydrolysis of ATP to a lesser extent than 2 mM EGTA, even in concentrations which removed as much Ca<sup>45</sup> as did 2 mM EGTA. However, it should be noted that since the activation of the

TABLE IV  
REMOVAL OF EXCHANGEABLE Ca FROM VARIOUS ACTOMYOSIN SYSTEMS BY RF WITH AND WITHOUT OXALATE

Actomyosin system		Ca				Superprecipitation
		RF	Added	Total†	Bound Ca*	
		mg N/ml	μM	μM	μmoles/gm	mg protein/ml ppt
Skeletal Mf	CP§	0	20	34	2.5	130
Skeletal Mf	CP§ + 2.0 mM EGTA	0	20	34	0.5	37
Skeletal Mf	CP§	0.1	20	41	1.0	26
Skeletal Mf	1.0 mM oxalate	0	20	33	2.4	116
Skeletal Mf	1.0 mM oxalate + 2.0 mM EGTA	0	20	33	1.1	29
Skeletal Mf	1.0 mM oxalate	0.26	20	60	1.1	30
Cardiac Mf	1.0 mM oxalate	0	20	27	1.3	87
Cardiac Mf	1.0 mM oxalate + 2.0 mM EGTA	0	20	27	0.4	43
Cardiac Mf	1.0 mM oxalate	0.08	10	22	0.6	42
Actomyosin	1.0 mM oxalate	0	10	22	1.3	120
Actomyosin	1.0 mM oxalate	0.09	10	25	0.1	25

\* Ca bound to 1 gm myofibrillar protein or actomyosin.

† Added + contaminations in solutions, actomyosin or myofibrils and RF.

§ CP + CPkinase, Mg-ATP only 1.0 mM.

|| Maximal value, since this particular RF had not been centrifuged prior to the assay and some Ca bound to precipitated RF might be included.

Myofibrils (Mf), 1.5 to 2.0 mg protein/ml, actomyosin 0.85 mg/ml; Γ/2 = 0.12 for Mf, 0.06 for actomyosin, 4 mM Mg-ATP, incubation with ATP 130 to 200 sec.

relaxing factor requires incubation with ATP, the relaxing factor becomes fully active only after a lag period (33), whereas the chelation of Ca, the basis for the EGTA effect (49), occurs instantaneously. Consequently, in the presence of the relaxing factor, the rate of ATP hydrolysis (measured from the time of ATP addition) included the initial uninhibited rate, before the relaxing factor produced its effect. By contrast, the amount of bound exchangeable Ca indicated the final state reached at the end of the incubation period. Therefore the data for ATPase activity and Ca-binding in Fig. 1 are not quite comparable, and do not show the correct relationship between ATPase activity and the exchangeable Ca bound to myofibrils.

This relationship is described more accurately by the data in Fig. 2. The amount of ATP hydrolyzed and the quantity of exchangeable Ca remaining bound to myofibrils were measured at successive intervals after the addition of ATP (from 28 to 150 sec.). From the quantity of ATP split during each interval, the average rate was calculated, and plotted at the time  $\frac{t_1 + t_2}{2}$ . The amount of bound Ca and the extent of superprecipitation were referred

