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Role of Magnesium and Calcium in the First and Second Contraction of Glycerin-Extracted Muscle Fibers*

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ABSTRACT: The role of Mg, Ca, and ATP in isometric contraction of well-washed, glycerin-extracted skeletal muscle was studied. Two contractions were obtained: (1) a rapid "first" contraction dependent only on Mg and ATP at [Ca^{2+}] below threshold for ATPase activation (less than 10^{-8} M Ca^{2+}); (2) a much slower "second" contraction requiring [Ca^{2+}] above 3×10^{-7} M in addition to Mg and ATP. The initial speed of the first contraction is related to [$MgATP^{2-}$] and follows a Langmuir adsorption isotherm. The speed is maximal at zero time. These results suggest that the initial event is the binding of [$MgATP^{2-}$] to the fiber. The fibers relax spontaneously when [Ca^{2+}] is below 10^{-7} M and after this relaxation a slower "second" contraction occurred upon raising the [Ca^{2+}] to above 3×10^{-7} M. The rate of the second contraction is related to the [Ca^{2+}] in the range of 10^{-7}–10^{-5} M, a range required for activation of fiber ATPase suggesting that Ca^{2+} activates ATPase and the second contraction is controlled by the rate of ATP hydrolysis and the subsequent binding of new MgATP. When Ca is present in the initial contraction mixture, the fibers remain contracted because MgATP is turned over continuously. The fact that the two contractions occur under different conditions may explain the controversies concerning the requirements for Ca^{2+} and for ATP hydrolysis in muscular contraction.

It is generally believed that the release of calcium by the sarcoplasmic reticulum is an intermediate step in the excitation-coupling mechanism of muscular contraction (Sandow, 1965; Nayler, 1967) and Ca^{2+} has been reported to be a requirement for the contraction of glycerin-extracted skeletal muscle (Seidel and Gergely, 1963; Filo *et al.*, 1965; Portzehl *et al.*, 1965; Schädler, 1967). Magnesium (Mg) and ATP were demonstrated early (Szent-Györgyi, 1946, 1947; Bowen, 1951) to be also a requirement for the contraction of glycerin-extracted muscle and this requirement is now generally accepted. The requirement for Ca is still controversial because in fibers which had been extracted for more than 12 weeks, Mg (and ATP) will cause a maximal contraction which is not enhanced by Ca (Embry and Briggs, 1966). Briggs and King (1962) who studied fibers which were extracted for more than 2 weeks in glycerine plus deoxycholate also concluded that there is no relationship between the Ca^{2+} in their contraction mixtures and the contractions. These authors even treated their chemicals with Chelex cationic exchange resin to remove traces of Ca and this treatment did not affect their contractions. They reported that they were able to reduce the Ca contamination in their contraction solutions to less than 7×10^{-9} M, and calculated that the free Ca concentration was probably less than 7×10^{-10} M. Watanabe *et al.* (1964) have also reported that glycerinated extracted fibers can be contracted completely in the absence of Ca.

Takahashi *et al.* (1965) studied the contraction of glycerin-extracted isolated sarcomeres which were resuspended several times and concluded that the MgATP complex is essential for these contractions and that Ca is nonessential. Kuribayashi (1969) found that Ca is not essential for ATP (Mg) contractions of glycerinated taenia coli muscle. This conclusion agrees

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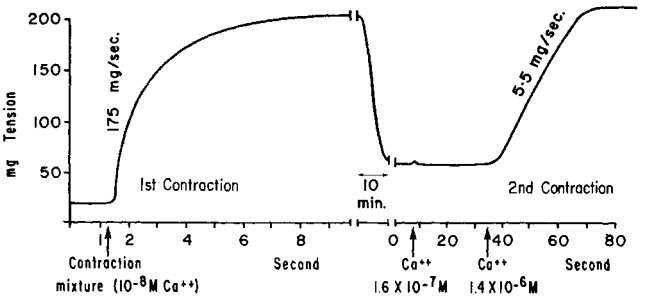

FIGURE 1: Ca was added to the original contraction mixture (5×10^{-3} M ATP, 5×10^{-3} M $MgCl_2$, 0.1 M KCl, and 10^{-3} M EGTA, pH 6) after spontaneous relaxation causing recontraction. Fiber diameter was about 300 μ.

with Tomita (1965) who studied glycerinated smooth muscle of the ileum.

Weber and Winicur (1961) who studied the superprecipitation reaction of actomyosin did not find Ca to be essential for this reaction and Bowen (1951) was able to show that Ca under certain conditions can inhibit the contraction of actomyosin threads (*cf.* Erdös, 1942).

The above observations are not in agreement with the more generally accepted opinion that trace amounts of Ca (in the order of 10^{-6} M) are always required for muscle contraction and for the contraction of glycerin-extracted muscle (Ebashi, 1961; Seidel and Gergely, 1963; Caldwell and Walster, 1963; Caldwell, 1964; Portzehl *et al.*, 1964, 1965; Weber *et al.*, 1964; Filo *et al.*, 1965; Schädler, 1967, and others).

In this article we report the finding that two contractions can be obtained with well-washed, glycerin–deoxycholate-treated rabbit psoas muscle fibers, a MgATP-dependent (Ca-independent) rapid first contraction and a Ca- and MgATP-dependent slower second contraction.

