A STUDY OF THE ADENOSINE TRIPHOSPHATASE ACTIVITY OF MYOSIN AND ACTOMYOSIN*

By W. F. H. M. MOMMAERTS and KRIKOR SERAIDARIAN

(From the Department of Biological Chemistry, The American University of Beirut, Beirut, Lebanon)

(Received for publication, December 19, 1946)

I

INTRODUCTION

The present study is devoted to an analysis of the enzymatic behavior of myosin and actomyosin preparations towards ATP under different conditions. Several of our experiments are repetitions of the work of previous authors, but our analysis takes full account of the interaction between different factors, a problem more or less neglected by other workers. The emphasis on these interactions reveals several consequences not previously discovered.

The study of the enzymatic dephosphorylation of adenosine triphosphate (ATP), first described by Jacobsen (15) and by Barrenscheen and Láng (8), received increased interest through the report by Engelhardt and Ljubimova (11) that the ATPase of muscle accompanies the myosin fraction in all attempts to isolate and purify this structure protein. They considered therefore that myosin and the ATP-splitting enzyme are identical, *i.e.* that the contracting structure protein is responsible for the enzymatic hydrolysis of ATP, which reaction was supposed to be the energy-yielding reaction of muscular activity.

That myosin preparations have ATPase activity which is retained during purification was confirmed by a number of authors (1, 2, 4, 7, 22, 23, 27, 31, 32, 39, 40). Several of them advocated the identity of ATPase and myosin without restriction. The methods for the purification of myosin, however, are rather uniform, and give no assurance that a separation of ATPase activity from the myosin is not possible by other means. That an aqueous extract of muscle which contains no myosin at all is active towards ATP (Lohmann, 18) seems to have escaped attention for 15 years.

The problem exceeds enzymological interest through its physiological implications. Of all coenzymes and metabolites involved in muscular metabolism ATP is believed to be the most closely related to the transfer of energy to the contracting structure, and its enzymatic hydrolysis is now generally believed to represent the energy-yielding reaction. That this reaction might be catalyzed by the structure protein of the contracting fibrils itself, so that the liberated energy would be taken up directly by the catalyzing and contracting protein,

* This investigation was supported by The Rockefeller Foundation.

seemed to open entirely new views not only on the problem of muscular contraction, but even on that of life processes in general.

It was the purpose of this investigation to make a survey of the ATPase activity of different myosin preparations under different circumstances. Other authors have already published data in this field, but we have tried to correlate results with the electrolyte composition of the muscle fibre, and hence have paid special attention to the problem of the Ca-Mg antagonism.

Furthermore we were struck by the many contradictory statements made by different authors. As far as minor differences are concerned these may be due to inappropriate kinetic conditions in the enzymatic tests, but when one experienced investigator finds a pH optimum of 6, and others of 9 (see section VI) there must be another reason. We found the explanation of such differences in the extreme sensitivity of myosin-ATPase towards certain electrolytes.

II

Myosin and Actomyosin

In the literature myosin is usually regarded as a well defined protein, the purity of which is accepted with no limitations other than those connected with protein purity in general. The work of Straub (3, 33, 34) and Szent-Györgyi (37) shows that this is not true. They find that "myosin" as usually prepared is a complex of this protein with smaller amounts (up to some 20 per cent) of another protein, actin. Actomyosin is the general name for such complexes, which have physiological significance since the muscle fibril seems to contain actomyosin rather than myosin. The presence of actin not only affects the physical behavior of the myosin, but also modifies the enzymatic properties in several respects. Among the possible actomyosins of varying composition two take a special position in preparative work. One of them (here called actomyosin A) is obtained by extracting coarsely ground fresh muscle with neutral or alkaline 0.6 M KCl for 15 to 30 minutes (6). It contains some 2 to 5 per cent actin. The other (here designated as actomyosin B), containing about 16 per cent actin, is obtained by a similar extraction lasting for at least 6 hours at room temperature, or 24 hours in the cold. Myosin was probably not prepared in pure form by any of the older workers. It was obtained by Szent-Györgyi (36) in crystalline form, and found to be also enzymatically active. (For further information, see a forthcoming review by the author, 25.)

Since the enzymatic properties of actomyosin A approach those of crystalline myosin (hereafter designated merely as myosin) we did not study the former extensively, but restricted ourselves to an investigation of the properties of myosin and of actomyosin B. We studied their effects upon ATP, making also a few observations on the splitting of inosine triphosphate (ITP). In the experiments described here, adenosine diphosphate (ADP) was not decomposed

by myosin or actomyosin B. Consequently only one mole of inorganic phosphate was liberated per mole of ATP, but in the presence of other factors the situation may be different.

III

Methods

ATP was prepared as the Ba salt from dog muscle by the standard procedures of this laboratory (Kerr, 16). Its purity was always nearly 100 per cent, but we did not find it necessary to remove the last traces of inorganic phosphate. The Ba salt was dissolved with 1 N HCl and water, the Ba precipitated by addition of a slight excess of K_2SO_4, and the solution neutralized with KOH under electrometric or colorimetric control. ATP concentrations are expressed in terms of easily hydrolyzable P (⅔ of the total P, and thus two times the P mineralized by myosin).

ITP was prepared from ATP by desamination with nitrous acid and purified by a method developed by Kerr.[1]

The myosin preparations were obtained usually from the hind leg muscles of dogs, and occasionally of rabbits. The dogs were killed in amytal narcosis, the muscles were cut out quickly, cooled in ice, and minced in a cooled meat grinder after removal of fat. For the preparation of actomyosin B the minced muscle was extracted for 6 hours at room temperature or 24 hours at 0° with 3 volumes of alkaline 0.6 M KCl solution (containing 0.04 M $NaHCO_3$ and 0.01 M Na_2CO_3). After the extraction the residue was removed by centrifugation, the extract filtered through gauze, and the actomyosin precipitated by adding 5 volumes of water (redistilled from glass vessels) (see page 404) and sufficient acetic acid under strong stirring to bring the pH to 6.5 (glass electrode). The actomyosin precipitate was separated on the centrifuge, washed with pure water, and dissolved by adding 2 M KCl and water until the final salt concentration was 0.5 M and the protein concentration from 0.5 to 1.0 per cent. This precipitation was carried out three times in all. The actomyosin content was determined by the Kjeldahl procedure, the N content of the solution being multiplied by 6 (see Bailey, 1). The actin content was estimated from the viscosimetric response towards addition of ATP (see a forthcoming paper). The myosin content was obtained by subtracting the amount of actin from the actomyosin.

Occcasionally actomyosin was prepared from pure myosin and actin, but we used this cumbrous method only for a few control experiments. Actomyosin B prepared according to the direct method described above is not entirely free from proteins which attack ADP (myokinase or ADP isomerase, 5), but the small amounts did not interfere with our experiments, and could be demonstrated only in an indirect way.

For the preparation of crystalline myosin the muscle was extracted for 10 to 15 minutes with a solution containing 0.3 M KCl and a 0.15 M potassium phosphate buffer of pH 6.5 or 6.8. We closely followed Szent-Györgyi's (36) directions, which could be applied to dog muscle with only minor variations. After the last crystallization the sedimented crystals were suspended in water and dialyzed overnight.

