A Study of the Effects of Substrate Concentration and Certain Relaxing Factors on the Magnesium-Activated Myofibrillar Adenosine Triphosphatase

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Marsh (1952) first showed that whereas the apparent rate of hydrolysis of adenosine triphosphate (ATP) by homogenates of fresh rabbit muscle in an isotonic solution of potassium chloride was comparatively low, after centrifuging and removal of the soluble protein extract a considerable increase in the rate of production of inorganic phosphate by the residue occurred when it was resuspended in fresh potassium chloride solution. On the basis of these findings Marsh concluded that there was present in muscle a factor which inhibited adenosine triphosphatase (ATPase) activity, and he further demonstrated that in the presence of this factor ATP caused an increase in the volume of the cell fragments in the suspension. Bendall (1953*a*) extended Marsh's findings and was able to bring about the relaxation of glycerated fibres by the application of ATP in the presence of preparations of the Marsh factor. He also confirmed the inhibitory action of the factor on the ATPase activity of homogenized glycerated fibres. Further investigations by Hasselbach & Weber (1953) on the ATPase activity of these preparations showed that at higher concentrations ATP was inhibitory, and in the presence of the Marsh factor the level at which ATP became inhibitory was considerably reduced.

It is clear from the investigations mentioned above and those of Bozler & Prince (1953) that, in general, relaxation in muscle models is associated with a low rate of ATP hydrolysis. Magnesium appears to be of special importance in the contraction-relaxation cycle, yet the precise role of this metal is far from clear. Although magnesium is essential for relaxation (Bendall, 1953*a*), reagents which form complexes with magnesium, namely ethylenediaminetetraacetate (EDTA) (Bozler, 1954; Watanabe, 1955) and inorganic pyrophosphate (Weber, 1951; Bozler, 1951; Bendall, 1953*b*) also bring about relaxation of glycerated fibres. Bendall (1953*b*) has commented on the similarity between the relaxing action of the Marsh factor and that of inorganic pyrophosphate, and it is conceivable that one of the properties possessed in common by these substances is the ability to complex with magnesium.

Glycerated fibres consist mainly of myofibrils, but studies on the ATPase activity of these fibres are not entirely satisfactory, as preparations may be contaminated with sarcoplasmic granules and nuclei which also possess ATPase activity, and with varying amounts of sarcoplasm. Furthermore, precise analysis of such experiments in terms of substrate and activator concentrations at any point in the fibre is difficult because concentration gradients will be produced by the diffusion of ATP to the myofibrils from the outside of the fibre. To throw more light on the enzymic behaviour of the myofibril a study has been made of the ATPase activity of isolated myofibrils, with particular reference to those conditions that bring about the low level of activity which is characteristic of relaxation. It has been shown that at a low ionic strength the marked inhibition of ATPase activity by ATP is dependent on the magnesium concentration and that this inhibition is not obtained when calcium is the activating metal, nor in the presence of magnesium when inosine triphosphate (ITP) is the substrate. No inhibition could be demonstrated with myokinase, but all the other relaxing factors tested reduced the ATPase activity of isolated myofibrils to a low level. With the exception of findings with EDTA the results are in agreement with the view (Perry, 1955, 1956) that the low rate of ATPase activity associated with relaxation may be due to the binding or removal of magnesium from the system, so that it is no longer available for the myofibrillar ATPase system.

A preliminary account of some aspects of this work has already been presented (Perry, 1955).

METHODS

Preparation of myofibrils

From fresh muscle. The method employed for large-scale preparations was similar to that reported earlier (Perry, 1952), except that the medium used (borate-KCl) contained 0.025 M-KCl and 0.039 M borate, pH 7.1. In this buffer less myofibrillar protein is leached out on standing, and the myofibrils are not so hydrated as those prepared in borate alone. For this latter reason the centrifuging times used differ from those originally given. All manipulations were

carried out at 0°, and preparations were controlled by frequent microscopical examination of the suspensions.