Materials and Methods

Preparation of Fibers. Fiber bundles from rabbit psoas muscle were extracted in aqueous solution of 50% glycerin and 3×10^{-3}, 5×10^{-3}, and 10^{-2} M deoxycholate for 1 day at 0°, then stored in a fresh solution of the same composition at −20° for a period of 3 months to 3 years. The deoxycholate was added to abolish the relaxing activity (Ca pumping) of the granules (Elison *et al.*, 1965) and electron micrographs made by Dr. R. D. Yates showed that the sarcoplasmic reticulum and mitochondria were largely removed from 10 mM deoxycholate-treated fibers. Before use, the fiber bundles were immersed for about 30 hr in 0.05 M EDTA and 10% glycerin at 0°, then washed twice for 30 min in 2.5×10^{-3} M EGTA[1] and 0.1 M KCl at room temperature (approximately 23°). The test preparations contained 6–10 fibers per fiber bundle which had an average diameter of 200–300 μ and a length of about 3 cm. A bundle of fibers was cut into 6- to 8-mm lengths so that four to five experiments were done with segments of the same bundle. The average diameter of a single fiber was about 50 μ. The bundles of fibers used had more the shape of ribbons than of cylinders, and nearly every individual fiber in the bundle was in direct contact with the bathing solution.

Apparatus. The glycerin-extracted fibers were mounted on an isometric myograph, slightly modified from the one described by Ranney (1954a,b), and an RCA "5734" transducer was used to record tension. The recording system required 250 msec for a full-scale deflection. For the experiments which determined the role of Mg and ATP, parts of the same fiber, approximately 6 mm long, were first glued with Testor's type A cement to small pieces of paper which were clamped into place in the myograph. In the experiment which studied the role of traces of Ca^{2+} in the first contraction a fiber 1 cm long was looped around the hook on the bristle of the transducer and the ends were clamped together in the myograph without using glue or paper. All fibers were attached to the myograph while immersed in fresh 0.1 M KCl, 2.5×10^{-3} M EGTA, or KCl treated with Chelex cationic exchange resin. All fibers were left for an additional 5–10 min under 30 mg of "resting" tension prior to the start of each experiment.

The bath was stirred by a stream of air or helium bubbles. The bath was emptied by suction and fresh solution was immediately added. All experiments were performed at room temperature (approximately 23°).

The initial rate of tension formation expressed in milligrams per second was obtained by recording at a paper speed of 30 in./min and drawing a tangent at the start of the change in tension.

Chemicals. Traces of calcium which contaminated the following chemicals were estimated in concentrated solutions with a Beckman atomic absorption spectrophotometer.

Na_2H_2ATP (99% pure) was obtained from two sources, Nutritional Biochemical Corp. and P-L Biochemicals, Inc. A solution of ATP was adjusted to pH 7 with NaOH and made up to 2×10^{-2} M. A 5×10^{-3} M ATP solution adjusted to pH 7, was found to contain 2×10^{-7} M Ca.

$MgCl_2 \cdot 6H_2O$ (Baker, Analyzed Reagent grade). A 5×10^{-3} M $MgCl_2$ solution was found to contain 2.5×10^{-7} M Ca.

KCl (Baker, Analyzed Reagent grade). A 0.1 M KCl solution was found to contain 1.4×10^{-7} M Ca.

In some experiments, traces of Ca^{2+} which contaminated these chemicals were largely removed by treating the solutions with Chelex cationic exchange resin according to the method of Briggs and King (1962). The residual amount of Ca^{2+} was again estimated using the Beckman atomic adsorption spectrophotometer in concentrated solution. In most experiments, however, EGTA was added in order to reduce the free Ca^{2+} concentration.

EGTA (Eastman Organic Chemicals 98+% pure). A 0.1 M solution was adjusted to pH 7 with NaOH.

EDTA (Fisher Scientific Co., ACS, 99.4% pure). A 0.1 M solution was adjusted to pH 7 with NaOH.

NaOH (Fisher Scientific Co.) certified 1 N solution.

Results

Two types of contractions were obtained with our glycerin-extracted fibers (see Figure 1). The first contraction, a very rapid contraction, occurred upon the exposure of the fibers to a contraction solution containing only Mg and ATP in 0.1 M KCl. Reducing the Ca ion contamination of these chemicals by additions of EGTA or treatment with Chelex cationic exchange resin to less than 10^{-7} M produces a similar rapid contraction which is now followed by a spontaneous relaxation. These relaxed fibers are very sensitive to Ca^{2+} and contract a second time upon the addition of this ion to the original contraction mixture. No other contractions were found.

The "First" Contraction. Exposure of the glycerin-extracted fibers to a contraction solution containing 5×10^{-3} M $MgCl_2$, 5×10^{-3} M ATP, and 0.1 M KCl at pH 7.0 produces an im-

[1] Abbreviation used is: EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetic acid.

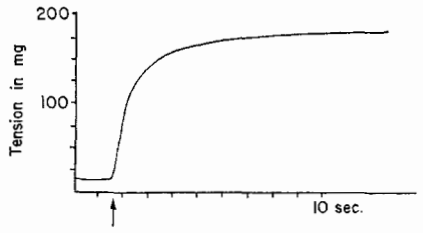

FIGURE 2: First contraction in 5×10^{-3} M ATP, 5×10^{-3} M $MgCl_2$, and 0.1 M KCl (pH 7.0). Fiber diameter about 300 μ.