As a general routine we adhered to the traditional low temperatures during all steps

[1] Kerr, S. E., unpublished data.

of the preparations. However, only during the summer was any damage observed when certain parts of the procedures were carried out at room temperature. We were very careful to work at low temperature, however, during certain steps of the crystallization. Our experience is that myosin is not as labile as is usually assumed. Repeatedly we precipitated actomyosin at pH values approaching 5.0 without loss in enzymatic activity (contrary to the experience of others, 32, 29). We were always careful, however, to use water redistilled in an all glass apparatus, since myosin accumulates copper when repeatedly treated with common distilled water (Szent-Györgyi, 38; Bailey, 1). We were able to decrease the stability of our preparations by addition of traces of $CuSO_4$.

During the warm summer months we observed several times irregularities in the transition of actomyosin A to B in the course of the preparation of actomyosin B. In such cases an actomyosin of lower actin content, often around 10 per cent, was obtained. Several experiments have been carried out with such preparations.

IV

Investigation of Enzymatic Activity

For the enzymatic tests myosin or actomyosin was suspended or dissolved in a medium of known ionic composition, incubated at the desired temperature, and mixed with the required amount of ATP. At intervals samples were pipetted into equal volumes of 10 per cent trichloroacetic acid. After centrifugation inorganic phosphate was determined by the Lohmann and Jendrassik modification (19) of the method of Fiske and SubbaRow, with a photoelectric spectrophotometer.

The experiments were made in test tubes suspended in a water bath at 37° unless indicated otherwise in the text. The total volume of the solution was always 10 cc. The volume of the samples taken for analysis was 3 cc., so that in every experiment three points of a curve were determined. Correction was always made for the initial low phosphate content. Phosphate values in the curves are given as milligrams of inorganic P in the total volume (10 cc.) of the experimental solution. The time of sampling varied between 1 and 30 minutes in different experiments, and was always adapted to the velocity of splitting in the experiment in question. We found the initial part of the curve always to be a straight line except in experiments where the medium was unfavorable to the stability of the enzyme (*e.g.* strongly alkaline), the enzyme being progressively destroyed during the course of the experiment. Most workers on ATPase have recorded only one determination of liberated phosphate after an arbitrary interval, a procedure which may give rise to inexact or incorrect conclusions.

Originally it was our intention to make an extensive survey of the kinetics of the reaction, with the object of obtaining information concerning the combination between ATP and myosin. When during the investigation we became doubtful that myosin and ATPase are identical (see section VIII),

so that no such information could be expected, we omitted these studies from the program. Consequently we do not propose or use any units of enzymatic activity. We restricted ourselves to a few formal investigations on the dependence of the velocity of hydrolysis on substrate and enzyme concentration.

Working with the low actomyosin concentration of 0.25 mg. per cc. in a moderately favorable medium (glycine buffer pH 7.0, KCl 0.10 M, $CaCl_2$

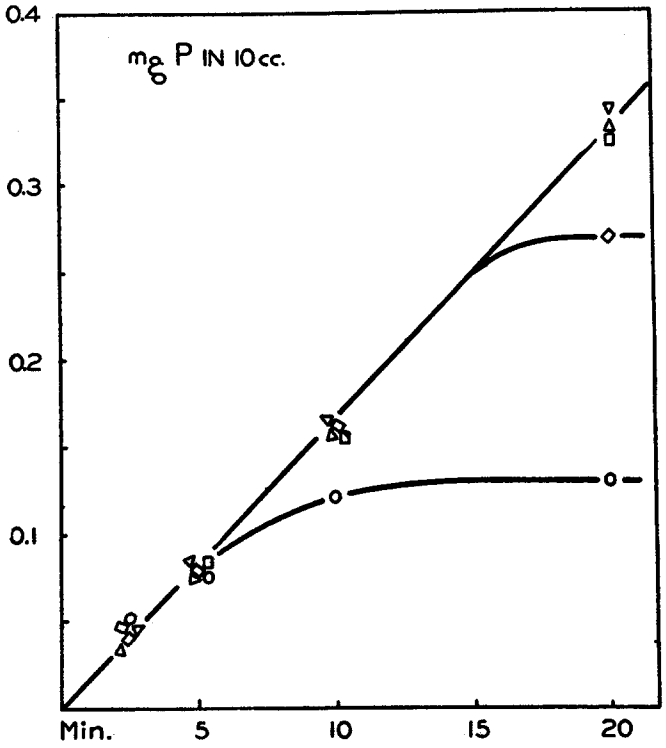

FIG. 1. Rate of phosphate liberation from ATP by actomyosin B (see text) at different substrate concentrations. ATP concentration in milligrams of hydrolyzable P in 10 cc. of reaction mixture: ○, 0.29; ◇, 0.58; ▽, 1.16; □, 1.74; △, 2.32.

0.01 M), we found the initial rate of ATP splitting to be independent of the substrate concentration down to an ATP concentration of 0.030 mg. of hydrolyzable P per cc. Lower concentrations were difficult to test, but in the range investigated the splitting had the character of a zero order reaction (see Fig. 1). This was not the case with ITP (see Fig. 2) where the reaction velocity depends on the substrate concentration over a wide range. This is of interest in relation to a statement made by Kleinzeller (17) who found the enzymatic splitting of ITP by myosin to proceed faster than that of ATP,

and concluded from this that ITP, not ATP, is the natural substrate of myosin. The higher reaction velocity with ITP is found only at very high substrate concentration, not at the low concentrations which are of physiological interest. We cannot accept his reasoning, moreover, and we find his conclusion that ITP is the natural substrate of myosin ATPase to be inadequately supported.

An experiment on the influence of the enzyme concentration (actomyosin) is presented in Fig. 3. It is seen that within the range studied the reaction

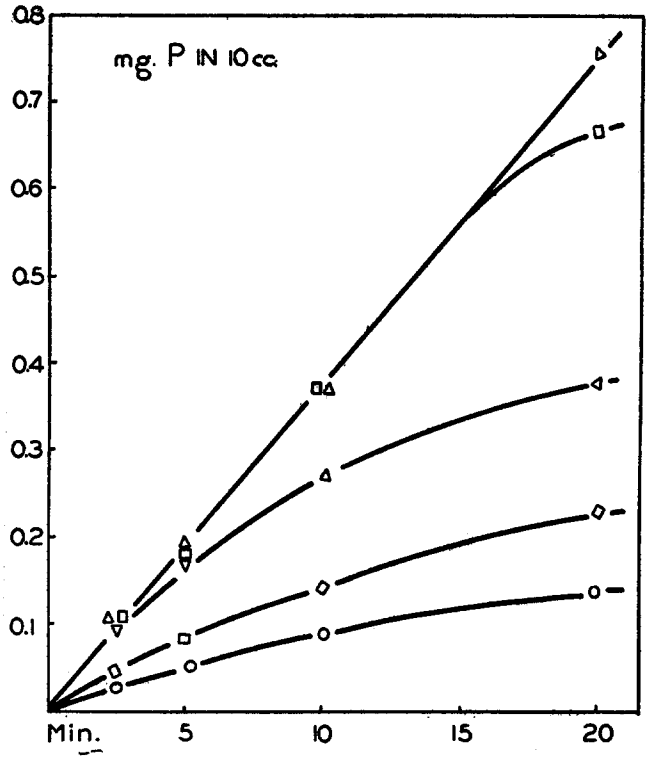

FIG. 2. The same as Fig. 1, with ITP as substrate. Concentrations as in Fig. 1.

velocity is proportional to the enzyme concentration. Since all subsequent experiments were made with the lower concentrations of this range, all changes in activity of the ATPase are reflected optimally in the rate of splitting, and are not obscured by limited accessibility of the substrate. This, it is true, has not been controlled in the optimal range of activity in glycine buffer with Ca, where the activity is very sensitive towards pH changes so slight as to be almost unavoidable if the protein concentration is varied. We can say, however, that here also deviations from the supposed kinetic behavior, if present at all, are negligible.

