THE REACTION BETWEEN ACTOMYOSIN AND ADENOSINE TRIPHOSPHATE*

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I

INTRODUCTION

The modern tendency in the biochemistry of muscle is to consider the enzymatic dephosphorylation of adenosine triphosphate (ATP) as the energy-yielding reaction of muscular activity. In a preceding paper by Mommaerts and Seraidarian (16) it was demonstrated that there are serious objections to this viewpoint, which currently seems to be untenable. The main experimental basis for this conclusion was the demonstration that the hydrolysis of ATP by myosin-ATPase can account at most for only a small percentage of the speed of breakdown of ATP in contracting muscle as actually observed.

Even before this development, the criticism could have been made that the proponents of what may be called the myosin-enzyme theory failed to make the mechanism of the energy transfer clear. It is stated repeatedly (*e.g.* Dainty *et al.*, 9) that the reaction energy of the hydrolysis of ATP, supposedly of the order of magnitude of 10,000 calories per mol phosphate, is somehow given over to the myosin, but no explanation of the mechanism of this transfer has been attempted.

Extremely valuable contributions have been made by A. Szent-Györgyi (23), independently of any theory. He showed that myosin reacts with ATP, and that various physical changes can be evoked in actomyosin as a result of this reaction. In an earlier publication (12) the author showed that this reaction is actually a combination between ATP and myosin, but the methods were imperfect and the mechanism was not worked out in detail.

It is the purpose of this paper to present a preliminary analysis of the combination between ATP and myosin, and of some of the physical phenomena resulting from it. The most striking of the effects discovered by Szent-Györgyi is certainly the contraction of actomyosin threads under influence of ATP (22, 23). For several reasons, however, this phenomenon is not well suited to

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quantitative analysis. More promising seems the effect of ATP upon the viscosity of actomyosin solutions (Banga and Szent-Györgyi, 3), a phenomenon studied by the author in previous approaches (12–14). This problem is apparently linked with that of the form and dimensions of the myosin molecules (13, 15) and with that of the forces causing their aggregation (14). These aspects will be reconsidered in the future. The present paper is devoted to a more formal study of the reaction between ATP and myosin, without any attempt to consider in detail the molecular mechanism and its implications.

As discovered by Banga and Szent-Györgyi (3), a solution of actomyosin with a sufficient actin content has a considerably higher viscosity than a solution of pure myosin, or than a solution of actomyosin with less actin. Upon the addition of ATP the viscosity of every actomyosin solution drops approximately to that of pure myosin. The difference in viscosity between actomyosin and myosin has been ascribed by the author (13) to the fact that the myosin molecules in actomyosin are aggregated in an end-to-end arrangement. The effect of ATP on the viscosity of actomyosin is then due to a dissociation of this complex protein (14). Somewhat similar effects have been observed by Dainty *et al.* (9), and were interpreted by them as a contraction of the dissolved myosin molecules. This interpretation was disproved in an earlier paper (14).

Although at present it is difficult to relate this viscosity effect directly to the problems of contraction, the effect seems to offer an advantageous approach to the study of the reaction between ATP and myosin. The main advantage is that the phenomenon can be studied in solution, and that it can be measured quantitatively.

II

General Description of the Observed Effects

In this study the viscosity was measured in viscosimeters of the Ostwald type. Measurements were done at 0°, or at temperatures between 16° and 24°. In most cases the actomyosin used was actomyosin B, prepared as described in an earlier paper (16). The viscosimeter usually contained 5 cc. of a solution of actomyosin in 0.5 M KCl, each cubic centimeter of the solution averaging 2 to 3 mg. protein per cc. ATP and other reagents were added through a capillary pipet, with complete mixing. They were dissolved in small enough amounts of fluid, 0.05 cc., that the dilution due to this addition did not in itself decrease the viscosity to a measurable degree.