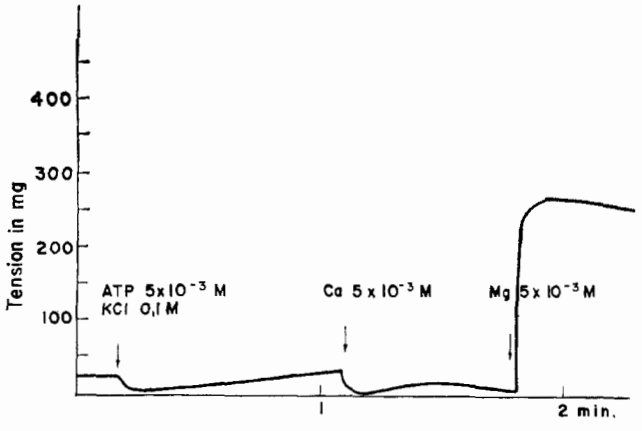

FIGURE 3: Inhibiting effect of Ca on slow initial contraction, presumably due to traces of Mg in ATP or KCl (see text). Fiber diameter 250 μ.

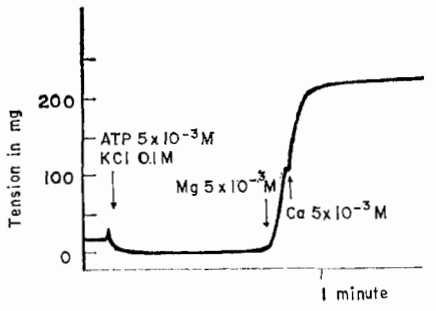

FIGURE 4: Absence of an effect by Ca on the speed of contraction following the addition of ATP and Mg. Fiber diameter about 250 μ.

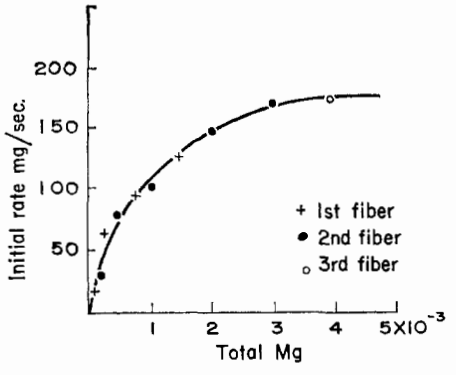

FIGURE 5: Initial speed of tension formation as function of total Mg concentration for 5×10^{-3} M ATP and 0.1 M KCl, pH set to 7 after each Mg addition. The maximum tension was about 250 mg.

mediate rapid contraction which reaches 50% of maximum tension within 1 sec and 100% maximum tension within 10 sec (see Figure 2). The initial rate of tension formation obtained with the 300-μ diameter fiber shown in Figure 2 was 178 mg/sec at zero time. A similar rapid first contraction is shown in Figure 1 where 10^{-3} M EGTA was added to the contraction mixture, reducing the 4×10^{-7} M Ca contamination in this contraction solution (measured with the atomic absorption spectrophotometer) to a free Ca concentration of less than 10^{-8} M at pH 7 (see Appendix), which is considerably below that needed to activate the fibrillar ATPase (Caldwell, 1964; Portzehl *et al.*, 1964; Schädler, 1967). Similar treatments of the contraction mixture with Chelex cationic exchange resin did not reduce the speed of this first contraction.

A typical maximum tension produced by a bundle of 4 fibers was approximately 200 mg, a calculated force of 2540 g/cm^2 correcting for space between the fibers. Similar values for maximum tension for glycerin-extracted skeletal muscle are reported by Ranney (1954a), Bowen and Martin (1958), Watanabe *et al.* (1964), and Helander (1962) who also corrected for space between the fibers.

The first contraction was found to be dependent on Mg because a contraction does not occur in mixtures which do not contain this ion. Figure 3 shows that only a very slow contraction is produced by fibers exposed to a contraction mixture which contains only 5×10^{-3} M ATP in 0.1 M KCl. This slow contraction is blocked by 5×10^{-3} M Ca. However, a subsequent addition of 5×10^{-3} M Mg (final concentration) will cause a rapid contraction. Repeating this experiment with 5×10^{-4} M EDTA added to the contraction solution blocks the slow contraction. Repeating this experiment with 5×10^{-4} M EGTA added to the contraction mixture does not block the slow contraction. These results suggest that this slow contraction is caused by traces of Mg in the contraction solution since EDTA binds both Mg and Ca (pK for Mg is 5.48 at pH 7.1) whereas EGTA only binds Ca strongly (pK for Mg is only 1.09 at pH 7.1). This was confirmed by finding (measured with atomic adsorption spectrophotometer) a 10^{-6} M contaminant of Mg in the Mg-free contraction solutions.

Additions of Ca to the contraction mxture do not alter the initial speed of tension formation nor the maximum tension of the first contraction (see Figure 4).