Chilled, minced, freshly excised rabbit muscle (200 g.) was homogenized for 1 min. with 5 vol. of borate-KCl and centrifuged for 15 min. at 600 g. The supernatant was discarded, the residue resuspended with a further 5 vol. of borate-KCl and homogenized for 2 min. After centrifuging for 15 min. at 600 g the supernatant was again discarded and the light-coloured upper layer of the sediment, consisting mainly of myofibrils, was removed with the aid of a little borate-KCl. The myofibril paste was diluted with borate-KCl to 1 l./200 g. of muscle taken, and coarse material removed by centrifuging for 3 min. at 400 g. The sediment was discarded and the suspension centrifuged again for 15 min. at 600 g to sediment the myofibrils. After three further resuspensions and centrifugings to remove sarcoplasmic proteins and granules the sediment was resuspended and centrifuged once more for 3 min. at 400 g. The sediment was again discarded and the turbid supernatant centrifuged to obtain a concentrated myofibril suspension. At this stage usually less than 1% of the protein was soluble. The myofibrils were transferred to a flask with a minimum amount of borate-KCl and stored at 0° in the presence of traces of toluene.

It was necessary, for some experiments, to prepare myofibrils rapidly from smaller amounts of muscle, e.g. 5 g. of rabbit psoas. Such preparations were carried out in borate-KCl and also in 0.1 M-KCl, although, in the latter medium, the endogenous ATP of the muscle appears to be more effective in causing contraction during homogenization. To minimize this effect the muscle was cut up with scissors and broken down into large cell fragments by homogenization with 20 vol. of medium for a few seconds in a small homogenizer of the Waring Blendor type fitted with an anti-foaming device. The suspension was centrifuged and the supernatant discarded. The residue was re-homogenized in 20 vol. of the medium until the cell fragments were almost completely broken down into myofibrils. After centrifuging the myofibrils were resuspended in 5–10 vol. of medium and the procedure was then similar to that followed in the large-scale preparation after the second homogenization. In solutions of ionic strength higher than that of the borate-KCl, myofibrils sedimented readily, and consequently lower speeds and shorter centrifuging times were used to remove unbroken cells and large myofibrillar bundles. It was advisable to follow this stage carefully by microscopical examination.

The stock suspensions contained 20–30 mg. of protein/ml. and were diluted with 4–5 vol. of borate-KCl for enzyme experiments. These preparations contained slight myokinase activity, which could be partly reduced by repeated washing and centrifuging. The myofibrils also possessed 5-adenylic-deaminase activity (Q_{NH_3} = 200–400 at pH 6.1 in succinate buffer at 20°) and very slight deaminating activity towards ADP. (Q_{NH_3} = μ l. of NH_3 /hr./mg. of protein.)

From glycerated fibres. Strips of rabbit psoas muscle which had been stored at -10° in 50% (v/v) aqueous glycerol were cut up into short lengths and homogenized with 20 vol. of the appropriate medium. The procedure was then as described above for the preparation of myofibrils from small amounts of rabbit muscle.

Myokinase

Myokinase was prepared by the method of Kalckar (1947) and stored at 0° as an $(NH_4)_2SO_4$ paste.

ATPase determinations

Incubations were usually carried out at 20.5° in a total vol. of 2 ml. containing 0.05 M aminotrihydroxymethylmethane (tris)-HCl buffer, pH 7.4, $CaCl_2$ or $MgCl_2$ as activator, ATP (Na salt), and other components as indicated. The reaction was started by the addition of 0.2 ml. of the myofibril suspension, and stopped by adding rapidly 1 ml. of 15% (w/v) trichloroacetic acid. The myofibril precipitate was removed by filtration through a loose pad of absorbent cotton wool, and phosphate estimated by the method of Fiske & Subbarow (1925) in a final vol. of 25 ml.

Low concentrations of inorganic pyrophosphate inhibit the development of colour under the conditions of the Fiske & Subbarow (1925) method, and in all experiments reported here care was taken to ensure that the final inorganic pyrophosphate concentration in the solution used for colorimetric measurements did not exceed 1 mM. At this concentration no inhibition of colour development was obtained, although 2 mM- $Na_4P_2O_7$ was found to give marked inhibition. Magnesium chloride did not have any significant effect on the final development in the presence of $Na_4P_2O_7$ or ATP in the concentrations used in the enzyme experiments.