The results of a series of experiments done with different amounts of ATP are given in Fig. 1. It is seen that the viscosity drops immediately after addition of the ATP, as far as can be judged by these experiments (no rapid technique has been devised yet, but the reaction certainly needs no more than a few seconds, probably much less). After some time, varying directly with the amount of ATP added, the effect of the reaction diminishes gradually, ap-

parently through hydrolysis of the ATP. With certain precautions it is possible to do the experiments in such a way that the original viscosity returns quantitatively. In experiments carried out with the routine technique, however, there usually was a residual effect: the viscosity did not return entirely to the original value. The magnitude of this difference is variable. The experiments of Figs. 1 and 2, done in the summer, constitute extreme examples. This effect is understandable when one realizes that after the splitting of ATP the actomyosin aggregates again, although under very different circumstances

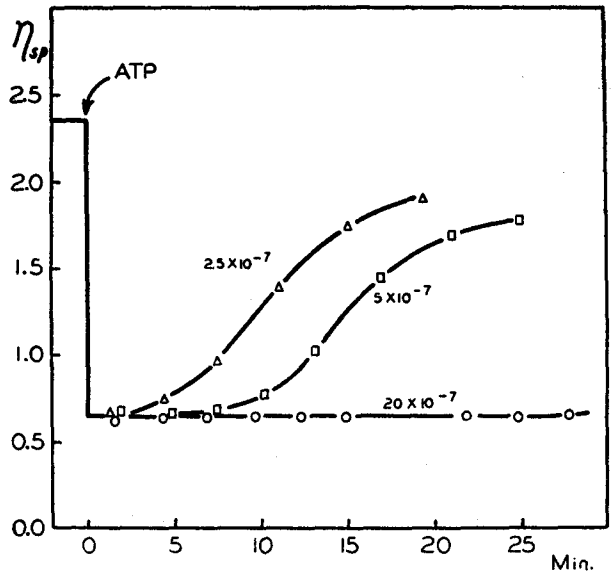

FIG. 1. Effect of ATP upon the viscosity of actomyosin solutions, containing 12.5 mg. protein in 5 cc. Temperature 0°C.

than when it was formed during the extraction of muscle. Actomyosin is certainly polydispersed, with respect to its degree of aggregation. The variation among the different individual aggregates may depend on the way in which they were formed. After the addition and removal of the ATP, the micellar structure of the actomyosin, and thereby the viscosity of its solution, have changed. A more complete analysis of this problem, in terms of skew or polymodal polydisperity, may be attempted at another time. In anticipation it may be said that the phenomenon indicates that the aggregates in actomyosin have a permanent character. This would mean that the high viscosity and other properties of actomyosin are not due to long range intermolecular forces, as assumed by Bernal and Fankuchen (4) for tobacco mosaic virus.

Returning to Fig. 1, we can describe the composite behavior shown in the

curves in terms of two separate reactions: the initial "viscosity response" and the subsequent "recovery effect." This recovery effect must be explained by the enzymatic hydrolysis of the ATP by the myosin, which has enzymatic activity in 0.5 M KCl solution (23, 16). The speed of this effect is diminished considerably at low temperatures.

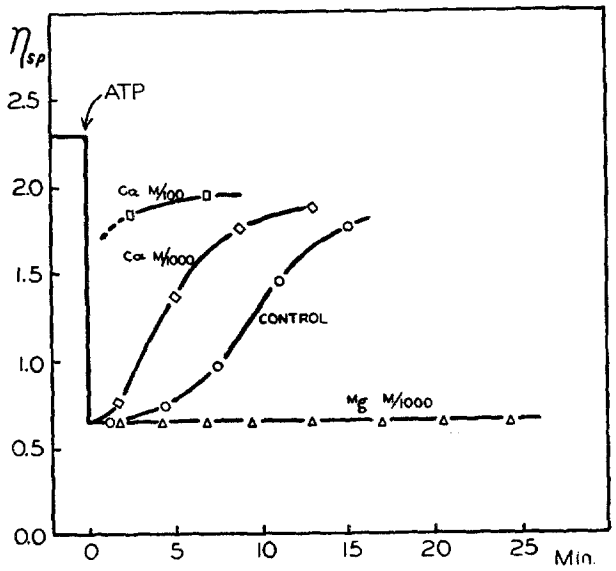

FIG. 2. Effect of ATP upon the viscosity of actomyosin solutions, as affected by Ca and Mg. Experimental data as in Fig. 1; 2.5 × 10^{-7} mol ATP added.