Initial Velocity of Tension Formation as a Function of the Mg Concentration. An interesting relationship was found between the initial rate of tension formation and the Mg (MgATP) concentration. A curve, Figure 5, was obtained by plotting the initial rate of isometric tension formation (taken relative to the maximum tension produced by the highest Mg concentration) of four to five segments of a long fiber and contracting them with contraction solutions with different Mg concentrations. In these experiments, the ATP concentration was kept constant at 5×10^{-3} M, KCl at 0.1 M, and the pH at 7.0. The curve resembles a Langmuir adsorption isotherm. Transformation of these data in the reciprocal coordinates produces a linear relationship, except at the higher Mg concentration, see open circles in Figure 6. Substitution of the calculated $MgATP^{2-}$ concentration for the total Mg concentration (closed circles in Figure 6) produces a very good linear relationship.[2] These data suggest that the substrate for the first

[2] For calculations, see Appendix. The calculated values for $MgATP^{2-}$ concentrations were plotted against total Mg in Figure 7.

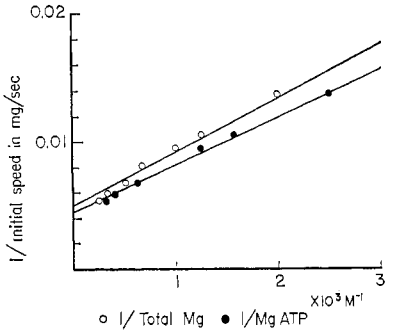

FIGURE 6: Data from Figure 5 plotted in reciprocate coordinates (open circles). Closed circles are the same data but plotted against the reciprocate of the MgATP concentration.

contraction is the $MgATP^{2-}$ complex. The concentration of $MgATP^{2-}$ which gives half-maximum speed of tension formation was found to be 8×10^{-4} M.

Experiments with Different Size Bundle of Fibers. Although a single fiber would give the best data, our transducer was not sensitive enough so that a bundle of fibers between 90 μ (3 fibers) and 325 μ (20–25 fibers) had to be studied. A contraction mixture containing 5×10^{-3} M ATP, 10^{-3} M $MgCl_2$, and 0.1 M KCl at pH 7 was used to obtain the data given in Table I. The initial rate of tension formation (IR) for the different sizes of fiber bundles was measured and related to its maximum tension (MT). Since all fiber bundles gave approximately the same value for IR/MT of 1.07 ± 0.16 (SD) sec^{-1}, it was concluded that the initial rate of tension formation measured for each bundle of fibers is equal to that of the sum of its individual fibers.

Spontaneous Relaxation. Fibers contracted in mixtures where the free Ca concentration was less than 10^{-7} M relaxed spontaneously in 3–10 min. Removal of the Ca contaminants from the chemicals used to make the contraction solutions with Chelex cationic exchange resin or chelation of this Ca^{2+} with EGTA also produced this effect. In Figure 1, this fiber bundle was contracted in a contraction solution (5×10^{-3} M ATP, 5×10^{-3} M $MgCl_2$, 0.1 M KCl, and 10^{-3} M EGTA at pH 6) which had a 6×10^{-7} M total Ca contaminant. The free Ca concentration of this mixture was reduced to less than 10^{-8} M by EGTA. In this contraction mixture, the rapid contraction is followed by a slow relaxation. Our impression is that, the lower the free Ca in the contraction solution, the more rapid is the slow relaxation. Fibers, which were contracted in mixtures containing Ca, sometimes maintained tension without relaxing for several days. This was especially true of fibers which were extracted for long periods in deoxycholate-free glycerine.

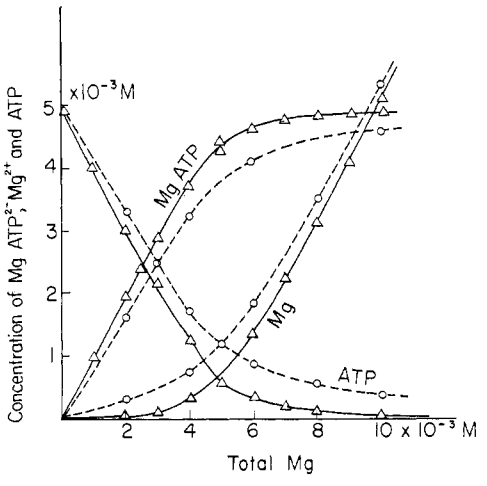

FIGURE 7: Concentrations of the $MgATP^{2-}$ complex and of free Mg^{2+} and ATP (ATP^{4-}, $HATP^{3-}$, and $KATP^{3-}$) as functions of the total Mg concentration for 5×10^{-3} M ATP and 0.1 M KCl, pH 7 (full curves) and pH 6 (dotted curves). Calculations, see Appendix.

TABLE I[a]

Width of Bundle (μ)	No. of Fibrils	IR (mg/sec)	MT (mg)	IR/MT (sec^{-1})
90	3	93	99	0.94
120	3	63	81	0.78
100	4	126	117	1.07
120	4	126	114	1.11
150	4	165	129	1.29
155	4–5	201	186	1.08
175	×	168	198	0.85
200	4	213	192	1.11
200	6–7	250	267	0.94
210	6–7	250	174	1.44
275	10[b]	249	222	1.12
285	10[b]	250	228	1.10
285	10[b]	250	256	1.03
325	×	300	288	1.04
325	×	330	294	1.15
			Av	1.07 ± 0.16[c]

[a] Since the fibers were looped around the bristle of the myograph and both ends fixed together, the values of maximum tension and initial rate were divided by two. The contraction mixtures all contained 5×10^{-3} M ATP, 10^{-3} M $MgCl_2$, and 0.1 M KCl. [b] Approximations; × = not counted. [c] Standard deviation.