Nucleotides

ATP, adenosine diphosphate (ADP), ITP and uridine triphosphate (UTP) were the Na salts (Sigma Chemical Co.). Neutralized solutions (0.05 M) were stored at -10°. Concentrations of the triphosphates were determined by assuming that 7 min. hydrolysis in N-HCl at 100° liberated two-thirds of the total organic P as inorganic P. For ADP the acid-labile P was taken as equal to half the total organic P. Chromatography with the isobutyric acid-ammonia solvent of Krebs & Hems (1953) indicated that the ATP was pure, whereas the ITP contained a trace of inosine diphosphate, and the UTP a small amount of another nucleotide which was probably uridine diphosphate.

Inosine monophosphate (IMP) was prepared from 5-adenylic acid (L. Light and Co. Ltd.) by the method of Embden & Schmidt (1929).

RESULTS

Effect of ATP concentration on the ATPase activity of isolated myofibrils

When $MgCl_2$ was the activator and in the absence of salts other than those present in the standard incubation medium (ionic strength, I , approx. 0.06) higher ATP concentrations caused a marked inhibition of the myofibrillar ATPase. The substrate level at which the ATPase activity began to decline was dependent on the $MgCl_2$ concentration (Fig. 1). In general, the results suggested that at low ionic strength inhibition began when the concentration of ATP exceeded that of $MgCl_2$. This was clearly so over the range 5–10 mM- $MgCl_2$, but with 2–3 mM- $MgCl_2$ the lowest relative concentrations of ATP required to produce inhibition were somewhat higher.

It is possible that this divergence at the lower range of $MgCl_2$ concentrations was due to small amounts of magnesium which may be present in the

myofibril preparations. The fact that well-washed isolated myofibrils possess appreciable ATPase activity in the absence of added MgCl_2 suggests that activating cations are still present in these preparations.

Fig. 1. Effect of MgCl_2 concentration on the inhibition of the myofibrillar ATPase activity by excess of substrate. Incubations were carried out in 0.05M tris buffer (pH 7.4) for 5 min. at 20.5° , and a different myofibril preparation was used for each level of MgCl_2 : Δ , 2.5 mM-MgCl_2 ; $\times$, 5 mM-MgCl_2 ; $\circ$, 7.5 mM-MgCl_2 ; $\square$, 10 mM-MgCl_2 .

Fig. 2. Comparison of activation of myofibrillar ATPase by MgCl_2 and CaCl_2 with increasing ATP concentration. Experiment carried out as those illustrated in Fig. 1. $\bullet$, No activator; $\circ$, 5 mM-MgCl_2 ; $\square$, 5 mM-CaCl_2 .

With 5 mM-MgCl_2 similar substrate-inhibition effects were obtained in 0.05M glyoxaline buffer pH 6.9 and 0.05M tris buffer pH 7.7.

The inhibitory action of higher concentrations of ATP was shown to be reversible in that addition of MgCl_2 to systems containing excess of substrate restored the enzymic activity to a high level. At pH 7.4, with 5 mM ATP and 2 mM-MgCl_2 , or with 7.5 mM ATP and 3.75 mM-MgCl_2 , the activity was low. On addition of MgCl_2 to bring the concentration equal to that of the substrate in each case the level of ATPase activity rose to approach that obtained in control experiments in which the ATP and MgCl_2 concentrations were equal throughout.

Inhibition by higher concentrations of substrate appeared to be characteristic of magnesium activation of the myofibrillar ATPase, for when MgCl_2 was replaced by 5 mM-CaCl_2 a very slight decrease in activity was obtained on increasing the ATP concentration over the range $2\text{--}10\text{ mM}$ (Fig. 2). Furthermore, under conditions in which the magnesium-activated ATPase was inhibited by excess of substrate, addition of Ca abolished the inhibition. In fact, with certain concentrations of CaCl_2 the ATPase activity may rise with increasing ATP concentration, as is shown in Fig. 3, which illustrates the results of experiments carried out in the presence of 5 mM-MgCl_2 and CaCl_2 ranging from 0.5 to 5 mM . It seems clear that this effect is not

Fig. 3. Effect of CaCl_2 on the substrate inhibition of Mg -activated myofibrillar ATPase. Conditions of enzyme experiment similar to those of Fig. 1, with $5 \times 10^{-3}\text{M}$ MgCl_2 present in all cases and the following further additions: $\circ$, none; $\blacksquare$, 0.5 mM-CaCl_2 ; $\bullet$, 2 mM-CaCl_2 ; $\square$, 5 mM-CaCl_2 .

simply due to the activating effect of CaCl_2 alone, for whereas 0.2 mM- CaCl_2 , in the presence of MgCl_2 , restores the activity to the level obtained when ATP is not in excess, this concentration of CaCl_2 produces little activation of the myofibrillar ATPase when it is the only added activator (Fig. 4).