After these preliminary kinetic studies we adopted an ATP concentration of 1.2 to 1.5 mg. of hydrolyzable P per 10 cc. of sample for all experiments. In case of reactions reaching completion half of this P is liberated by the myosin

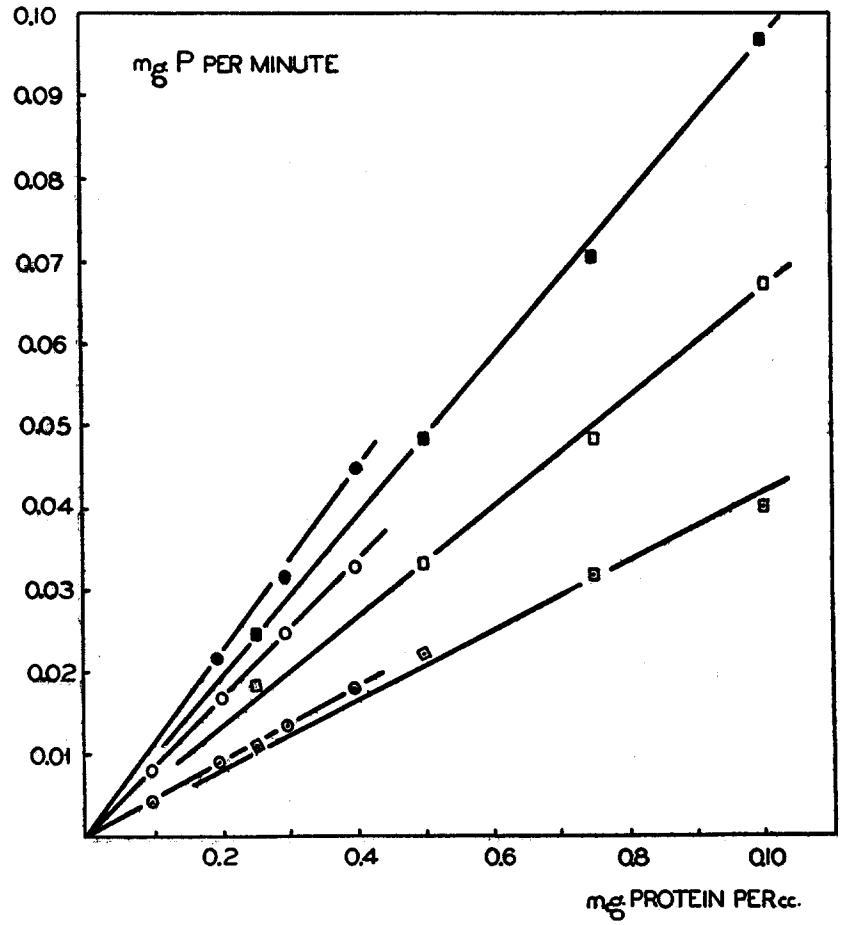

FIG. 3. Hydrolysis of ATP, 1.4 mg. of hydrolyzable P per 10 cc., by actomyosin B at different enzyme concentrations. Two different myosin preparations were used (squares and circles). Medium: 0.1 M KCl; glycine buffer pH 6.5; $CaCl_2$ to a final concentration of 0.001 M (⊙, ⊡), 0.005 M (○, □), and 0.020 M (●, ■).

(compare Fig. 1), showing that ATP is broken down only to ADP, as stated in the introduction.

We did not choose a standard enzyme concentration for all cases but adapted this to the needs of each experiment. Usually, however, 5 mg. of myosin or less were present in the 10 cc. reaction mixture so that the enzyme quantity

limited the reaction. Curves representing single experiments are given as they were obtained. Curves summarizing and generalizing the results of experimental series give the rate of P liberation in milligrams per minute per milligram of protein ("K").

V

Enzymatic Activity in Simple Salt Solutions

The activating effect of neutral salts upon the enzymatic activity of myosin-ATPase has been noticed by most authors in this field. The most extensive experimental material has been collected by Banga (4) and Szent-Györgyi (37) who also noticed several differences in the behavior of actomyosins A and B, and later of pure myosin. Our work is less extensive, since we only studied the effects of K, Ca, and Mg. Our results do not differ materially from Banga's, but there are minor deviations which we ascribe mainly to the fact that she made only one determination of P in each experiment, whereas we were always able to follow the course of P liberation, thus permitting finer quantitative distinctions. Moreover, we found some interactions not investigated by other authors.

Under the experimental conditions studied, enzymatic activity seems to be demonstrable only in the presence of salts. This, however, is difficult to prove directly, since the presence of ATP at approximately neutral reaction necessarily implies the presence of cations. Nevertheless the activity in the absence of any additional salts was so low that the above generalization is permissible.

That potassium in appropriate concentrations stimulates the enzymatic activity has been found by all authors who studied the effect of this ion. There is, however, some difference of opinion regarding the quantitative relations. Mehl and Sexton (23) found decreasing enzymatic activity over a range of concentrations of 0.1 to 0.6 M KCl (at pH 7.35, with 0.001 M $CaCl_2$ present). Their data are compatible with the assumption of a KCl optimum at or below 0.1 M, but lower concentrations were not studied by them. Singher and Meister (32) found a decrease of activity only at concentrations of KCl above 0.7 M, in experiments in which Ca was present at pH 8.5. Szent-Györgyi (37) and Banga (4, 5) on different occasions found a KCl optimum somewhat above 0.2 M, both for actomyosin (B, or with still higher actin contents) and for actin-free myosin.

Our own experiments confirm the fact that actomyosin B and myosin behave similarly towards KCl. We find (Fig. 4) a very pronounced effect if $CaCl_2$ is simultaneously added. In the absence of $CaCl_2$ we find a well pronounced optimum at about 0.3 M KCl for pure myosin, and a somewhat broader optimal range around this concentration for actomyosin B, similar to the results of Banga and Szent-Györgyi. In the presence of 0.001 M $CaCl_2$, however, the

K-optimum is shifted to much lower concentrations, and much higher activities are reached. This result can also be described as a strong activation by 0.001 M $CaCl_2$ (see below), and a K-Ca antagonism at higher K concentrations.