Second Contraction. Relaxed fibers are very sensitive to Ca^{2+} and will contract a second time when small amounts of this ion are added to the original contraction mixture. This second contraction is much slower than the first contraction, less than one-tenth as fast, even though both contractions reach the same final tension, see Figure 1.

The range of Ca^{2+} necessary to cause a second contraction was estimated by adding small amounts of Ca to a contraction mixture bathing a relaxed fiber until a contraction occurred. It was found that the minimum level of Ca^{2+} necessary to induce a slow second contraction was in the range of 3×10^{-7} M at pH 6.0. In Figure 1, when only a very small amount of Ca is added giving a final free Ca concentration of 1.6×10^{-7} M, this concentration of Ca does not cause a second contraction. However, when a larger amount of Ca is added to a final free Ca concentration of 1.4×10^{-6} M, a second contraction is induced. Its initial rate of tension formation was 5.5 mg/sec as compared to an initial rate of tension formation of 175 mg/sec for the first contraction. Adding a larger amount of Ca will increase the initial rate of tension formation of this second contraction only slightly. Both Mg and ATP are also necessary for the second contraction.

Discussion

The observation that two contractions can be obtained with long-extracted, deoxycholate-treated, well-washed psoas muscle fibers may explain some of the controversies concerning the role of Ca ions in muscle contraction. The first contraction, dependent only on Mg and ATP, occurs in mixtures deficient in the Ca ion. This contraction is rapid and if the Ca ion concentration is sufficiently low, it is followed by a slow relaxation. These relaxed fibers are very sensitive to Ca and will contract again much slower to the same maximum tension when this ion is added to the bathing mixture.

Apparently some investigators have studied our first contraction and others our second contraction. For example, Embry and Briggs (1966), Briggs and King (1962), and Takahashi *et al.* (1965) obtained contractions without traces of Ca in their contraction mixtures. These authors used long-extracted well-washed fibers similar to our fibers. On the other hand, Seidel and Gergely (1963), Schädler (1967), and Portzehl *et al.* (1965) preincubated their fibers in ATP and Mg before inducing a contraction with small additions of Ca. Actually, we preincubated our fibers during the spontaneous relaxation period and we were also able to induce a contraction with small additions of Ca.

Magnesium appears indispensable for both the first and the second contraction. Watanabe *et al.* (1964) who have emphasized the importance of Mg, found that no significant contraction occurred in the absence of Mg, and we found that a second contraction will not occur with addition of Ca if Mg is removed from the bath.

Ca does not alter the initial speed of tension formation of the first contraction if Mg is added to the contraction mixture; however, additions of Ca inhibit the very slow contractions usually observed with contraction mixtures containing ATP without added Mg. A similar finding was reported by Erdös (1942) and Bowen (1951). CaATP probably competes with trace amounts of MgATP for a binding site on the fiber because this slow contraction can also be inhibited by EDTA, but not by EGTA which does not bind Mg appreciably (Chaberek and Martell, 1959). Moreover the absence of an effect of Ca on the initial speed in the presence of Mg disagrees with the effect of Ca on the myofibrillar ATPase (Schädler, 1967).

An interesting relationship was found between the initial rate of tension formation and the MgATP concentration in the contraction solutions. Plots of the initial rate of tension formation as a function of the MgATP concentration give curves similar to the Langmuir adsorption isotherm. In other words, the first contraction starts with maximum velocity and this initial speed is dependent on the MgATP concentration similar to any binding reaction (Nanninga and Mommaerts, 1960; Nanninga, 1962). This suggests that this initial rate is a function of the fraction of sites bound. Ranney (1954a,b) also found that the rate of isometric tension formation increased with the ATP (MgATP since Mg was in excess) concentration. Blum *et al.* (1957) found a similar correlation when measuring the shortening of the glycerinated fibers. These authors concluded that the splitting of the ATP was probably not involved in the shortening. Mommaerts (1948, 1950, 1956) and Mommaerts and Hanson (1956) who measured the initial velocity of the dissociation of actomyosin concluded that the binding and not the splitting of the ATP (MgATP again, Mg was in excess) caused this dissociation. Similar binding hypotheses have been presented by Morales and Botts (1952), Morales (1956), and Bowen (1952).

Calcium activates the ATPase of glycerinated fibers (Weber *et al.*, 1964; Elison *et al.*, 1965; Schädler, 1967). Calcium also can inhibit under certain conditions the shortening of actomyosin threads (Bowen, 1951) and glycerin-extracted fibers (Bowen and Martin, 1958). Bowen and Martin (1964) obtained considerable shortening with methylated glycerin-extracted fibers which did not split ATP, Kaldor and Gitlin (1963) inhibited 60–80% of the ATPase activity with quinacrine (atabrine) without any changes in the contractions. Portzehl (1954) nearly completely inhibited the speed of shortening with salyrgan when only 25% of the ATPase activity was inhibited. Burshtein *et al.* (1965) obtained enhancement of both the tension and the enzyme-substrate complex concentration with acridine orange and they concluded that "the molecular basis of the process of muscular contraction is formation of the enzyme–substrate complex." While it is recognized that Ca is involved in the *in vivo* excitation-coupling mechanism (Sandow, 1965) and is essential for the second contraction, we conclude that the first contraction is caused by the binding of MgATP to the fiber.