III

The Effect of Calcium and Magnesium

The study of these ions is indicated because of their profound influence upon the enzymatic activity of myosin-ATPase, and still more because of their effects upon contractility of muscle and of actomyosin threads. It may be remembered that enzymatic activity is promoted by Ca and inhibited by Mg (see reference 16) whereas contraction of actomyosin threads is inhibited by Ca and enhanced by Mg (see Szent-Györgyi, 22, 23).

The influence of Ca and Mg ions upon the viscosity effects is shown in Fig. 2 which refers to experiments done at 0°. It is found that, provided sufficient ATP is added, the magnitude of the initial viscosity response is entirely unaffected by the presence of these ions, but that the recovery effect is strongly accelerated by Ca and inhibited by Mg.

This could be taken as an indication that the combination between myosin and ATP which leads to the viscosity response, is not affected by these ions.

However, such a conclusion is not valid, since ATP was in excess in these experiments. A possible promotion by Mg and a possible inhibition by Ca, should reveal themselves only if the available quantity of ATP is not large enough to overcompensate inhibition. Such experiments could not be done with the technique employed in this study, due to the difficulties mentioned in section VI. However, there is already evidence that the primary reaction between ATP and myosin is indeed activated by Mg and inhibited by Ca. In fact, the position of the curve referring to a $CaCl_2$ concentration of M/100 in Fig. 2 has to be explained in this way. These problems will be dealt with in a special publication.

The influence of Ca and Mg upon the recovery effect is at least partly due to the fact that the enzymatic hydrolysis of the ATP by myosin-ATPase is inhibited by Mg and increased by Ca. Thus, in the presence of Ca, the quantity of ATP in the system will be more quickly reduced below an effective minimum needed for a full effect, whereas in the presence of Mg the opposite will occur. This is however, not the only reason. At the same subminimal ATP concentration the effect will be further decreased due to the presence of the inhibiting Ca, whereas in the presence of the promoting Mg the decrease will be entirely or partly compensated. The situation in the case of Mg is further complicated by the presence of myokinase or ADP-isomerase, which reconverts ADP into active compounds (see section IV). In this way the viscosity effect is further protracted.

IV

The Specificity of the ATP Effect

It seems reasonable to suppose that ATP influences myosin through interaction of some part of its molecule with some group of the myosin. The question arises then whether substances similar or related to ATP affect myosin in the same way. This leads to the study of inosinetriphosphate (ITP) in which the purine nucleus is changed; adenosine diphosphate (ADP) and monophosphate (AMP) which contain less phosphorus; and inorganic substances with the pyrophosphate configuration.

ADP was prepared from ATP through enzymatic dephosphorylation with crystallized myosin and purification as Ba salt. ITP was prepared from ATP through deamination with nitrous acid. Inorganic Na triphosphate was put at my disposal through the kindness of Dr. A. Deutsch, Kemiska Institutionen, Lund, Sweden. Pyrophosphate was a commercial preparation.

Adenosine diphosphate (see Fig. 3) has only a very slight influence which might be ascribed to small amounts of admixed ATP. If Mg is added, a strong viscosity response is the result. This differs, however, from the ATP effect in that it requires time to develop fully. The same result can be obtained in

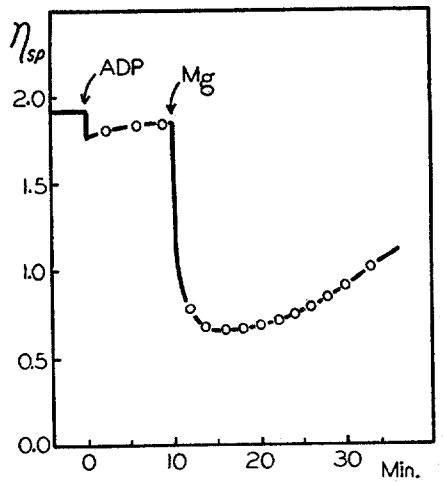

FIG. 3. Effect of ADP (approximately 10^{-6} mol) and Mg ions upon the viscosity of actomyosin solutions (12 mg. protein in 5 cc; temperature 16°).