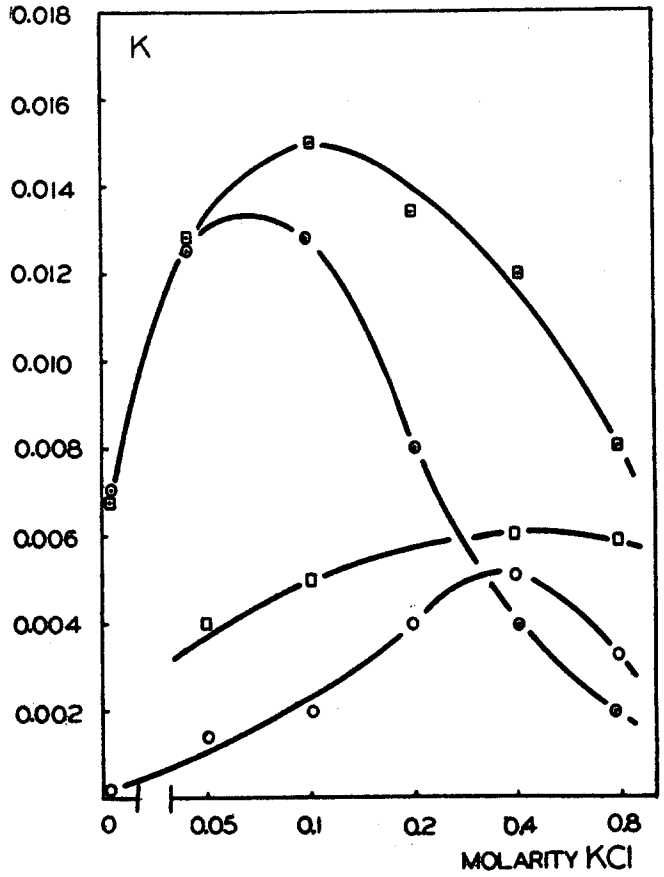

FIG. 4. Hydrolysis of ATP by crystallized myosin (circles) or by actomyosin B (squares) in the presence of different amounts of KCl. Upper curves (figures with dots): with 0.001 M $CaCl_2$; lower curves (figures without dots): without $CaCl_2$. K = mg. P per minute per mg. myosin.

The activating effect of Ca mentioned above was also noticed by several investigators (1, 4, 11). As shown in Fig. 5 (compare also Fig. 4) the Ca effect is evident both in the presence and in the absence of KCl, but as stated before high concentrations of KCl counteract the Ca activation.

The effect of Ca at different pH values will be discussed in the next section.

Concerning the effect of Mg conflicting statements are given in the literature,

varying from activation to inhibition. That the behavior is complex follows from the results of Banga and Szent-Györgyi, which we were able to confirm. The enzymatic action of crystallized myosin was found to be always inhibited by Mg under the conditions so far studied. At sufficient concentrations (dependent on the presence and concentration of other ions) the inhibition is practically complete. Actomyosins of low actin content behave like myosin.

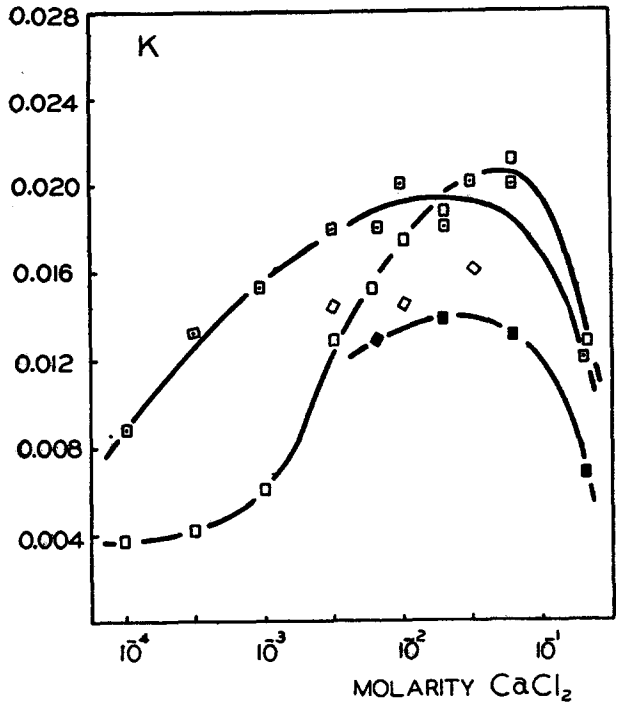

FIG. 5. Hydrolysis of ATP by actomyosin in dependence on the $CaCl_2$ concentration. Various amounts of KCl: □ no KCl; ⊡ 0.1 M; ◇ 0.12 M KCl, 0.05 M NaCl; ■ 0.5 M KCl.

With higher actin contents, however, (*e.g.* in the case of actomyosin B) Mg activates but only if other ions are absent, or present in small amount (*e.g.* 0.01 M KCl). In the presence of 0.1 M KCl, for example, Mg always inhibits. The same is true in the presence of Ca, but this case will receive special attention in section VII.

VI

Effect of pH on Enzymatic Activity

The dependence of enzymatic activity on pH has been studied by several investigators. Whereas most of them (Engelhardt and Ljubimova, 11;

Bailey, 1; Singher and Meister, 32) agree that the pH optimum is situated in the neighborhood of 9.0, Banga (4) finds an optimum around 6.0. Later Engelhardt and Ljubimova (12) observed two maxima, but did not specify the conditions under which these appear.

It soon became obvious to us that the behavior of myosin-ATPase is too complex to be described in terms of a constant or approximately constant pH optimum. It is general experience in enzymology that the relation between enzymatic activity and H ion concentration is modified by the specific action of other ions present in the buffer system, but in the present case we found effects much stronger than those found in studies on other enzymes. We observed moreover that the stability of the enzyme complex varies markedly in different buffer solutions, notably in the alkaline range. Consequently the temperature has a profound influence, and in some buffers we found a qualitatively different behavior when the experimental temperature was lowered from 37° to 25°. We present our results with actomyosin in detail. The behavior of myosin follows the same general pattern.

The results of the experiments with actomyosin are summarized in Figs. 6, 7, and 8. We first present the results with borate buffers (Fig. 7); the final concentration of the buffer was 0.05 M. As in the other experiments described in this section, the pH was determined with the glass electrode at the temperature of the experiment, after all the components were mixed and before the first sample was taken. In all experiments on the influence of pH, there was a basic concentration of KCl of 0.1 M in addition to the ions of the buffer system.

In the absence of Ca (Fig. 7, lower curve) the pH optimum is 6.5 or slightly below this point. The activity diminishes sharply on the acid side, but the curve extends far into the alkaline range, showing a hump which may indicate a less pronounced optimum at alkaline reaction.

In the presence of Ca however (Fig. 7, upper curve) the situation is essentially different. It is true that here also the acid optimum is present but, in addition, a much more pronounced optimum appears around pH 9.0. The curve as a whole is on a higher level, but it is seen that the Ca activation is much stronger at alkaline reaction.

Next we describe our findings with glycine solutions which have been described by Bailey (1) as a medium particularly favorable for myosin-ATPase. As one of the causes of the high activity in the presence of glycine, Bailey mentions the ability of this buffer to combine with damaging heavy metal ions, notably Cu. We do not deny the possible significance of this effect but it cannot have played a rôle in our experiments, since we took especial care to avoid contamination with traces of Cu, as explained in section III. We fully agree with Bailey's discussion of the other effects involved.

We used Sörensen's glycine buffers, 5 cc. of which were contained in 10 cc. of reaction mixture, so that the final concentration of the buffers was 0.05 N.