The dissociation constant for binding of the MgATP complex to the fiber in the first contraction is about 8.3×10^{-4} M. The binding constant of ATP to the ATPase enzymatic site on the myosin is about 3×10^{-7} M, measured by Nanninga and Mommaerts (1960a,b). These two differing values support the hypothesis of Levy and Ryan (1967a,b) that (Mg) ATP binds to two sites, an enzymatic site which hydrolyzes ATP requiring Ca and a nonhydrolytic site which can be inhibited with dinitrophenol. The latter site could be the site for tension formation in the first contraction where Mg could act as a bridge between myosin and the terminal P of ATP (to which Mg is bound in the complex) as in the metal peptidase theory proposed by Smith (1948).

Our binding hypothesis assumes that diffusion is not rate limiting in our experiments. We found that diffusion was not a factor as far as the number of fibers in a fiber bundle was concerned. Electron micrographs of these fibers showed that the 10 mM deoxycholate had almost entirely removed the sarcoplasmic reticulum and that the large spaces between the fibers allowed the bathing solution direct contact with most of the surface of each fiber. Hayashi and Tonomura (1968) similarly found that diffusion of ATP into glycerin-extracted fiber bundles was not rate limiting when they were 300 μ or smaller. However, we did not study the diffusion within the individual fibers. We can only guess based on calculations using Hill's equation (Hill, 1928) for diffusion into a cylinder, using the diffusion coefficient measured by Bowen and Martin (1963) for diffusion of ATP into glycerin-extracted muscle fibers, that diffusion is probably not the limiting factor in tension formation in the first contraction. The second contraction, being much slower, is less likely affected by diffusion.

The spontaneous relaxation which occurs in contraction mixtures when the free Ca concentration is below 10^{-7} M has been noted by Bozler (1951, 1952) and by Ranney (1954a,b). Since additions of Ca prevented relaxation, Bozler postulated that the hydrolysis of ATP is required for the maintenance of tension. Relaxation sites have been postulated by Eisenberg and Moos (1965) for well-washed, homogenized muscle and by Levy and Ryan (1967a,b) for actomyosin suspensions which bind additional ATP.

Ca-dependent contractions have been studied by Seidel and Gergely (1963), Portzehl *et al.* (1965), and Schädler (1967). Seidel and Gergely admitted that they were unable to explain the contractions obtained by Briggs and King (1962) with Ca-free mixtures. Abbott (1966) who obtained contractions in the absence of Ca, explained this apparent contradiction by

suggesting that the glycerinated fibers are in an "active state" independent of the presence of Ca and that relaxation with EGTA will occur only after the fibers are further contracted with Ca. The troponin–tropomyosin complex now considered to be a crucial protein complex in the regulation of the contraction–relaxation cycle by the Ca^{2+} (Ebashi and Endo, 1968), may have been inactivated during our extraction procedure since our fibers contracted in the absence of Ca. This contraction would be analogous to superprecipitation reactions of "synthetic" actomyosin which has been shown by Ebashi and Ebashi (1964) to be devoid of troponin and tropomyosin and similarly insensitive to Ca^{2+}. Following the first contraction our fibers not only lost tension (relaxed) but also developed a Ca^{2+} sensitivity. Ebashi and Ebashi (1964) also showed that a similar Ca^{2+} sensitivity is developed when troponin and tropomyosin are added to synthetic actomyosin, converting it into Ca^{2+}-sensitive "natural" actomyosin (myosin B). Additions of Ca^{2+} to our relaxed fibers probably inactivates a regenerated troponin–tropomyosin activity causing them to recontract. This second contraction would be analogous to the Ca-sensitive reactions of "natural" actomyosin (myosin B).

We obtained Ca-sensitive fibers by preincubating them in a Ca-free, MgATP mixture during our spontaneous relaxation period. Schädler (1967) preincubated his fibers in Mg and ATP. These fibers were extracted only a short period of time and probably contained an active sarcoplasmic reticulum. Portzehl *et al.* (1965) and Seidel and Gergely (1963) obtained Ca-sensitive fibers by preincubating in mixtures containing (Mg) ATP and EGTA.

The concentration range of free Ca required for our second contraction is similar to that needed for the activation of fibrillar ATPase (Weber and Hertz, 1963), the contraction of glycerinated Maia (crab) fibers (Schädler, 1967), glycerin-extracted rabbit psoas fibers (Filo *et al.*, 1965; Schädler, 1967), glycerin-extracted fibers from other species (Schädler, 1967), and for the contraction of intact Maia muscles (Caldwell, 1964; Portzehl *et al.*, 1964). The concentrations of free Ca is between pCa 7 and pCa 5.