Effect of ionic strength. On increasing the ionic strength of the incubation medium to approx. 0.16 by the addition of KCl, the activity of the magnesium-activated ATPase of myofibrils isolated in borate-KCl was markedly reduced. Furthermore, for a given MgCl_2 concentration the inhibitory effect of ATP became apparent at lower concentrations as the ionic strength increased (Fig. 5a).

It was considered that exposure of the isolated myofibrils to solutions of lower ionic strength during preparation might modify their response to increasing ATP concentration. Therefore, to investigate the significance of the substrate inhibition effect under the ionic conditions prevailing *in vivo*, and in experiments with glycerated fibre models (I, 0.1–0.16), myofibrils were isolated in 0.1 M-KCl from fresh and glycerated psoas muscle. Fig. 5b shows the results obtained with myofibrils from glycerated muscle. The ATPase was much less sensitive to increasing KCl concentration—a general finding with myofibrils from both fresh and glycerated muscle which were isolated in 0.1 M-KCl. With

Fig. 4. Comparison of the activating effect of CaCl_2 on the myofibrillar ATPase in the absence and presence of MgCl_2 . All tubes contained 7.5 mM ATP and incubations were carried out as for Fig. 1. ○, 2.5 mM- MgCl_2 ; □, MgCl_2 absent.

Fig. 5. Effect of ionic strength on the substrate inhibition of the myofibrillar ATPase. (a) Myofibrils prepared from fresh muscle in borate-KCl; (b) myofibrils prepared from glycerated muscle in 0.1 M-KCl. Conditions of enzyme experiments similar to those of Fig. 1, with 5 mM- MgCl_2 present in all cases and the following further additions: ○, no added KCl; □, 0.05 M-KCl; ●, 0.1 M-KCl.

these myofibrils the effect of increasing ATP concentration in an incubation medium of I about 0.16 is very similar to that obtained with borate-KCl prepared myofibrils in a medium of I about 0.06. At the latter ionic strength with myofibrils prepared in 0.1M-KCl the concentration of ATP required to produce inhibition was higher than that needed at I of 0.16, and, over the range investigated, the effect obtained was much less.

It is clear that the inhibitory effect of higher substrate concentrations is shown by myofibrils isolated from fresh and glycerated muscle, but differences exist in the sensitivity of the systems to increasing ionic strength. These differences are related to the previous history of the myofibrils, in particular to the ionic conditions to which they have been exposed.

Effect of pyrophosphate

Bendall (1954) reported that inorganic pyrophosphate inhibited the magnesium-activated ATPase activity of suspensions of homogenized glycerated fibres; he suggested that the lowered rate of ATPase activity associated with relaxation could be explained by production of pyrophosphate. The inhibitory effect of pyrophosphate was investigated when myofibrils isolated from fresh muscle were used, with particular emphasis on the relative concentrations of $MgCl_2$, $Na_4P_2O_7$, and ATP. In general the $MgCl_2$ concentration was equal to, or greater

than, the ATP concentration. In the former case addition of increasing amounts of pyrophosphate produced progressive inhibition, whereas in the latter case the concentration of $Na_4P_2O_7$, at which inhibition began was dependent on the concentration of $MgCl_2$ (Fig. 6). Although inhibition was not so marked as with excess of ATP, the same general relation appeared to hold in that the onset of inhibition occurred when the total pyrophosphate group concentration, i.e. ATP + inorganic pyrophosphate, exceeded the $MgCl_2$ concentration.

Effect of inosine triphosphate and other nucleotide phosphates

It was noted that certain marked differences existed between the ITPase and ATPase activity of myofibrils. With activator and substrate concentrations both equal to 5 mM, myofibrils split ATP at a high rate in the presence of $CaCl_2$ or $MgCl_2$, the activity with Mg^{2+} being somewhat lower than with Ca^{2+} . The rate of hydrolysis of ITP, however, with $MgCl_2$ was four times that with $CaCl_2$. In general, the rate of hydrolysis of ITP with magnesium was approximately one-third of that of ATP under similar conditions.