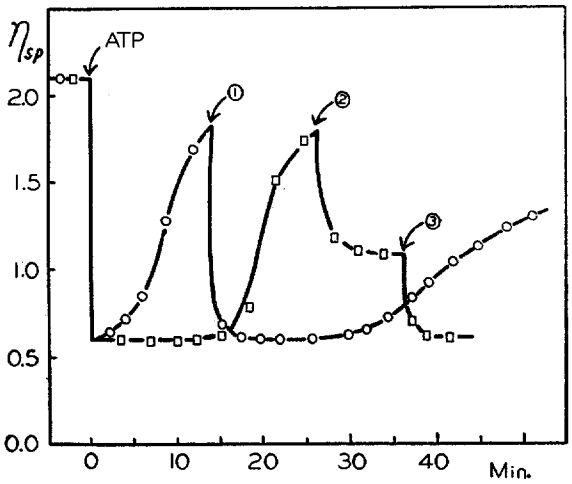

FIG. 4. Effect of addition of Mg after completion of the recovery effect, due to regeneration of ATP from ADP by myokinase. Two experiments, with addition of 5×10^{-7} mol ATP (circles) and 15×10^{-7} mol ATP (squares) (temperature 16°). At 1 and 3, addition of Mg to a concentration of 0.01 M, at 2 to a concentration of 0.001 M (12 mg. protein in 5 cc.).

a somewhat different way by first adding ATP in the absence of Mg (see Fig. 4). After the recovery effect is complete or in other words after the ATP is decomposed to ADP, Mg is added; this causes a second drop in the viscosity, but again this effect is not immediate.

The explanation of these phenomena is that actomyosin, if prepared directly from muscle, is not entirely pure but contains small amounts of an enzyme which in the presence of Mg ions causes a change in the ADP. Whether this must be interpreted as a conversion of 2 ADP into 1 ATP and 1 AMP under influence of myokinase (Kalckar, 10), or as an isomerization of ADP into a form which affects myosin as does ATP (Banga's "ADP-isomerase", 2) can be left undecided. In any case, the phenomenon is restricted to actomyosin B prepared directly from muscle. If myosin is crystallized as such, and combined with actin prepared according to Straub (19, 20), the effect does not occur (compare Szent-Györgyi, 23).

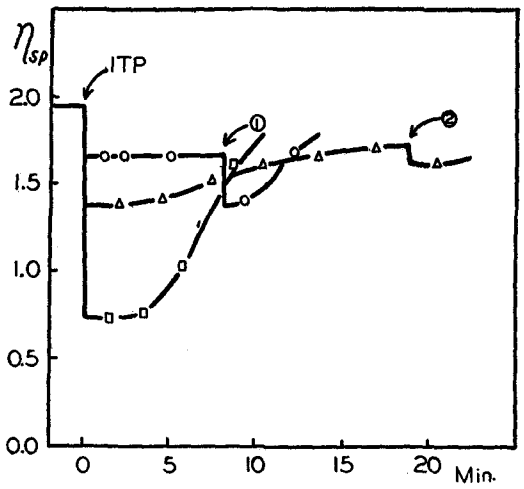

FIG. 5. Effect of ITP on the viscosity of actomyosin (14 mg. in 5 cc.). Lowest curve (squares) in the presence of 0.01 M $MgCl_2$. The other experiments first without Mg; addition of Mg to a concentration of 0.01 M at 1 and 2. Temperature 20°C.

Adenosine monophosphate (Na or K salt of muscle adenylic acid) had no effect under the conditions studied.

Inosinetriphosphate, which is hydrolyzed by myosin-ATPase (Kleinzeller, 11; Mommaerts and Seraidarian, 16) is not able to induce the viscosity response at room temperature, unless Mg is present. The enzymatic splitting of ITP does not require Mg ions. Consequently the magnitude of the effect depends on the time of addition of the Mg (Fig. 5). If added with the ITP, the effect is shown in its maximal degree. If the Mg is added after the ITP, the effect is less, due to the splitting of the ITP which has taken place in the meantime. At low temperature, however, ITP shows activity without Mg ions (Fig. 6), but in this case the reaction is sluggish. As long as the maximal effect has not been reached, it may be enhanced to its final value by Mg. ATP causes no further increase.