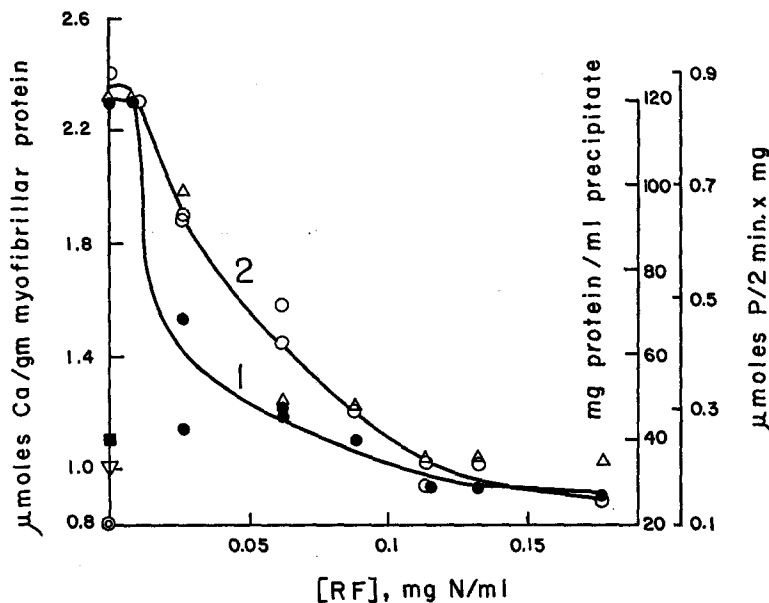


FIGURE 1. Exchangeable Ca bound to myofibrils, ATPase, and superprecipitation of myofibrils as a function of the concentration of RF. Ca binding, left ordinate: curve 1 and, in presence of 2 mM EGTA, filled square; ATPase, right ordinate: curve 2 and, in presence of 2 mM EGTA, double circle; superprecipitation, inset ordinate: open triangles and, in presence of 2 mM EGTA, inverted triangle. 1.98 mg/ml myofibrillar protein, 1.0 mM oxalate, 0.02 mM  $\text{Ca}^{48}\text{Cl}_2$ , 4 mM Mg-ATP, incubation 110 to 130 sec.

to the end of the time interval, which coincided with the time of complete separation of the myofibrils from the assay solution. This time could not be determined quite accurately, but was estimated by interpolation from a series of experiments measuring precipitation times as described under Experimental Procedure. Fig. 2 shows that the ATPase activity decreased sharply when relatively small amounts of Ca had been removed from the myofibrils, in a manner similar to the fall in ATPase activity on removal of bound Ca by lowering the concentration of ionized Ca with Ca buffers (49). Fig. 2 shows that a reduction in the amount of bound exchangeable Ca to about 75 per cent reduced the ATPase activity to about 20 per cent as

compared to about 40 per cent in the absence of factor (49). The difference between the values in the absence and in the presence of factor is within experimental error (see Experimental Procedure). For the fall of ATPase activity with time in the absence of the relaxing factor shown in Fig. 2, see references 4, 47.