In contrast to the effect obtained with ATP (cf. Fig. 1), increasing the ITP concentration from 2 to 10 mM produced only a slight progressive fall in activity of the myofibrillar ITPase. The results illustrated in Fig. 7 were obtained when 5 mM-

Fig. 6. Effect of $MgCl_2$ concentration on the inhibition of the myofibrillar ATPase by inorganic pyrophosphate. Additions common to all enzyme tubes were 0.05M tris buffer, pH 7.4, and 5 mM ATP. Different myofibril preparations used for each $MgCl_2$ concentration. □, 10 mM- $MgCl_2$; ×, 7.5 mM- $MgCl_2$; ○, 5 mM- $MgCl_2$.

Fig. 7. Effect of substrate concentration on the splitting of ITP, UTP and ATP by myofibrils. 0.05M tris buffer, pH 7.4, and 5 mM- $MgCl_2$ common to all incubation tubes. ○, ITP; ●, UTP; □, ATP; ■, 5 mM ATP + ADP.

MgCl₂ was added, but similar results were obtained with 2 and 7.5 mM-MgCl₂. On the other hand, when uridine triphosphatase (UTPase) activity was studied under similar conditions, increasing the substrate concentration caused a progressive increase in inhibition. The significance of this effect is not clear, as the UTP preparations contained appreciable quantities of inorganic P (equivalent to 7 % of the acid-labile P) and further investigation was prevented by the shortage of UTP.

The inhibitory action of ADP was also investigated in a similar way to that employed in the experiments carried out with pyrophosphate, namely by adding ADP to a system containing MgCl₂ and ATP in approximately equimolar quantities. Some inhibition was obtained on increasing the ADP concentration, but this is much less than that obtained with excess of ATP (Fig. 7). In similar circumstances AMP and IMP were not inhibitory.

Effect of myokinase

Bendall (1954) has reported that, during the isolation of relaxing-factor preparations from rabbit-muscle extracts, myokinase and factor activity are closely correlated at all stages of the purification. These preparations also inhibited the ATPase activity of homogenates of glycerated fibres, and Bendall's results suggest that under certain conditions myokinase itself can inhibit the myofibrillar ATPase.

At pH 7.4 in tris buffer, added myokinase had no significant effect on the initial rate of ATPase activity of washed myofibrils. Varying amounts of enzyme were used in a system containing 5 mM-MgCl₂ and 5 mM ATP, and the ATP concentration was varied between 2 and 10 mM in the presence of 2, 5 and 10 mM-MgCl₂. On addition of 0.05 and 0.10 M-KCl to a system containing 5 mM-MgCl₂ and 5 mM ATP, myokinase likewise failed to produce inhibition of the ATPase.

This lack of effect cannot be attributed to the myokinase in the myofibril suspensions, since they probably contain very much less than glycerated fibres with which the inhibitory effect of myokinase was demonstrated by Bendall (1954).

Effect of ethylenediaminetetraacetate

Recently several studies (Friess, 1954; Friess, Morales & Bowen, 1954; Bowen & Kerwin, 1954) on the effect of EDTA on myosin ATPase have been reported. For the most part the conditions of study do not readily allow us to relate these results to the investigations (Bozler, 1954; Watanabe, 1955) which have been carried out on the relaxing action of this chelating agent on the glycerated-fibre model. Some experiments on the effect of EDTA on the myofibrillar ATPase are reported here, as they bear

particularly on this problem and the role of magnesium in the relaxation process. They form part of a general study of EDTA on the interaction of ATP, actin and myosin which is now in progress and which has been reported (Perry & Grey, 1956).