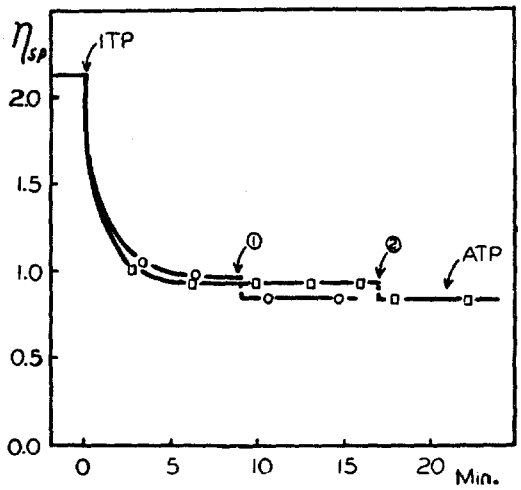

FIG. 6. Effect of ITP at low temperature (0°C.). In the beginning no Mg; addition of $MgCl_2$ to a concentration of 0.01 M at 1 and 2. Actomyosin 15 mg. in 5 cc.

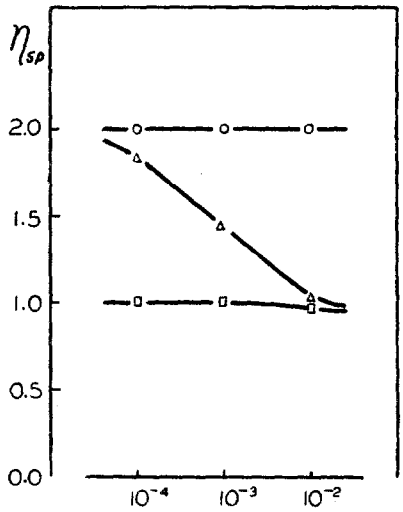

FIG. 7. Effect of inorganic pyrophosphate and Mg on the viscosity of actomyosin (30 mg. in 10 cc.). In all samples, 0.0005 M Na pyrophosphate. Upper curve (circles): before addition of Mg. Middle curve (triangles): after addition of Mg, to the final concentrations indicated on the abscissa. Temperature 17°. Lower curve (squares): the same, after cooling to 0°C.

The behavior of inorganic pyrophosphate is still more complicated. At room temperature, it may induce the viscosity response in the presence of Mg, but unless very high concentrations of Mg are present, the effect remains in-

complete. It takes time to develop fully. At low temperature, 0°C., pyrophosphate reacts more easily. The reactions are almost instantaneous, and the full effect is reached even at low concentrations of Mg. Fig. 7 summarizes the results of an experimental series, in which the viscosities of actomyosin solutions containing 0.0005 M sodium pyrophosphate were measured 30 minutes after the addition of the Mg salt. Then the solutions were cooled and the viscosities were measured again without further waiting. If the measurements at room temperature are made in less than 30 minutes, the differences between these and the results at 0° are still more pronounced. Straub (21)

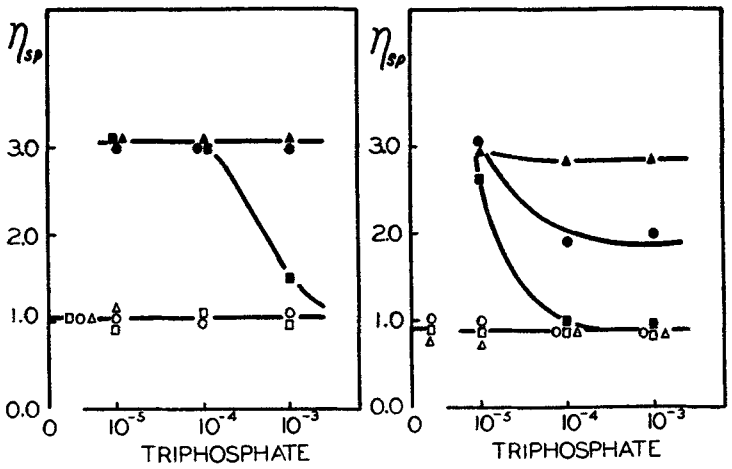

FIG. 8 *a* FIG. 8 *b*

FIG. 8, *a* and *b*. Effect of inorganic triphosphate on the viscosity of actomyosin (15 mg. in 10 cc.). Fig. 8 *a* at 18°, Fig. 8 *b* at 0°C. Solid symbols: without addition of ATP. Open symbols: after adding ATP in addition to the other substances present. Squares: in presence of $MgCl_2$, 0.01 M; triangles: with $CaCl_2$, 0.01 M; circles: without Mg or Ca.

found that pyrophosphate has an effect at low, but not at high temperatures. If his system contained some Mg, his observations fit well with the above analysis.