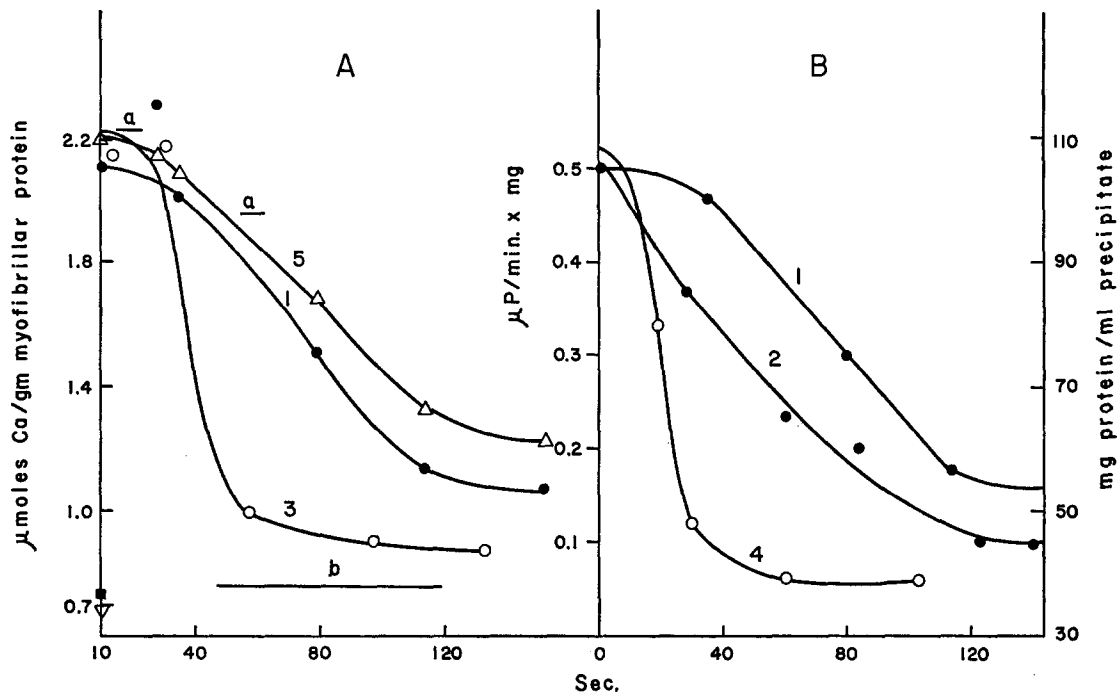


FIGURE 2. Exchangeable Ca bound to myofibrils, ATPase, and superprecipitation of myofibrils in the presence of RF as a function of the length of the incubation period with ATP. Ca binding, left ordinate, curves 1 (RF 0.03 mg N/ml) and 2 (RF 0.05 mg N/ml); ATPase, middle ordinate, curves 3 (RF 0.03 mg N/ml) and 4 (RF 0.05 mg N/ml); superprecipitation, right ordinate, curve 5 (RF 0.03 mg N/ml). In the absence of RF: Ca binding without and with 2 mM EGTA filled circle and square, respectively, on left ordinate; ATPase, without and with 2 mM EGTA, horizontal lines *a* and *b*, respectively; superprecipitation, without and with 2 mM EGTA triangle and inverted triangle, respectively, on left ordinate. 1.9 mg/ml myofibrillar protein, 1.0 mM oxalate, 0.02 mM  $\text{Ca}^{45}\text{Cl}_2$ , 4 mM Mg-ATP.

Fig. 2 shows furthermore that the relaxing factor not only inhibited ATPase and prevented superprecipitation but actually reversed superprecipitation. Whereas after only 40 sec. incubation of myofibrils and relaxing factor with ATP superprecipitation was still nearly complete, it was reversed by nearly 50 per cent after incubation for 150 sec. Both the relaxing factor and EGTA in the presence of ATP not only relax contracted glycerol-extracted muscle

fibers (2, 13), but also are capable of partially reversing the superprecipitation of myofibrils (48) and both are capable of dissolving actomyosin gels (31, 51).

The relationship between the ATPase activity and the degree of superprecipitation with various ratios of  $\frac{\text{EGTA}}{\text{Ca-EGTA}}$  (49, Fig. 1 and 2) on the one hand and after incubation with relaxing factor on the other is not quite comparable because superprecipitation had been prevented with EGTA whereas it had been reversed with the relaxing factor. Since superprecipitation was found to be only partially reversible (also with EGTA (48)), the difference between the data of Fig. 2 and those published elsewhere (49)

TABLE V  
PARALLELISM BETWEEN ADP INHIBITION  
OF RELAXING EFFECT AND REMOVAL OF EXCHANGEABLE  
Ca FROM MYOFIBRILS BY RF

Addition	RF	Ca		Total	Bound Ca	Superprecipitation
		Added				
	<i>mg N/ml</i>	$\mu\text{M}$	$\mu\text{M}$	$\mu\text{moles/gm}$	<i>mg protein/ml ppt</i>	
None	0.11	10	38.5	1.2	31	
0.5 mM ADP	0.11	10	40*	1.5	45	
None	0.05	10	32	1.1	35	
1.0 mM ADP	0.05	10	35	2.2	110	
1.0 mM ADP ‡	0	10	29	2.1	120	
2.0 mM EGTA ‡	0	10	26.5	0.9	30	
0.5 mM EGTA ‡	0	10	26.5	1.4	30	

\* ADP was contaminated by Ca.