Fig. 8 shows the effect of the addition of increasing amounts of EDTA to the myofibrillar ATPase system activated by MgCl₂ or CaCl₂. In the presence of MgCl₂, and when the concentration of the chelating agent was 0.2–0.5 mM, pronounced inhibition, largely independent of the MgCl₂ concentration, was apparent. For example 0.5 mM EDTA produced 75–80 % inhibition, whether the MgCl₂ concentration was 1 or 10 mM. On the other hand, much higher concentrations of EDTA were required to produce similar degrees of inhibition of the calcium-activated ATPase under otherwise similar conditions. In the latter case the quantitative aspects of the inhibition were such to suggest that it was caused by the removal from the system of the major part of the added calcium as the EDTA complex. It does not seem possible to explain the results with MgCl₂ on a similar basis, however, for EDTA has a stronger affinity for Ca²⁺ than for Mg²⁺ ions (Calvin, 1954), and is strongly inhibitory when the amount of EDTA present is such that only a very small fraction of the Mg²⁺ ions would be expected to be bound. These results with magnesium may explain Watanabe's (1955) finding that EDTA induced relaxation of glycerated fibres when present

Fig. 8. Effect of EDTA on the Ca- and Mg-activated ATPase of myofibrils. Incubations carried out for 5 min. at 20.5° in the presence of 0.05 M tris buffer, pH 7.5, 5 mM-ATP. ○, 5 mM-CaCl₂; ●, 5 mM-MgCl₂.

in one-tenth of the concentration of the MgCl_2 , for although only a small fraction of the magnesium in the system would be present as a complex under these conditions, the ATPase activity of the myofibrils will be low.

DISCUSSION

With the exception of myokinase all the substances that are known to produce relaxation in the glycerated-fibre models studied in this investigation have been shown to bring about a marked inhibition of the magnesium-activated ATPase of isolated myofibrils. Although the results obtained with myokinase do not confirm those obtained by Bendall (1954) with glycerated-fibre preparations, they are in agreement with the recent findings of Kumagai, Ebashi & Takeda (1955), who have provided evidence that myokinase alone was not adequate to produce relaxation in well-washed fibres.

The inhibition obtained with either ATP or $\text{Na}_2\text{P}_2\text{O}_7$ is clearly dependent on the magnesium concentration, and the effect at low ionic strength produced by excess of substrate on myofibrils isolated in borate-KCl resembles in a striking way the effect on pyrophosphatase (cf. Bauer, 1937; Bailey & Webb, 1944; Heppel & Hilmo, 1951; Gilmour & Calaby, 1953; McElroy, Coulombre & Hays, 1951; Bloch-Frankenthal, 1954; Robbins, Stulberg & Boyer, 1955). The last two groups of workers have both concluded, from analysis of their data, that the substrate for the pyrophosphatase enzyme is $(\text{MgP}_2\text{O}_7)^{2-}$ and that free inorganic pyrophosphate inhibits the enzyme. Superficially the similarities between the pyrophosphatase and myofibrillar ATPase systems are sufficiently close to suggest that a similar mechanism may exist when magnesium is the activating cation for the ATPase, i.e. that Mg-ATP is the true substrate, whereas free ATP is not split but is bound at the enzymically active sites of the actomyosin system and causes inhibition.

It should be emphasized, however, that such a function of magnesium would appear to be confined to the myofibrillar actomyosin system, since the ATPase of myosin is not activated by magnesium. Both calcium and magnesium activate the myofibrillar ATPase, but the myosin ATPase requires calcium and it is clear that considerable differences exist in the characteristics of ATP hydrolysis by the myofibril in the presence of these two metals. Earlier work (Chappell & Perry, 1955) has shown that the stimulation of myofibrillar ATPase activity by magnesium disappears at 0° , and at higher temperatures as the ionic strength increases (Perry, 1951). The present study provides further examples of differences, in that, with calcium as activator, substrate inhibition is not obtained over the range

at which this phenomenon is obtained with magnesium, and that the magnesium-activated myofibrillar ATPase is very much more sensitive to EDTA. It is possible that the difference in the effect of excess of substrate on activity of the myofibrils in the presence of calcium and magnesium reflects differences in the complex-forming properties of these metals with ATP rather than in fundamental mechanism. From this point of view it may be significant that, whereas with 5 mM ATP optimum activation is obtained with 5 mM- MgCl_2 , with CaCl_2 the optimum concentration is much higher than the ATP concentration.