An appropriate question is: Which contraction occurs *in vivo?* Infante and Davies (1962) were the first to show that ATP is split during the contraction phase in a single twitch, but they also found ATP splitting during relaxation. Mommaerts and Wallner (1967) found that ATP is split only during the contraction phase. These data plus the need for Ca ions in whole muscle contractions indicate that the Ca-sensitive contraction may be the one occurring *in vivo*. The fibrils in the cell are probably always surrounded by MgATP and the repressor activity of the troponin–tropomyosin system (Ebashi and Endo, 1968) is required for relaxation. Ca would inactivate this system for contraction (Ebashi and Endo, 1968; Yasui *et al.*, 1968). Although the speed of our first contraction more closely resembles the contraction in whole muscle, the slower speed of the Ca-dependent contraction could be caused by partial inactivation of the ATPase by the glycerin–deoxycholate treatment because Barany (1967) who studied the relationship between the speed of contraction of intact muscle and the myosin ATPase activity in different species, found that the ATPase activity was inversely proportional to the contraction time. He has suggested that the ATPase activity determines the speed of contraction *in vivo*. We postulate that our glycerin-extracted fibers remain contracted when Ca^{2+} is present in the initial contraction mixture because Ca^{2+} inactivates any troponin–tropomyosin activity that is regenerated by MgATP. In the absence of Ca^{2+}, on the other hand, the slowly regenerated troponin–tropomyosin repressor activity would dissociate the actin and myosin and produce a Ca-sensitive, relaxed fiber. This binding site may be the "relaxation site" postulated by Eisenberg and Moos (1965) and Levy and Ryan (1967a,b). This hypothesis is also compatible with a similar hypothesis suggested by Yasui *et al.* (1968).

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Appendix

Free Ca as Function of pH and Concentration of EGTA. Complex formation occurs between Ca^{2+}, Mg^{2+}, H^+, and $EGTA^{4-}$, forming $CaEGTA^{2-}$, $MgEGTA^{2-}$, $HEGTA^{3-}$, and H_2EGTA^{2-}.[3] The formation of $CaHEGTA^-$ is neglected because there is a 10^7 fold difference between the association constants of Ca^{2+} to $EDTA^{4-}$ and to $EDTAH^{3-}$.[4] It is assumed that a similar difference exists for EGTA. The calculation of the apparent association constant (K') of Ca^{2+} to $EGTA^{4-}$ takes into account the competing effects of Mg^{2+} and H^+ (see below).

$$(EGTA)^{4-}(Ca)^{2+} = K'(EGTACa)^{2-}$$

$$(Ca)^{2+} = K'(EGTACa)^{2-}/(EGTA)^{4-}$$

$$-\log Ca^{2+} = -\log K' + \log (EGTA)^{4-}/(EGTACa)^{2-}$$

$$pCa = pK' + \log\left[\frac{\Sigma EGTA - EGTACa^{2-}}{EGTACa^{2-}}\right]$$

$$pCa = pK' + \log\left[\frac{\Sigma EGTA}{\Sigma Ca} - 1\right] \text{ if } \Sigma EGTA \gg \Sigma Ca$$

K': apparent dissociation constant of $EGTACa^{2-}$

K: true dissociation constant of $EGTACa^{2-}$ (10^{-11})[4]

K_2: true association constant of $EGTAMg^{2-}$ ($10^{5.2}$)[4]

K_3 and K_4: true association constants of the first and second[3] bound H^+ of EGTA ($10^{9.43}$ and $10^{8.85}$)[4]

$$K' = \frac{(Ca)^{2+}(EGTA^{4-} + EGTAMg^{2-} + EGTAH^{3-} + EGTAH_2^{2-})}{(EGTACa)^{2-}}$$

$$= \frac{(Ca)^{2+}(EGTA)^{4-}[1 + K_2(Mg^{2+}) + K_3(H^+) + K_3K_4(H^+)^2]}{(EGTACa)^{2-}}$$

$$= K[1 + K_2(Mg^{2+}) + K_3(H^+) + K_3K_4(H^+)^2]$$

	pH 7	pH 6
With Mg^{2+}[5]	$K' = 2 \times 10^{-7}$ $pK' = 6.70$	1.9×10^{-5} 4.72
No Mg^{2+}:	$K' = 1.93 \times 10^{-7}$ $pK' = 6.715$	1.9×10^{-5} 4.72

Since in a mixture of 5×10^{-3} M Mg, 5×10^{-3} M ATP, and

[3] The binding of the other two H^+ to $EGTA^{4-}$ can be neglected since their association constant is more than 10^6 times lower.

[4] Chaberek and Martell (1959).

[5] 5×10^{-3} M.

10^{-3} M EGTA nearly all Mg is bound to ATP, "no Mg" values were used for the calculation of pCa in this mixture.

In general:

$$pK' = 2\,pH - 7.28$$

$$pCa = 2\,pH - 7.28 + \log\left[\frac{\Sigma EGTA}{\Sigma Ca} - 1\right] \text{ for } \Sigma EGTA \gg \Sigma Ca^{6}$$

Apparent Association Constant of MgATP. True association constants of $MgATP^{2-}$: 24,000 (Nanninga, 1961); of $KATP^{3-}$: 10 (Melchior, 1954; Smith and Alberty, 1956) and of $HATP^{3-}$: $10^{6.9}$ (Melchior, 1954; Smith and Alberty, 1956). The apparent association constant is the true constant divided by $1 + K_K \cdot (K) + K_H(H) = 2 \times 10^{-0.1}$ at pH 7 and $2 + 10^{0.9}$ at pH 6. This gives 8600 and 2400, respectively. The formation of $MgHATP^-$ from Mg^{2+} and $ATPH^{3-}$ was neglected since its association constant is 100 × less than that for $MgATP^{2-}$ (Martell and Schwarzenbach, 1956). Also the formation of $MgHATP^-$ from H^+ and $ATPMg^{2-}$ with $pK = 4.5$ (Martell and Schwarzenbach, 1956) can be neglected since at pH 7 $(MgHATP^-) = 3.1 \times 10^{-3}$ M $(MgATP^{2-})$. The competitive binding of H^+ and K^+ to ATP^{4-} has, however, been taken into account. $MgATP^{2-}$ is the only stable complex between Mg and ATP at pH 7 (Martell and Schwarzenbach, 1956; Burton, 1959; Liebecq, 1959; Nanninga, 1961).