‡ ADP was without influence in the absence of RF.

Skeletal Mf 2.04 mg protein/ml, 1.0 mM oxalate, 4.0 mM Mg-ATP. The incubation period with ATP of 70 to 120 sec. was preceded by an incubation with ADP for 60 sec.

should be expected. The reversibility of superprecipitation decreased with the length of time myofibrils had remained superprecipitated before the relaxing agent was added or took effect.

Inhibition of the relaxing factor by ADP (12, 20) affected its relaxing effect and its capacity to remove Ca<sup>45</sup> from myofibrils to a comparable degree (Table V).

If most of the bound exchangeable Ca dissociated from the myofibrils in presence of the relaxing factor because the factor accumulated all Ca present (11, 14, 19), the factor must have lowered the concentration of ionized Ca to less than 0.1  $\mu\text{M}$  (see Ca binding as a function of ionized Ca (49)). We found that in the presence and absence of oxalate (provided creatine phosphate and phosphokinase had been added to avoid the accumulation of ADP (21)), the relaxing factor was capable of lowering the concentration of total

exchangeable Ca from an initial concentration of 28  $\mu\text{M}$  to a final value of 0.05  $\mu\text{M}$ , corresponding to a concentration of ionized Ca of about 0.008  $\mu\text{M}$  (Table VI). Five out of seven different factor preparations (from 0.17 to 0.018 mg N/ml) reduced the concentrations of total exchangeable Ca from the initial concentrations of 10 to 30  $\mu\text{M}$  to values of 0.05 to 0.3  $\mu\text{M}$  with corresponding concentrations of ionized Ca of 0.01 to 0.04  $\mu\text{M}$ . (The final concentrations of total and ionized Ca produced by the other two preparations were much higher with nearly 1  $\mu\text{M}$  for total and 0.3  $\mu\text{M}$  for ionized Ca.) When the initial concentrations of Ca were higher, 100 to 200  $\mu\text{M}$ , only one preparation (0.19 mg N/ml) out of three (0.13 and 0.17 mg N/ml) lowered

TABLE VI  
EFFECT OF VARIOUS ADDITIONS  
ON THE REMOVAL OF EXCHANGEABLE Ca  
FROM THE MEDIUM BY RF

Preparation	RF	Additions	Total exchangeable Ca	Ca in supernatant Per cent of total	Ionized Ca in supernatant
	<i>mg N/ml</i>		$\mu\text{M}$		$\mu\text{M}$
1*	0.065	CP‡ + 1.0 mM oxalate + 2 mM Mg-ATP	28	0.17	0.008
	0.065	CP‡ + 2.0 mM Mg-ATP	28	0.21	0.01
	0.065	1.0 mM oxalate + 4.0 mM Mg-ATP	28	0.37	0.018
	0.065	4.0 mM Mg-ATP	28	38.0	1.8
2§	0.13	CP‡	28	0.3	0.02
	0.13	CP‡ + 2.0 mM Mg-EDTA	28	15.5	0.005
	0.13	CP‡ + 0.2 mM Mg-EDTA	28	3.8	0.01

\*  $\Gamma/2 = 0.12$ .

‡ CP + CPkinase; 1 hr. at 100,000 g.

§  $\Gamma/2 = 0.15$ , 1.0 mM Mg-ATP.

the total Ca to 0.14  $\mu\text{M}$  and the ionized Ca to 0.02  $\mu\text{M}$ ; the two others left the concentrations of Ca much higher, 4 to 5  $\mu\text{M}$ , 1 to 1.4  $\mu\text{M}$  respectively.

The variability of the results might be explained by Table VII, which illustrates one of the difficulties inherent in the method of determination. To measure the Ca left in the solution by the relaxing factor, the factor must be completely removed from the supernatant by a centrifugation of sufficient speed and duration. However, it appears that an adequate centrifugation of 60 min. resulted in the partial inactivation of the factor, if its concentration was low, possibly because the ratio Ca/relaxing factor was increased. On the other hand, a centrifugation of 30 min. might not be adequate to precipitate the last traces of the factor. After 30 min. the supernatant still contained a small amount of protein (about 3 to 5 per cent of the total) which was heavy enough to spin down during the second 30 min.