The results obtained when inorganic pyrophosphate is introduced into the ATPase system can be explained by assuming either that free inorganic pyrophosphate inhibits the hydrolysis of the substrate, Mg-ATP, or that the inorganic pyrophosphate preferentially binds Mg^{2+} ions, leaving ATP in excess to produce inhibition. $(\text{MgP}_2\text{O}_7)^{2-}$ ions do not inhibit the enzyme, since if there is enough magnesium to combine with both ATP and inorganic pyrophosphate the high level of activity is maintained. It is well known that ATP (Hers, 1952) and inorganic pyrophosphate (Rogers & Reynolds, 1949) form complexes with Mg^{2+} ions, but as adequate information is not available to assess the relative affinities of these two substances for the metal under the conditions of the enzyme experiments, it is not possible to decide which of these two alternatives is more likely.

ITP and UTP differ in behaviour from ATP in that a progressive fall in activity was obtained with increasing UTP concentration, whereas little inhibition was obtained when ITP was used as substrate. If the mechanism for the hydrolysis of ITP in the presence of magnesium is similar to that suggested above for ATP, these results would imply that free ITP has little affinity for the active centres of the myofibrillar ATPase. From this point of view the absence of the amino group in the 6-position of the purine ring may be a relevant factor.

Most of the studies described have been carried out with myofibrils isolated in borate-KCl, the magnesium-activated ATPase of which appears to be particularly sensitive to an increase in the ionic strength. Nevertheless, the results can be related to events occurring *in situ* where the ionic strength is about 0.16, for myofibrils freshly isolated in 0.1 M-KCl from both fresh and glycerated muscle, which have been subsequently studied in an incubation medium of *I* approx. 0.16, behave with respect to increasing ATP concentration very much like borate-KCl myofibrils at *I* approx. 0.06. It appears that, after isolation of myofibrils and subsequent storage in the borate-KCl medium, the magnesium-activated ATPase becomes more sensitive to the inhibitory effects of higher ionic strength.

With both preparations, however, the level of ATP which is inhibitory at a given MgCl_2 concentration falls as the ionic strength is increased. With fructokinase, Hers (1952) found that the optimum ATP:Mg ratio fell from 2 to 1 with increasing ionic strength.

Hasselbach (1952) has reported that the addition of 0.1 mM- MgCl_2 lowers the concentration at which ATP begins to inhibit the ATPase activity of actomyosin gels. These results appear to be contrary to those reported here, but they are perhaps not strictly comparable, as in all cases the ATP concentration (1–10 mM) was much greater than that of the MgCl_2 .

It seems likely that the low rate of hydrolysis of ATP characteristic of the myofibril in relaxation could be due to the presence of ATP in excess of the available magnesium in the muscle. Strong suggestion that substrate inhibition of the type described above occurs in relaxation (at least in the fibre models) is given by the fact that in the presence of calcium, which relieves the inhibition of the myofibrillar ATPase, it is impossible to make glycerated fibrils relax (Bendall, 1953*a*). Furthermore, the failure of Hasselbach (see Weber, 1955) to demonstrate relaxation with ITP in the presence of the relaxing factor could be explained by the finding that the substrate inhibition effect was not obtained when this nucleotide was split by myofibrils. The relative concentrations of magnesium and ATP in muscle are such that if all the magnesium present were available to the myofibrillar ATPase system, then the enzymic activity would be high. It has been suggested (Perry, 1955, 1956) that in relaxed muscle most of the magnesium is bound to the relaxing factor to ensure that the ATP concentration is in the relative excess necessary to produce inhibition. This view is supported by the experiments with inorganic pyrophosphate, the relaxing actions of which can be explained on the basis that it forms a complex with the magnesium with the result that the concentration of the available magnesium is exceeded by that of the ATP. It appears that with EDTA, however, inhibition is obtained when only a very small fraction of the magnesium would be bound as a complex and when its level would not be reduced sufficiently to produce any appreciable inhibition due to excess of ATP. It is possible that the EDTA complex is not with magnesium but with small amounts of some other cation present in the system which is essential for magnesium activation. The results with EDTA support the general hypothesis that relaxation is associated with a low rate of ATP hydrolysis, but the fact that this is achieved when the amount of EDTA present is adequate to form a complex with only a small fraction of the magnesium present reflects some unique aspect of the mechanism of ATPase activity of the myofibril.