Due to the low buffer capacity in the acid range, we did not investigate the optimum at pH 6.5 in this case. In the absence of Ca the activity of the

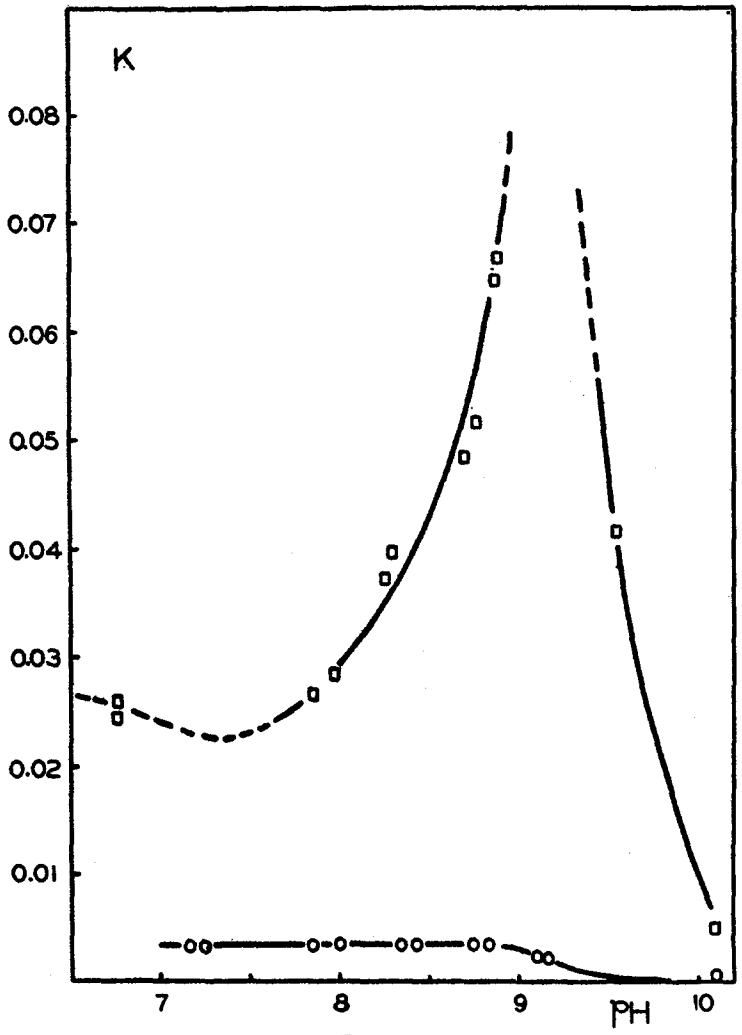

FIG. 6

FIGS. 6 to 8. Enzymatic activity of actomyosin in dependence on pH; □ with 0.005 M $CaCl_2$; ○ without $CaCl_2$. Explanation in text. Fig. 6. Glycine buffer. Fig. 7. Borate buffer. Fig. 8. Veronal buffer. Figs. 6 and 7 are on the same scale, but in Fig. 8 the vertical scale is five times larger.

actomyosin ATPase in glycine buffer is very similar to that in borate (Fig. 6); with Ca, however, much higher activities are reached, and the sharp optimum at pH 9.2 represents the highest activity obtained in our experiments.

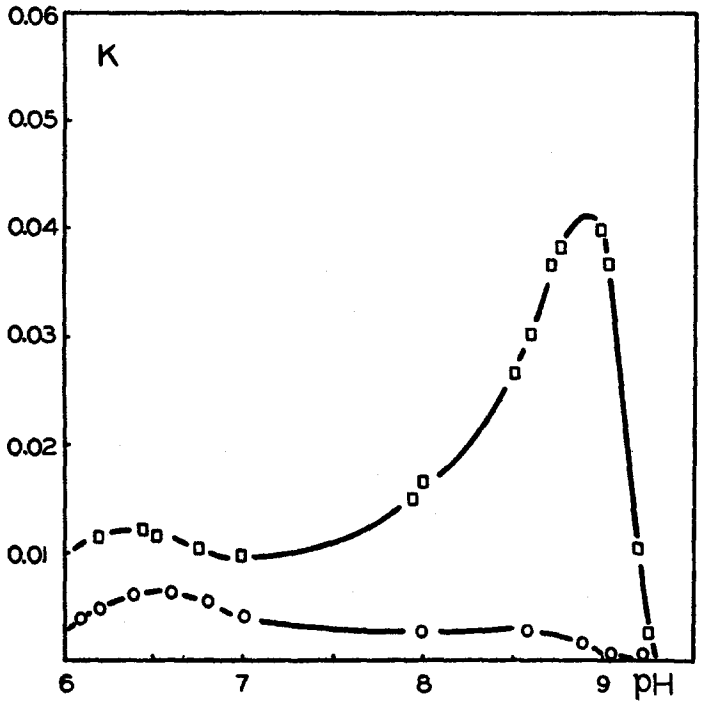

FIG. 7

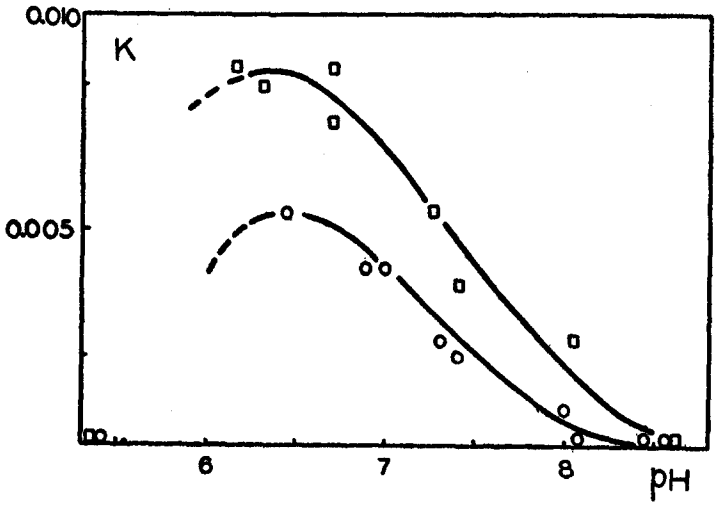

FIG. 8

It will be seen that the alkaline pH optimum in glycine buffer (9.2) differs from that in borate (pH 9.0). At lower temperature, however, the optimum

in borate shifts to the alkaline side. This is probably explained by decreased stability of the enzyme in the alkaline medium. Indeed, the time course of the liberation of P in the alkaline mixtures indicated a progressive destruction of the enzyme.

The third buffer used in our investigations was Michaelis' veronal-acetate mixture. Wherever possible we used it in $2\frac{1}{2}$ times the concentrations employed by Michaelis, so that the final concentration in the reaction mixture was 1/14 M. The concentration was slightly less in the most acid solutions employed. The results with veronal buffers (Fig. 8) seem to be very different from those with the other systems. Both in the absence and in the presence of Ca there is only one optimum, that at pH 6.5, with no indication of an alkaline optimum.

Notwithstanding the total absence of the alkaline optimum at 37°, the behavior in veronal buffers is not fundamentally different from that in other media. At 25° the alkaline optimum is well developed (we did not determine its exact position), so that the absence of the optimum at 37° must be due to lack of stability of the enzyme complex in this kind of buffer.