Since inorganic pyrophosphate is not decomposed by myosin-ATPase, the viscosity drop is not followed by any recovery effect.

The behavior of inorganic triphosphate, $Na_5P_3O_{10}$, resembles that of pyrophosphate. At room temperature (Fig. 8) the triphosphate has an effect at rather high concentration, if Mg is present. At low temperatures the effects in the presence of Mg are stronger. Sometimes an effect was found at 0° without addition of Mg, but these results were not regularly reproducible. Such reactions seemed to have complicated time relations. In all experi-

ments with Na triphosphate, long reaction times were allowed before the measurements were taken. The time course of the development of the effects with triphosphate has not yet been investigated.

V

Enzymatically Inactive Actomyosin

With regard to the questions raised in section II concerning the explanations of the nature of the ATP effect it would be of interest to know whether the effect is also possible under conditions which exclude the enzymatic activity of the myosin. The experiments in the presence of Mg are not sufficient for this purpose. An attempt was therefore made to prepare actomyosin which would be devoid of any enzymatic activity.

From the literature one would get the impression that this is an easy matter. It is reported that enzymatic activity of myosin preparations can be abolished, by precipitation at or below pH 6 (17) without denaturing the myosin, and also by oxidation with H_2O_2 (24). Notwithstanding repeated efforts this was never confirmed. Actomyosin kept its enzymatic activity even on repeated precipitation at pH 5.2, and was not inactivated by H_2O_2 in moderate quantities.

The reason for this difference is probably the total absence of heavy metals in the present experiments. If common distilled water is used for the preparative work, myosin will gradually accumulate Cu. The actual concentration of this ion in common distilled water may be very low, but if myosin is repeatedly precipitated with large amounts of water, Cu will accumulate. Indeed, Bailey (1) could demonstrate the presence of this metal in ashed myosin. As explained in the previous publication (16), heavy metal impurities were carefully excluded during the preparation by the use of water redistilled from an all glass apparatus.

It turned out that it was possible to inactivate myosin-ATPase by the manipulations mentioned above, if traces of Cu sulfate were added. This procedure was not without difficulties since excessive amounts of the metal caused denaturation. The inactivation by hydrogen peroxide was studied in some detail. It was reproducible if H_2O_2, Cu salt, and ATP were present together. After purification however, the inactivated actomyosin behaved as an actomyosin of diminished actin content. Apparently part of the actin was destroyed. If the protein was incubated with Cu salt and peroxide without ATP, the inactivation of the enzyme was very incomplete. In some experiments an attempt was made to inhibit the ATPase with *p*-chloromercuribenzoate (kindly made available to me by Dr. Leslie Hellerman of Johns Hopkins University). Here, very unexpected complications were met with, to which a special investigation will be devoted.

Finally, experiments were done to study the effect of ATP added directly in

the viscosimeter to actomyosin in the presence of Cu and H_2O_2. In one experiment at low temperature, this procedure gave clear cut results. The viscosity response was normal but the recovery effect was entirely absent. Nevertheless, this experiment was still not satisfactory for several reasons. The problem will be followed up again in the near future.

VI

The ATP Dissociation Curve of Myosin

In an earlier study (12) the stoichiometric proportions in which ATP and actomyosin react were investigated. This was done by measuring quantitatively the viscosity-lowering effect of subminimal doses of ATP. These measurements met with great experimental difficulties, since at such low ATP concentrations the recovery effect sets in immediately, and the initial response is much diminished before the first measurement is done. The problem was tentatively solved by extrapolating the viscosity-time curves towards zero time, making certain rather arbitrary assumptions concerning the form of these curves. From such studies it was concluded that myosin reacts with ATP in units with a weight of 10^5 relative to the hydrogen atom, and that ATP and myosin form a sparingly dissociated compound. Similar results were obtained by Straub (18) with actomyosin A under somewhat different conditions.