Some of this protein could conceivably have been relaxing factor. In that case some of the Ca remaining in the supernatant would not have been soluble Ca but Ca bound to vesicles. These findings suggest that a more adequate method of separating the factor from the solution might give more consistent and more accurate results. Attempts to use filtration methods (Millipore) failed: either a considerable fraction of the relaxing factor or no solution at all passed through the filters.

TABLE VII  
VARIABILITY IN THE EXTENT TO WHICH Ca  
WAS REMOVED FROM THE MEDIUM AFTER TWO DIFFERENT  
PERIODS OF CENTRIFUGATION

RF	Total exchangeable Ca	Ca in supernatant Per cent of total	Time elapsed	
			Before*	During centrifugation
<i>mg N/ml</i>	$\mu\text{M}$		<i>min.</i>	<i>min.</i>
0.013	21	0.5	14	74
0.013	21	1.2	12	42
0.0065	21	18.5	14	74
0.0065	21	0.7	12	42
0.0013	20	34	14	74
0.0013	20	7	12	42

\* From the addition of ATP to the moment 100,000 *g* was reached.  
 $\Gamma/2 = 0.12$ , 1.0 mM oxalate, 2.0 mM Mg-ATP, CP + CPkinase.

TABLE VIII  
COMPARISON OF THE EXTENT OF THE REMOVAL OF EXCHANGEABLE  
Ca FROM MYOFIBRILS WITH THE REDUCTION IN THE CONCENTRATION  
OF IONIZED Ca BY RF

Experi- ment No.	Additions	EGTA	RF	Total exchangeable Ca	Ionized Ca	Bound Ca*
1	1.0 mM oxalate	0	0	33	4	2.2
	1.0 mM oxalate	2.0	0	33	0.02	0.7
	1.0 mM oxalate	0	0.065	41	0.02‡	0.9
	None	0	0.065	41	1.8‡	2.0
	1.0 mM oxalate	0	0.013	35	0.04‡	1.6
2	1.0 mM oxalate	0	0	32	4	1.6
	1.0 mM oxalate	2.0	0	32	0.02	0.7
	1.0 mM oxalate	0	0.12	44	0.4§	1.1

\* Bound to 1 gm myofibrillar protein.

‡, § As determined in controls in the absence of Mf.

‡ 30 min. centrifugation.

§ 60 min. centrifugation.

Mf, 19 mg protein/ml (Experiment 1) and 22 mg/ml (Experiment 2), 4 mM Mg-ATP, incubation of RF and Mf with ATP 120 to 140 sec.



In Table VIII a comparison is made between the extent to which the factor removed Ca from myofibrils and the extent to which it lowered the concentration of ionized Ca under similar conditions. The two were fairly well correlated in three out of four experiments. The exception occurred with the low factor concentration of Experiment 1 in Table VIII. Whereas the factor lowered the concentration of ionized Ca to  $0.04 \mu\text{M}$ , *i.e.* about as much as 2 mM EGTA, the binding of Ca by the myofibrils was nearly 70 per cent complete. This result might be rationalized by assuming that the short period of incubation (2 min.) of the factor and myofibrils with ATP was inadequate for the accumulation of Ca by such a small amount of factor, particularly since the myofibrils liberated ADP to a final concentration of 1.5 mM during this period. Although ADP is much less inhibitory if added after ATP than if added before (21), it still inhibits the rate of Ca accumulation by the factor considerably (21). The controls determining the lowering of the ionized Ca by the factor lasted much longer (12 min. before centrifugation, followed by 30 min. at 100,000 *g*) and allowed much less ADP to accumulate since the myofibrils had been omitted.