Considering the data in the literature, we may conclude that our analysis explains the difference of opinion mentioned previously. The authors who found the optimum around pH 9.2 all worked in the presence of Ca, which they consider essential for activity. The low optimum at pH 6.5 is often not observed, because it is almost negligible compared with the peak at pH 9.2. Banga (4) on the other hand worked without Ca; her optimum is situated at pH 6.0, but since according to her report the pH of the buffer was controlled before addition of the protein and the ATP, we may consider the lower value to be the result of this difference in experimental procedure.

VII

Calcium-Magnesium Antagonism

That Mg ions, although under certain conditions able to activate the enzymatic activity of actomyosin, always counteract the effect of other ions, was mentioned in section V. Although this fact is not unknown in the literature (Szent-Györgyi, 37; Ljubimova and Pevsner, 20; Du Bois *et al.*, 10; Greville and Lehmann, 14) its consequences require further investigation. We will discuss them in section VIII, but it may be noted here that the muscle fibre contains significant amounts of Mg. Since the fibre contains also Ca, which strongly activates the enzymatic activity, a study of the Ca-Mg antagonism is evidently of physiological interest.

Fig. 9 presents a typical experiment in which actomyosin was studied (5 mg. in 10 cc.) at the Ca and Mg concentrations indicated. The inhibition by Mg is apparently very effective, the residual activity diminishing to about 6 per cent of the original activity. In this experiment the solution contained 0.12 M

KCl and 0.05 M NaCl besides the Ca and Mg. The efficiency of the inhibition calculated for a certain Mg:Ca ratio decreases somewhat with decreasing concentrations of these ions. We did such experiments with several variations, using both myosin and actomyosin, Ca and Mg in different ratios and concentrations, both in the presence and absence of other ions (*e.g.* glycine buffers),

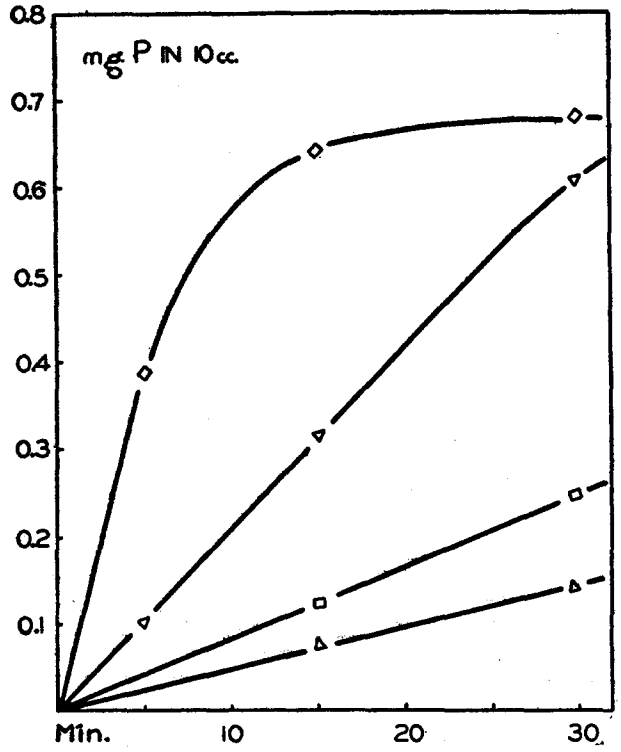

FIG. 9. Example of an experiment on the inhibition of actomyosin-ATPase by Mg ions (0.5 mg. actomyosin B per cc.). Composition of medium: 0.032 M $CaCl_2$, 0.12 M KCl, 0.05 M NaCl. Concentration of $MgCl_2$: △, 0.05 M; □, 0.01 M; ▽, 0.002 M; ◇, no $MgCl_2$.

at neutral reaction, and at pH 9. The strong inhibition of Ca activation was confirmed in all these cases. At Ca:Mg ratios around unity myosin was always practically completely inhibited, whereas the residual activity of actomyosin usually amounted to only a few per cent of the original activity. The Ca concentration was thereby varied between 0.0003 M and 0.032 M.

We summarize the results of these experiments in Fig. 10. Mg:Ca ratios higher than 5 are not represented in this figure; experiments with Ca concentrations below 0.003 M, in which no residual activity was detected, are also

omitted. Every point represents the initial velocity, evaluated from a 4 point curve, and expressed as fraction of the velocity in an experiment with the same amount of Ca but without Mg. It must be stressed that the finer differences between the curves are certainly within the range of experimental

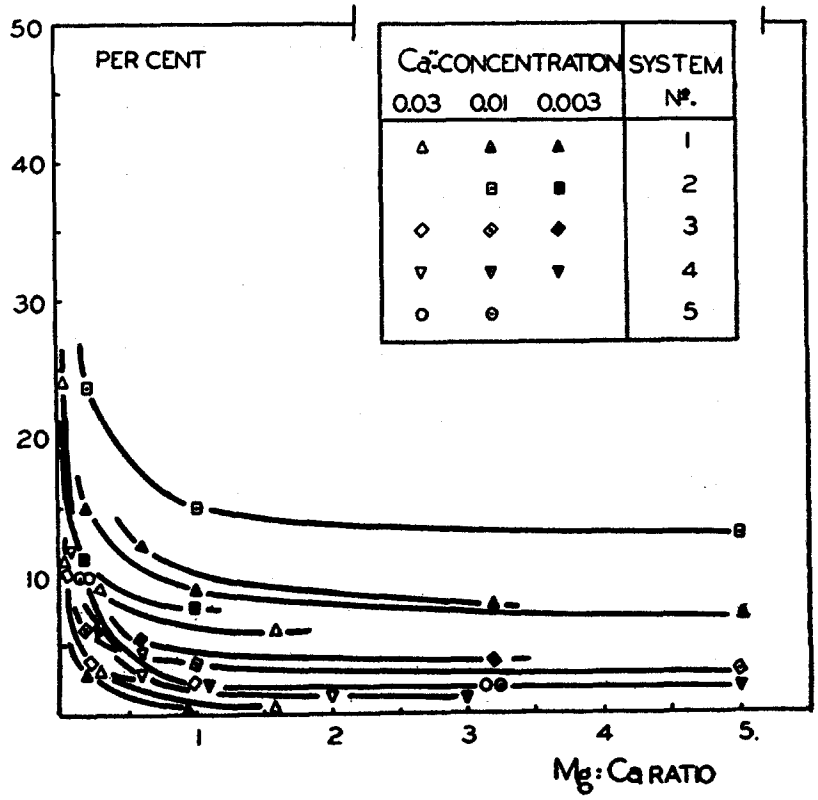

FIG. 10. Summary of experiments on Ca-Mg antagonism. The curves show the enzymatic activity as percental fraction of the activity without Mg, as a function of the Mg: Ca ratio. Composition of the system: (1) Actomyosin, 0.12 M KCl, 0.05 M NaCl. (2) Actomyosin in glycine buffer pH 7. (3) Myosin in glycine buffer pH 7. (4) The same as 3, pH 9. (5) Myosin in 0.12 M KCl, 0.05 M NaCl, or in 0.12 M KCl alone, or in 0.24 M KCl, 0.05 M NaCl.

error. Two general conclusions are apparent. First, that at a sufficient Mg:Ca ratio the residual enzymatic activity is only a small fraction of the original activity. Second, that the degree of inhibition is almost constant over a wide range above Mg:Ca = 1, and even well below this value. This is a fortunate circumstance with regard to the uncertainty concerning the concentration of cations in muscle, discussed in the next section.