That the extrapolation procedure was inaccurate was fully realized in 1942, and this became even more obvious during the present study. An attempt was made to improve the experiments by the construction of special viscosimeters with reservoirs of only about 1 cc. and correspondingly short outflow times. However, no real improvement was arrived at in this way.

With the same method, an attempt was made to obtain an ATP-myosin dissociation curve in the presence of 0.02 M $MgCl_2$. Under these circumstances the recovery effect is retarded considerably, and the method becomes much more reliable, although certainly not yet satisfactory. The results of one experimental series are represented in Fig. 9 in the form of a dissociation curve. The abscissa gives the total concentration of ATP in the solution, the ordinate gives the magnitude of the initial viscosity response, expressed as per cent of the maximal effect; it represents at the same time the percentage of the myosin units present as ATP complex. The curve, which is a distinct improvement over the results obtained in 1942, confirms that ATP and myosin form a sparingly dissociated compound. However, the molecular weight of the unit of the molecule which combines with ATP is decidedly more than 100,000. From analysis of the results obtained a unit weight of about 360,000 was computed.

Since in the presence of Mg the recovery effect is so strongly delayed, these

newer results are certainly more reliable than the experiments in the absence of Mg. It should be realized however, that with Mg the situation is not the same as in the absence of this ion.

Further studies with more rapid techniques will be devoted to the analysis of these questions. A quantitative discussion of the results will also be postponed.

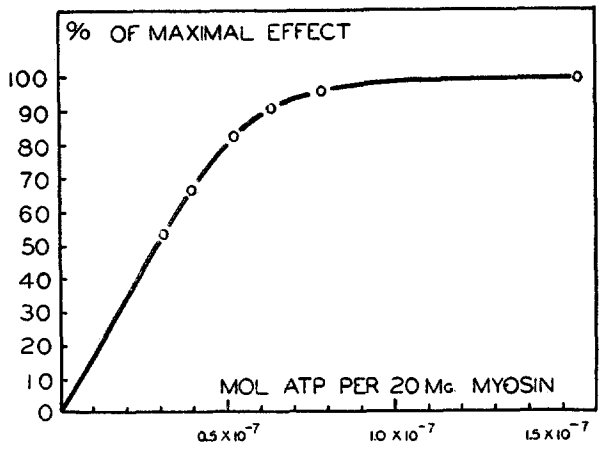

FIG. 9. Actomyosin-ATP dissociation curve in the presence of 0.02 M $MgCl_2$. Explanation in the text.

VII

DISCUSSION OF THE RESULTS

The main phenomenon studied in this investigation was the decrease of the viscosity which actomyosin solutions undergo after addition of ATP. As described above, these effects have a typical time course, in which one has to distinguish the immediate drop in viscosity, here called viscosity response, and its subsequent reversal here called recovery effect. The latter effect was ascribed to continuously proceeding enzymatic decomposition of the ATP.

It should be said that two fundamentally different explanations of these processes seem possible. On the one hand, it may be supposed that the viscosity response is due to a combination between myosin and ATP, and that the recovery effect is due to a disappearance of the ATP through the action of ATPase. But it is also conceivable that the viscosity response is caused by a continuous transfer of reaction energy from the splitting of ATP towards the myosin. The recovery effect means then that, as the ATP concentration drops, gradually less and less myosin molecules are involved in the reaction. For the first type of explanation it is immaterial whether myosin itself is the ATP-hydrolyzing enzyme or not. The second explanation is intelligible only

if myosin and ATPase are identical. This second interpretation is not in disagreement with the type of ATP-myosin dissociation curve discussed in section VI, since this curve can very well be looked upon as an enzyme-substrate saturation curve.

The current ideas concerning the rôle of ATP and myosin-ATPase, although not developed in detail, belong to the second group of interpretations. They all assume that the enzymatic hydrolysis of ATP by myosin is the cause of muscular activity, and that in this process the free energy liberated in the dephosphorylation of ATP is transferred to the enzymatically active contractile structure. On the other hand, this type of explanation is in disagreement with the results of the investigation of Mommaerts and Seraidarian (16), according to which the enzymatic activity of myosin-ATPase *in vivo* cannot account for the liberation of inorganic phosphate during muscular activity.