#### DISCUSSION

Our results indicate not only that the relaxing factor removes as much exchangeable Ca from actomyosin and myofibrils as does EGTA, but they suggest also that the quantitative relationship between the ATPase activity and the amount of exchangeable Ca bound to myofibrils might be independent of the presence of the relaxing factor. Thus it appears that the relaxing factor acts on actomyosin systems by causing the dissociation of Ca from actomyosin and not by directly affecting the ATPase activity or superprecipitation. This conclusion is corroborated by the finding that the superprecipitation of "non-relaxing" actomyosins (49, 51), which are insensitive to Ca removal, cannot be inhibited by the relaxing factor (50).

The relaxing factor might achieve this dissociation of Ca from actomyosin in different ways. On the one hand, a soluble relaxing substance (produced by the relaxing factor (7, 8, 34, 36) might combine with actomyosin and lower its affinity for Ca, or might act as a chelating agent for Ca. On the other hand, the vesicles of the relaxing factor might be able to accumulate Ca from the medium to such an extent that the concentration of ionized Ca falls to values lower than the dissociation constant of the Ca-actomyosin complex. Since the relaxing factor is capable of lowering the concentration of soluble and ionized Ca below  $0.1 \mu\text{M}$  and  $0.01 \mu\text{M}$ , respectively, it does not appear necessary to postulate any additional mechanism—such as a soluble relaxing substance—to explain its effect on actomyosin systems. An un-

equivocal answer would be provided by a comparison of the time course of Ca removal from the medium with the time course of Ca removal from actomyosin.

Hasselbach and Makinose (21) compared the ability of the relaxing factor to remove Ca from the medium with its ability to inhibit the ATPase activity of myofibrils under a variety of conditions. Whereas usually both effects of the relaxing factor are influenced to a similar degree, they found two notable exceptions. Caffeine prevented the depression of myofibrillar ATPase by the factor without impairing either the extent (personal communication) or the rate (21) of Ca removal from the medium by the factor. We cannot offer any explanation for this finding. We were unable to produce any effect of caffeine on the factor: even after 30 min. preincubation with 10 mM caffeine the factor still fully inhibited superprecipitation. In addition no correlation was found between Ca removal and relaxing activity by Hasselbach and Makinose with cardiac factor (21). Although cardiac factor inhibited myofibrillar ATPase, it removed only very little Ca from the medium. Since, however, each measurement of Ca uptake by the factor requires a prolonged centrifugation, it cannot be excluded that the cardiac factor, which is particularly labile (8), released previously accumulated Ca during this period (compare Table VII). It should be pointed out that the critical parameter for its relaxing effect is not the factor's *maximal capacity* for Ca accumulation but its ability to *reduce* the concentration of *ionized* Ca present in the medium to below 0.5 to 0.25  $\mu\text{M}$ , the required value for the inhibition of superprecipitation and ATPase activity which varies in dependence on the ionic strength to which the myofibrils are exposed (48, 49, 51). The preparations of Hasselbach and Makinose (20) from skeletal muscle lower the concentration of ionized Ca to 0.1  $\mu\text{M}$ , of soluble Ca to 1  $\mu\text{M}$ , which accounts for their inhibitory action on myofibrillar ATPase activity.

The tenfold difference between the preparations of Hasselbach and Makinose (20) and ours with respect to the extent to which they lower the Ca of the medium might be explained by a difference in experimental conditions: oxalate, ATP, and Mg, each 5 mM, in their experiments, *versus* no or 1 mM oxalate and maximally 4 mM ATP and Mg in ours. Hasselbach and Makinose pointed out that in the presence of 5 mM oxalate, 5 mM ATP is overoptimal and somewhat inhibiting compared to 1 mM ATP (19). Furthermore, it cannot be excluded that the extensive precipitation of Ca oxalate (Hasselbach and Makinose, personal communication) in the interior of the vesicles of their factor impairs the ability of the vesicles to pick up Ca, when the Ca concentration in the medium falls to low levels. However, since our preparations in this respect compare favorably also with those of Ebashi (11, 14), who did not use oxalate, we should like to mention that we consider it im-

portant that we removed the mitochondria quite thoroughly, and assayed at pH 6.5 which we found to be more favorable than pH 7.