VIII

DISCUSSION

The discovery of the ATPase activity of myosin preparations, mentioned in the introduction of this paper, was followed by numerous discussions on the mechanism of muscular contraction. The point in common in all these considerations is that muscular activity is believed to be due to hydrolysis of ATP by the myosin itself, and during this reaction an exchange of energy between substrate and enzyme is supposed to occur, causing the myosin to react mechanically.

Without questioning the ingenuity of several of these special interpretations we are of the opinion that the general position of such hypotheses is weak. Discussions on the validity of these special hypotheses can hardly begin until three conditions are fulfilled:—

1. It must be proven that hydrolytic splitting of ATP is really the energy-yielding reaction of muscular activity. We do not doubt that ATP is intimately related to contraction or relaxation, but we cannot exclude the possibility that this relationship may prove to be something other than hydrolysis of the ATP.

2. It must be demonstrated that the ATPase activity shown by myosin preparations is possible under the special conditions (particularly the electrolyte medium) prevailing in muscle. The interior of the muscle fibre may constitute a medium unfavorable for the activity of myosin-ATPase.

3. The identity of myosin and ATPase must be proven more conclusively. If they were found to be different proteins, many of the recent hypotheses would be deprived of their starting point and only argument. Of course, in this case one could try to save the situation by the assumption that the supposed energy transfer relations hold for the enzyme, and that this initiates contraction through its close connection with myosin or other contractile factors. This, however, would bring us back to the early days of muscle biochemistry, in which it was also known that some metabolic reaction "somehow" provides the energy for the muscular mechanism (24). Unless the nature of the connection between ATPase and contractility had been specified, no material progress would have been made.

Concerning the first point we might direct attention to experiments of Cori and Cori (9) which support the view that in muscular activity ATP is not decomposed by simple hydrolytic dephosphorylation. This we are investigating at present. Regarding the third condition we might mention that our experience with myosin and ATPase has gradually strengthened the conviction that the two proteins are different. We obtained undenatured myosin preparations without ATPase activity, but this might be interpreted as a topochemical inactivation of active groups without changes in other properties of the molecule. We observed also (as Lohmann did some 15 years ago, 18;

see also Sakov, 30), ATPase activity in watery muscle extracts, but the activity of these extracts might be of a complex nature. We have not yet attempted the preparation of pure ATPase from myosin, so that we shall postpone the discussion of this problem to a later occasion.[2]

Although thus myosin and ATPase may not be identical,[2] their relationship seems to be closer than one of accidental combination. This follows from the study of the enzymatic activity in single salt solutions. We present the results of this investigation without discussing the mechanism of the salt effects. Price and Cori (29) found no effect of Ca upon myosin-free ATPase. On the other hand, the extreme sensitivity of the physical behavior of myosin towards salts has been demonstrated by Szent-Györgyi (37). The available data do not yet allow a correlation between the effects of ions on enzymatic activity and on the physical properties of myosin, but it seems that ions affect the ATPase through the myosin rather than directly. This would involve a unique case of interaction between two proteins, the nature of which is not yet understood.

Instead of attempting to explain the mechanism of these salt effects in simple solutions and in mixtures, we wish to discuss the consequences and physiological implications of our results. We have demonstrated that Mg strongly inhibits the enzymatic activity of myosin-ATPase, thus counteracting the activating effect of other ions. This is true within a wide range of concentrations of the different ions, an inhibition of over 90 per cent being reached at a Mg:Ca ratio of only 0.2, and hardly enhanced by further increasing Mg concentration. Calculated for the total water, the concentrations of these ions in muscle are about 0.012 M Mg and 0.003 M Ca, so that the Mg:Ca ratio in muscle is about 4.

However, not all this Mg and Ca is present in a free condition. Part of it seems to be bound, and concentrated in the A bands, which suggests that it is combined with the myosin. This causes considerable uncertainty concerning the actual concentrations of Mg and Ca with which the myosin in the muscle is in equilibrium. The same binding of Ca and Mg by myosin probably took place in our experimental solutions, but here much less myosin was present relative to salt than in muscle.

There is uncertainty not only as to the total concentration of Ca and Mg, but also with regard to the Mg:Ca ratio in equilibrium with the myosin *in*

[2] During the preparation of this publication we read a preliminary paper by Price and Cori (29) who actually separated ATPase from myosin. We were unable to reproduce their results, but we ascribed this to the brevity of their description of the experimental procedure. Price and Cori's experiments would definitely demonstrate that myosin and ATPase are different substances. Compare also Polis and Meyerhof (28). We prefer, however, to postpone our judgment until more details are available.

situ. Fortunately, we can arrive at some conclusions on the basis of the experiences of Szent-Györgyi (37) with the binding of cations by myosin. He found that myosin has a stronger affinity for Ca than for Mg, and calculated that in muscle the total Ca available is much less than that which can be bound by myosin. Considerable amounts of Mg therefore can also combine with it. In our experimental solutions on the other hand there was a large excess of salt solution, with more than enough Ca to saturate the myosin. Therefore, if there is any difference between the behavior of myosin in the muscle and in the enzymatic experiment, it can only be that in muscle the conditions are more favorable for complete inhibition by Mg than in our experiments. This inhibition is fully developed with a Mg:Ca ratio less than 1, whereas in muscle a ratio of 4 is present; hence we may safely conclude that in muscle the inhibition of myosin-ATPase by Mg must be at its maximum.

On the basis of our quantitative measurements of the ATPase activity of myosin under different conditions we can estimate the velocity with which the myosin in muscle can hydrolyze ATP. As a starting point we take our experiments in glycine buffers, since according to Bailey (1) sarcosine and related substances present in muscle can cause an activation similar to that of glycine. The pH is difficult to define in a system such as muscle, but seems to be close to the neutral point, on the acid side (Fenn, 13). Ca is present in an amount favorable for enzymatic activity, and, neglecting for a moment the inhibitory action of Mg, we conclude from Fig. 6, upper curve, that the rate of ATP splitting may amount to some 0.030 mg. P per mg. myosin per minute. Accepting a 90 per cent inhibition by the Mg ions, a velocity of 0.003 mg. P per mg. myosin per minute remains.

This calculation involved as a simplification the assumption that ATP splitting in the muscle takes place under the same kinetically favorable conditions as in our experiments. This is certainly not true, the myosin concentration in the muscle fibril being so high that the rate of splitting per milligram of myosin is probably far from optimal.

We can, on the other hand, calculate the velocity with which inorganic phosphate is liberated from creatine-phosphate (*via* ATP) in muscular activity. The order of magnitude can be estimated from the work of Nachmansohn (26), but the most accurate basis is formed by the work of Lundsgaard (21) on the rate of splitting of phosphocreatine in the presence of iodoacetate, since here resynthesis does not disturb the picture. Taking only the shortest (2 seconds) tetani for our purpose we find (Lundsgaard, 21, page 59) that a frog muscle (usually somewhat less than 1 gm.) liberates from 0.47 to 0.91 mg. of H_3PO_4 in 2 seconds. Selecting the maximum figure we find a velocity of about 0.15 mg. P per second per gm. of frog muscle, at room temperature. For 37° we may double this value (compare the experience of Singher and Meister (32) on ATPase activity at different temperatures). This calculation

ignores the fact that mammalian muscle may have a higher rate of metabolism and also that tetanus is a discontinuous process, in which the rate of metabolism during the periods of activity will be higher than the over-all rate. Assuming the myosin contents to be 100 mg. per gm. of muscle we arrive at a rate of P liberation of roughly 0.200 mg. P per mg. myosin per minute. The simplifying assumptions made in this calculation all tended to give a low figure for the rate of P liberation.