The present study leads to a direct conclusion regarding the effect of ATP upon myosin through several equivocal arguments.

First, the effect of bivalent inorganic cations will be discussed. The enzymatic activity of myosin-ATPase is activated by Ca, and under almost all conditions inhibited by Mg. As shown in this paper, the viscosity response of actomyosin to which ATP is added is not inhibited, probably promoted by Mg, and is not promoted, probably inhibited by Ca. According to Szent-Györgyi (22, 23), the contraction of actomyosin threads is enhanced by Mg and inhibited by Ca. It is seen therefore that enzymatic splitting of ATP by myosin-ATPase, and the effect of ATP upon the physical behavior of myosin, are influenced by Mg and Ca in exactly the opposite way, a fact certainly not to be expected if the first of these two processes is the immediate cause of the second one.

Further arguments arise from the study of the effects of different substances related to ATP. Inosinetriphosphate is hydrolyzed by myosin-ATPase as readily as ATP, and the free energy effect of this reaction should be about the same as that of the splitting of ATP. Nevertheless the effect of ITP upon actomyosin is very much less pronounced than that of ATP, and is much more dependent on special conditions. Inorganic pyrophosphate and triphosphate, which are not hydrolyzed, affect actomyosin as ATP does, but at higher concentration and under more specialized conditions.

Finally the experiments described in section V show that after inactivation of the ATPase the viscosity response upon addition of ATP takes place undiminished. As shown by the absence of the recovery effect the enzymatic activity was destroyed completely.

These three independent groups of evidence allow us to conclude that the effect of ATP upon the physical behavior of myosin is entirely independent of the ATPase activity. The formation of a myosin-ATP compound through a topochemical reaction takes place as a result of which certain molecular proper-

ties of the myosin are changed. Whether myosin and ATPase are identical, or are two different proteins remains an important problem but has partly lost its significance with respect to the question of the energy transfer.

From these considerations it is not clear which rôle actin has. In the present experiments, actomyosin was used because under the circumstances studied myosin gives an observable effect only if it is combined with actin. Szent-Györgyi (23) seems to take the standpoint that changes in actomyosin are reflections of changes in the myosin moiety. According to him actin has no "active" rôle, it serves merely to keep the myosin in a proper condition. This deserves further study.

The physiological implications of the present results do not yet seem ripe for discussion. It has been shown that by combination with myosin, without hydrolysis, ATP induces physical changes in the myosin. According to the experiments with actomyosin threads such physical effects may reveal themselves as a contraction, if the conditions of the system are suitable. One arrives at the conclusion that the primary process consists of a combination between ATP and myosin; what follows this primary process depends on the conditions.

The applicability of the results of these studies on myosin solutions to the physiological process of contraction is confirmed by the studies of Buchthal *et al.* (5–7) concerning the contractions evoked by ATP and other substances in intact muscle fibers. The gap between Buchthal's work and the present investigation with actomyosin solutions is bridged by the studies of Szent-Györgyi (23) on frozen and water-extracted muscle slices, and on actomyosin threads. It should be remembered that according to Caspersson and Thorell (8) in the resting muscle the ATP is not present in the contractile A bands.

Further consideration of the mechanism of contraction at this moment, when so many fundamental aspects of the process have not yet been sufficiently investigated, seems untimely.

SUMMARY

1. A study is made of the effect of adenosine triphosphate (ATP) upon the viscosity of solutions of actomyosin in 0.5 M KCl.

2. The observed effects are discussed in terms of an initial drop of the viscosity (viscosity response) and its subsequent slow reversal (recovery effect). The latter is ascribed to a decrease in the ATP concentration through enzymatic hydrolysis.

3. The recovery effect is inhibited by Mg, activated by Ca, in accordance with the effect of these ions on the activity of myosin-ATPase.

4. The viscosity response is not inhibited, probably promoted by Mg. It is not promoted, probably inhibited by Ca.

5. The viscosity response is induced not only by ATP, but to a certain ex-

tent also by inosinetriphosphate, inorganic triphosphate, and inorganic pyrophosphate, not by adenosine diphosphate or monophosphate.

6. The viscosity response could be obtained with enzymatically inactive myosin.

7. It is concluded that the effect of ATP upon myosin does not depend on its enzymatic hydrolysis.

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