The maximal ratio between Ca accumulated in the vesicles and Ca in the medium, in the complete absence of oxalate (CP and CPkinase present) was  $10^6$  (three different preparations), 24 mM Ca inside and 0.0003 mM Ca outside (ionized Ca 0.05  $\mu\text{M}$ ). The Ca concentration in the interior of the vesicles follows from the binding of 0.19  $\mu\text{mole}$  Ca per mg dry weight of the factor which occupies maximally 8  $\mu\text{l}$  when wet.<sup>2</sup> Assuming Ca to be free in the vesicles it would require 6,900 calories per mole or the hydrolysis of 1 ATP per Ca to move Ca against such a concentration gradient if the efficiency were 100 per cent. However, the average value for the energy required to *establish* this concentration gradient must be smaller since Ca is transported against a gradient increasing from 1 to  $10^6$ . With a value of 1,000 in our experiments for the ratio of extra- to intravesicular volume (this ratio determines the increment by which the gradient increases per fraction of a mole transported) we arrived at an average value of 4,200 calories per mole Ca, *i.e.* the hydrolysis of 1 ATP per Ca (19), averaged over the whole period of transport, would provide more than sufficient energy. Since in these experiments the rate of ATP hydrolysis was such that per minute 10 times more ATP was split than the total Ca transported (we have no direct data on the duration of the Ca transport, but myofibril experiments suggest several minutes), the formation of such a concentration gradient cannot be excluded on thermodynamic grounds. To reduce the concentration ratio by one order of magnitude, 90 per cent of the accumulated Ca must have been bound, either by the substance of the vesicles (1 mole Ca per 5,000 gm) or conceivably as a Ca-phosphate precipitate.

The deduction that the vesicles might contain some substances which bind Ca very strongly, *e.g.* a carrier substance and possibly other components, might be used to explain the existence of a soluble relaxing substance (7, 8, 34, 36) which can be inactivated by Ca. It is conceivable that under certain conditions some of the vesicular components might be solubilized and able to act as Ca-chelating agents. Evidence for a soluble relaxing substance was first obtained by Parker and Gergely (36) and independently by Briggs (8) and later by others (7, 34). Its presence was deduced from the fact that in the supernatant of ATP-incubated factor solution, glycerinated fibers relax (7, 8, 34) and myofibrillar ATPase is partially inhibited (34, 36). These effects must be caused by a soluble relaxing substance provided three other possibilities, which we should like to stress, have been excluded. Since the incubation with factor removes Ca from a solution, it has to be excluded

<sup>2</sup> Determined after 1 hr. centrifugation at 100,000 g.

first, that the glycerinated fibers or the myofibrils were not inhibited just by a lack of Ca in these solutions, in contrast to untreated solutions contaminated by Ca. This possibility is excluded in Briggs' experiments (8), because he introduced so much Ca with his myofibrils (49) that the Ca content of his solutions was irrelevant. Second, after the pretreatment of his actomyosin systems with desoxycholate (25), no reactivation of intrinsic factor (frequently contained in actomyosin systems (3, 33) by soluble cofactor (9, 17) could have taken place. Nagai *et al.* (34), who found no protein connected with their soluble substance, thereby excluded only the third possibility, the presence of a very small contamination by the vesicles of the relaxing factor.