We find thus that the possible rate of P liberation from ATP by the muscle's myosin is at most 0.003 mg. P per mg. myosin per minute, whereas in muscular activity the actual liberation of inorganic P takes place at a speed of at least 0.200 mg. P per mg. myosin per minute. This shows convincingly that the hydrolytic splitting of ATP by myosin-ATPase cannot be the source of the liberated phosphate nor of the energy for the contraction or relaxation.

Whereas this conclusion is definitely unfavorable towards many of the recent hypotheses concerning the rôle of myosin and ATP in muscular activity, we think it necessary to remark that a somewhat different position is taken in the investigations of Szent-Györgyi (35, 37). It is true that he also accepted the identity of myosin and ATPase. But Szent-Györgyi discovered various interactions between ATP and myosin, and in his work stress is laid upon these interactions rather than on the enzymatic effects only.

We prefer to withhold any suggestions regarding the hydrolysis of ATP by agents other than myosin-ATPase, or about other processes in which ATP may be involved. We recognize the fact that hydrolysis of ATP may have a physiological function other than causing contraction or relaxation. Finally, we keep in mind that ATP exerts profound effects upon the physical properties of myosin but, as will be demonstrated in forthcoming papers, these effects have nothing to do with its enzymatic properties.

SUMMARY

1. An experimental study was made on the adenosine triphosphatase action of crystalline myosin and actomyosin preparations under different conditions.

2. No enzymatic activity was found in the absence of salts. Activation was given by KCl and $CaCl_2$, whereas $MgCl_2$ in the presence of other ions inhibited.

3. The effect of pH is complex. In stabilizing buffers or at low temperature, there are two optima (pH 6.2 to 6.5 and pH 9.2) provided Ca is present. Without Ca only the acid optimum is found. The highest activities are reached in glycine buffer at pH 9.2 in the presence of Ca.

4. The study of the Mg-Ca antagonism revealed that the inhibition due to Mg is fully developed with Mg:Ca ratios less than 1, the inhibition usually exceeding 90 per cent.

5. It is shown that in the muscle the myosin-ATPase is most probably also subjected to the inhibitory action of the Mg ions.

6. From data in the literature it is calculated that the liberation of inorganic phosphate during muscular activity takes place at a rate of at least 0.200 mg. P per mg. myosin per minute.

7. From the results of the present study it is found that the myosin in the muscle can liberate inorganic phosphate from ATP at a rate of at most 0.003 mg. P per mg. myosin per minute.

8. It is concluded therefore that myosin-ATPase cannot be responsible for the liberation of the main part of the phosphate in contracting muscle, and therefore cannot have the rôle in muscular metabolism ascribed to it in recent hypotheses and discussions.

LITERATURE

1. Bailey, K., *Biochem. J.*, 1939, **36,** 87.
2. Bailey, K., in Advances in Protein Chemistry, (M. L. Anson and J. T. Edsall, editors), New York, Academic Press, Inc., 1944, **1,** 289.
3. Balenovic, K., and Straub, F. B., *Studies Inst. Med. Chem., Univ. Szeged*, 1942, **2,** 17.
4. Banga, I., *Studies Inst. Med. Chem., Univ. Szeged*, 1942, **1,** 27.
5. Banga, I., *Studies Inst. Med. Chem., Univ. Szeged*, 1943, **3,** 64.
6. Banga, I., and Szent-Györgyi, A., *Studies Inst. Med. Chem., Univ. Szeged*, 1942, **1,** 5.
7. Banga, I., and Szent-Györgyi, A., *Studies Inst. Med. Chem., Univ. Szeged*, 1943, **3,** 72.
8. Barrenscheen, H. K., and Láng, S., *Biochem. Z.*, 1932, **253,** 395.
9. Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, 1936, **116,** 119, 129.
10. Du Bois, K. G., Albaum, H. G., and Potter, V. R., *J. Biol. Chem.*, 1943, **147,** 699.
11. Engelhardt, W. A., and Ljubimova, M. N., *Nature*, 1939, **144,** 668.
12. Engelhardt, W. A., and Ljubimova, M. N., *Biokhimia*, 1942, **7,** 205.
13. Fenn, W. O., *Physiol. Rev.*, 1936, **16,** 450.
14. Greville, G. D., and Lehmann, H., *Nature*, 1943, **152,** 81.
15. Jacobsen, E., *Biochem. Z.*, 1931, **242,** 292.
16. Kerr, S. E., *J. Biol. Chem.*, 1941, **139,** 121.
17. Kleinzeller, A., *Biochem. J.*, 1942, **36,** 729.
18. Lohmann, K., *Biochem. Z.*, 1928, **203,** 172.
19. Lohmann, K., and Jendrassik, L., *Biochem. Z.*, 1926, **178,** 419.
20. Ljubimova, M. N., and Pevsner, D., *Biokhimia*, 1941, **6,** 178.
21. Lundsgaard, E., *Biochem. Z.*, 1930, **227,** 51.
22. Mehl, J. W., *Science*, 1944, **99,** 518.
23. Mehl, J. W., and Sexton, E. L., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52,** 38.
24. Meyerhof, O., Die chemische Vorgänge in Muskel, 1930, Berlin, Julius Springer.
25. Mommaerts, W. F. H. M., papers in preparation.
26. Nachmansohn, D., *Biochem. Z.*, 1928, **196,** 73.
27. Needham, D. M., *Biochem. J.*, 1939, **36,** 113.
28. Polis, B. D., and Meyerhof, O., *J. Biol. Chem.*, 1946, **163,** 339.
29. Price, W. H. and Cori, C. F., *J. Biol. Chem.*, 1946, **162,** 393.
30. Sakov, N. E., *Biokhimia*, 1940, **6,** 163.

31. Singer, T. P., and Barron, E. S. G., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56,** 120.
32. Singher, H. O., and Meister, A., *J. Biol. Chem.*, 1945, **159,** 491.
33. Straub, F. B., *Studies Inst. Med. Chem., Univ. Szeged*, 1942, **2,** 3.
34. Straub, F. B., *Studies Inst. Med. Chem., Univ. Szeged*, 1943, **3,** 23.
35. Szent-Györgyi, A., *Studies Inst. Med. Chem., Univ. Szeged*, 1942, **1,** 17.
36. Szent-Györgyi, A., *Studies Inst. Med. Chem., Univ. Szeged*, 1943, **3,** 76.
37. Szent-Györgyi, A., *Acta physiol. Scand.*, 1945, **9,** suppl. **25.**
38. Szent-Györgyi, A., personal communication.
39. Szent-Györgyi, A., and Banga, I., *Science*, 1941, **93,** 158.
40. Ziff, M., *J. Biol. Chem.*, 1944, **153,** 25.