Free Mg in Mixtures of $MgCl_2$ and ATP.

$$(Mg)^{2+}(ATP)^{4-} = K'(MgATP)^{2-}$$

K': apparent dissociation constant

$$\Sigma ATP = MgATP^{2-} + ATP^{4-} = MgATP^{2-}(1 + K'/Mg^{2+})$$

$$Mg^{2+} = \Sigma Mg - MgATP^{2-} = Mg^{2+} - \frac{\Sigma ATP}{1 + K'/Mg^{2+}} \text{ which gives}$$

$$Mg^{2+} = {}^1/_2\{(\Sigma Mg - \Sigma ATP - K') + \sqrt{4K'\Sigma Mg + (\Sigma Mg - \Sigma ATP - K')^2}\}$$

Binding $MgATP^{2-}$ to Sites on the Fibrils.

r: occupied sites ($MgATP^{2-}$ bound)
n: total available sites
c: concentration of the $MgATP^{2-}$ complex
k: dissociation constant

$$\frac{(n - r)c}{r} = k$$

$$r = \frac{nc}{k + c}\text{: Langmuir's adsorption curve}$$

$$\frac{1}{r} = \frac{1}{n} + \frac{k}{nc}$$

k from slope (k/n) and intercept $(1/n)$ in Figure 6 (lower line) gives 8.3×10^{-4}. This equals the concentration which gives half-maximal adsorption.

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[6] When ΣEGTA is not ≫ΣCa, Ca^{2+} follows from ΣEGTA, ΣCa, and the apparent dissociation constant of CaEGTA, similar to the calculation for Mg^{2+} mentioned sub 2. For ΣEGTA = ΣCa = 10^{-3} M and for pH 6, 5×10^{-3} M Mg, $K = 1.9 \times 10^{-5}$, and $Ca^{2+} = 2.6 \times 10^{-4}$ M or pCa = 3.6.

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Mechanism of Alkylation of Rabbit Muscle Glyceraldehyde 3-Phosphate Dehydrogenase*

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ABSTRACT: The kinetics and stoichiometry of alkylation of the active-site thiols of glyceraldehyde 3-phosphate dehydrogenase (GPD) by iodoacetate (IAc) and iodoacetamide (IAM) were studied under a wide variety of conditions. The adherence to second-order kinetics (under most conditions), the maximum stoichiometry of 4 moles of inhibitor (IAc and IAM) per mole of enzyme tetramer, and the linear dependence of enzymic activity on the extent of alkylation, all strongly support a model for the enzyme in which all four thiols react equivalently and independently with the two inhibitors. Further supportive evidence for such a model is that enzyme species that are only partially alkylated undergo further alkylation at a rate similar to native, unmodified enzyme. These results are discussed with regard to the known functional heterogeneity of the active sites in the reaction with substrates and coenzyme (NAD^+). The kinetics deviate from second-order under some conditions: a hyperbolic dependence of the alkylation rate of NAD^+-bound GPD on the concentration of IAc indicates that IAc forms a reversible complex with GPD prior to the irreversible inactivation. On the other hand, the alkylation rates are linearly dependent on IAM concentration over the accessible concentration range. The alkylation rates are strongly dependent on the concentration of NAD^+. When the concentration of NAD^+ and GPD are comparable, the kinetics in the presence of a large excess of either IAc or IAM deviate from pseudo-first order. The rate of alkylation by IAc increases as the reaction proceeds whereas the rate of alkylation by IAM decreases as the reaction proceeds. These results are qualitatively consistent with the hypothesis that there is a redistribution of NAD^+ among alkylated and unalkylated subunits during the course of the inactivation process and that bound NAD^+ can be a positive or negative effector of reactivity dependent on the charge of the reactant. The pH–rate profile for the carboxymethylation of GPD suggests that the rate depends on the ionization of groups of pK_a = 5.5 and 5.2 in NAD^+-free and NAD^+-bound enzyme, respectively. The rate of inhibition by IAM depends on an acidic group of pK_a = 8.0–8.1 (although the rates are unexpectedly large at low pH). This dichotomy of pK_a's has also been found in studies of the pH dependence of enzyme acylation with neutral and anionic substrates. Evidence is presented that the active-site thiols do have an apparent pK_a = *ca.* 8.0, but that reaction with anionic inhibitor (IAc) and anionic substrate (FAP) is strongly influenced by enzymic equilibria other than the simple ionization of the essential thiols.

Recently, the properties of rabbit muscle GPD[1] have been studied extensively. The complex kinetics and the binding properties of NAD^+ have indicated that the active sites are functionally nonequivalent and perhaps not independent. NAD^+ binding studies on the rabbit muscle enzyme have shown the existence of two or more different NAD^+

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[1] Abbreviations used are: GPD, D-glyceraldehyde 3-phosphate dehydrogenase; IAc, iodoacetate; IAM, iodoacetamide; bicine, *N,N*-bis[2-hydroxyethyl]glycine; FAP, β-(2-furyl)acryloyl phosphate.