The studies with isolated myofibrils indicate that, with certain combinations of ionic strength, substrate concentration and activator concentration which are comparable to those occurring in the living muscle, it is possible to obtain, in the absence of the recognized relaxing factors, the low levels of ATPase activity characteristic of relaxation. In previous investigations with glycerated-fibre models the importance of the relative concentrations of ATP and magnesium has not been recognized, although Bendall (1953*a*) noted that the magnesium concentration necessary for the demonstration of contraction was very critical.

If it is accepted that low enzymic activity, together with the presence of a plasticizer to reduce the interaction of actin and myosin, are the requirements of relaxation, then on the basis of the findings reported in this paper relaxing factors can be divided into two classes: (1) those which bind metal activators, e.g. (a) inorganic pyrophosphate, which binds magnesium, and (b) EDTA (the effect of EDTA is exceptional in that it produces marked inhibition of magnesium-activated ATPase at concentrations that are sufficient to bind only a small fraction of the total magnesium); (2) those substances or systems which act by maintaining the ATP at the inhibitory level, e.g. creatine phosphokinase, and perhaps myokinase.

It appears that the Marsh factor acts by binding a metal activator, but there is not yet enough evidence to decide whether this factor is a specific protein or whether it is some protein or enzyme system already recognized in muscle.

SUMMARY

1. At ionic strength about 0.06 and pH 6.9–7.6 the concentration at which adenosine triphosphate (ATP) became inhibitory for the adenosine triphosphatase (ATPase) of myofibrils isolated in 0.039M borate buffer (pH 7.1)–0.025M-KCl, was dependent on the concentration of magnesium chloride added to the system. At 5 mM- MgCl_2 inhibition occurred when the molar concentration of ATP exceeded that of magnesium.

2. The substrate inhibition of the magnesium-activated ATPase did not occur in the presence of calcium chloride in concentrations one-tenth to one-twentieth of that of the magnesium chloride.

3. Myofibrils prepared in 0.1M-KCl from fresh and glycerated muscle exhibited similar substrate inhibition effects at *I* about 0.16 to those obtained at *I* about 0.06 with myofibrils isolated in borate-potassium chloride.

4. Inorganic pyrophosphate inhibited the magnesium-activated myofibrillar ATPase when the total concentration of ATP and pyrophosphate exceeded that of magnesium chloride.

5. Adenosine diphosphate (ADP) was less inhibitory than ATP when it replaced part of the latter; inosine triphosphate had little inhibitory effect. Over a similar range, increasing concentration of uridine triphosphate produced progressively increasing inhibition.

6. No inhibitory action on the magnesium-activated myofibrillar ATPase could be demonstrated with myokinase preparations.

7. Ethylenediaminetetraacetate strongly inhibited the magnesium-activated myofibrillar ATPase in concentrations which were only one-twentieth of that of the added magnesium chloride. Calcium activation of the ATPase was much less sensitive to ethylenediaminetetraacetate.

8. These findings are discussed in relation to the relaxation studies by other workers on glycerated muscle fibres.

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The Phospholipase B of Liver

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Previous isotopic experiments have indicated that the free glycerylphosphorylcholine (GPC) and glycerylphosphorylethanolamine (GPE) present in liver tissue are intermediates in the metabolic breakdown of phosphatidylcholine and phosphatidylethanolamine respectively (Dawson, 1955). In support of this conclusion an enzyme, GPC diesterase, has been found in liver tissue which specifically hydrolyses GPC and GPE into glycerophosphoric acid and the free base (Dawson, 1956a). The present paper reports some observations on a phospholipase B (nomenclature of Zeller, 1952) which occurs in liver, forming GPC and probably GPE by the splitting of the single saturated fatty

acid from lysolecithin or lysophosphatidylethanolamine.

The enzyme phospholipase B has a comparatively wide distribution in nature. Contardi & Ercoli (1933), studying rice bran, were probably the first to recognize that it was a specific enzyme which did not attack lecithin until one fatty acid had been removed by phospholipase A. The enzyme has also been found to occur in moulds (Fairbairn, 1948) and bacteria (Hayaishi & Kornberg, 1954). Schmidt, Hershman & Thannhauser (1954) and Schmidt, Greenbaum, Fallot, Walker & Thannhauser (1955) observed the accumulation of the ethanol-soluble GPC in autolysing pancreas and intestine, but not