In conclusion, we would like to speculate how Ca movements within the muscle might regulate its state of contraction or relaxation. This speculation is based largely on the results of studies on isolated actomyosin systems and relaxing factor which are applied to living muscle under the following assumptions. First, superprecipitation of actomyosin or syneresis of myofibrils is supposed to represent the *in vitro* equivalent of muscular contraction. Second, the reversal of the syneresis of myofibrils or the solubilization of an amorphous actomyosin gel is supposed to represent the *in vitro* equivalent of relaxation, a justifiable assumption since these phenomena occur under conditions which produce relaxation in contracted, glycerol-extracted (see Results) muscle fibers. Since any agent which produces relaxation or the reversal of superprecipitation logically must by the same mechanism prevent contraction of muscle fibers or superprecipitation of actomyosin, the degree of prevention of superprecipitation by agents which are known to produce relaxation, may be considered a result of their relaxing activity. (By contrast, the inhibition of superprecipitation or of ATPase activity (see below) as isolated phenomena by agents which have not been shown to reverse ATP-induced contractile processes does not indicate their relaxing activity.) If it has been established that the relaxing effect is accompanied by a simultaneous inhibition of actomyosin ATPase activity (this has been shown for all known relaxing agents), this inhibition alone also may be considered an expression of its relaxing activity. It might be noted at this point that a plausible theory exists for the mechanism of this inhibition of ATPase activity which may be summarized as follows (27). The ATPase activity of actomyosin in the presence of Mg results from the effect of actin on myosin, whereby actin in the manner of an enzymatic cofactor greatly increases the very low rate of ATP hydrolysis by myosin alone (we found that on saturation with actin the rate increased twentyfold). Since it appears to be characteristic of the relaxed state of living and glycerol-extracted muscle that bonds between actin and myosin are broken—thin and thick filaments may easily be

pulled apart (18)—relaxing agents presumably prevent actin from activating the myosin ATPase without necessarily influencing the ATPase activity of myosin alone. It has actually been found that the myosin ATPase activity is not altered by either the relaxing factor (27) or by the removal of Ca by EGTA (unpublished observations).

Based on these premises, the following findings are applied to living muscle. In the presence of more than  $10^{-5}$  M ATP actomyosin and myofibrils superprecipitate only if they have formed a complex with Ca (49). If the concentration of ionized Ca has been lowered sufficiently for the complex to dissociate, ATP causes a partial reversal of the syneresis of myofibrils (48) and the dissolution of an amorphous actomyosin gel (51). It was shown in this paper that fragmented sarcoplasmic reticulum effects the dissociation of the Ca-actomyosin complex, presumably by lowering the concentration of ionized Ca through its mechanism of Ca accumulation (14, 19). It has been demonstrated (35, 38, 39) that the introduction of Ca into living muscle fibers results in a reversible contraction of myofibrils.

Based on these findings, we would like to suggest the following as the sequence of events in living muscle. After stimulation, during the latent period, Ca is released into the cytoplasm and combines with actomyosin. The saturation of actomyosin with Ca would require the release of at least  $0.1 \mu\text{mole}$  of Ca per gm muscle. The intensity of the active state (45) begins to decrease when the sarcoplasmic tubules have reduced the concentration of ionized Ca in the sarcoplasm sufficiently for the Ca-actomyosin complex to begin to dissociate. Since the ratio of myofibrils to sarcoplasm is high, the interval from the beginning to the final state of dissociation, *i.e.* from the end of the plateau of the active state (23) to complete relaxation, could be relatively long. It would be determined by the rate of Ca accumulation by the tubules. During this interval, attending the accumulation of Ca by the tubules, about  $0.1 \mu\text{mole}$  of ATP per gram of muscle should be broken down according to the data of Hasselbach and Makinose (19), although from the point of view of energetics this amount could be considerably smaller, depending on the concentration gradient between cytoplasm and tubules during the removal of the bulk of the Ca. During rest, the concentration of ionized Ca would be low enough for maximal inhibition of actomyosin ATPase, presumably around  $0.2 \mu\text{M}$  (depending on ionic strength, pH, etc. of cytoplasm). This implies that the cytoplasmic enzyme systems functioning during recovery should either not be Ca-activated or bind Ca strongly enough to remain saturated at such low concentrations of Ca.

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