REACTIONS OF INOSINE- AND ADENOSINETRIPHOSPHATES WITH ACTOMYOSIN AND MYOSIN

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Ever since the presence of adenosinetriphosphate (ATP) in muscle was recognized by Lohmann (1), many muscular phenomena have been associated with it. Myosin threads (myosin B) prepared according to the method of Weber (2) and the glycerol-treated muscle fibers of Szent-Györgyi (3) contract on addition of ATP (4). This nucleotide also causes a reduction in viscosity of actomyosin solutions in 0.5 m KCl (5) and a contractile precipitation, termed superprecipitation by Szent-Györgyi, of actomyosin solutions in 0.15 m KCl.

Besides the adenosine nucleotides, the inosine nucleotides were also found in frog muscle by Lohmann (6). Needham et al. (5) state that inosinetriphosphate (ITP) is the only one of many phosphates and phosphorylated compounds, other than ATP, which reduces apparent specific viscosity and flow birefringence of myosin (myosin B). Kleinzeller (7) found that myosin splits ITP to inosinediphosphate (IDP) and phosphate 3 times faster than it splits ATP at pH 7.2. The possibility that inosine nucleotides play a role in reactions in actin and actomyosin in vitro is stressed by the finding that inosine nucleotides are prominent in actin (8). These considerations led us to investigate the effects of ITP on the phenomena caused by ATP in the various preparations from skeletal muscle. The results obtained prompted further investigation of the inosinetriphosphatase (ITPase) compared with the adenosinetriphosphatase (ATPase) activity of myosin and actomyosin.

Materials and Methods

Inosinetriphosphate was prepared from commercial ATP\(^1\) by the procedure given by Kleinzeller (7). Tests indicated that the amount of phosphate liberated from the ITP by incubation with myosin or by 10 minutes hydrolysis in molar HCl gave calculated values of ITP which were consistent with those derived from the \(D_{260} \text{ mu}\), by use of Kalekar’s (9) value for the absorption coefficient of inosine at 250 \(\text{mu}\). All experiments were done with the sodium, or potassium salt, of ITP or of ATP.

\(^1\) Purchased from the Nutritional Biochemicals Corporation and the Sigma Chemical Company.
Myosin A was prepared according to Szent-Györgyi (4) with further purification by addition of ATP to remove actomyosin according to a recently modified procedure (10).

Actin was prepared by the most recently recommended method of Feuer and coworkers (11). Glycerol-treated fibers were prepared from rabbit psoas muscle according to Szent-Györgyi (3). Myosin B was prepared by extraction of ground rabbit muscle with Weber's solution (2). Aqueous muscle extract was prepared by extracting autolyzed, ground, rabbit muscle with 2 volumes of cold water. This was used without further dilution. Myokinase was prepared according to Kalckar's method (12). The water used in all experiments was distilled from glass vessels.

Superprecipitation of actomyosin was carried out in Wassermann tubes partially immersed at 25° ± 1° in a water bath with flat glass sides. Actomyosin was prepared in the Wassermann tubes by adding 1 mg. of myosin in 0.1 ml. of 0.6 M KCl, 1.7 ml. of 0.1 M KCl, and 0.6 to 1.1 mg. of F-actin in about 0.1 ml. of 0.1 M KCl in the order given. Then the test substance (ATP or ITP) was added, and the tube was inverted once and returned to the rack for observation.

EXPERIMENTAL

Superprecipitation by ATP—When 0.5 mg. of sodium ATP in about 0.1 ml. of solution is added to an actomyosin preparation as described above, there appears in 5 to 20 seconds a heavy precipitate which contracts within 30 to 60 seconds into a small plug. The ratio of the dimensions of this plug remains the ratio of the dimensions of the solution, but the volume of the plug is reduced to about one-eighth that of the solution.

Superprecipitation by ITP—As has been indicated in an earlier report (13), the addition of ITP instead of ATP causes no superprecipitation, but when muscle extract or even ashed muscle juice is added with the ITP, superprecipitation does occur. The activity of the ash is probably due to Mg, for magnesium chloride in a concentration of 0.001 M with 1.2 mg. of ITP caused superprecipitation equal in magnitude to that caused by ATP (Fig. 1). The amount of Mg required is in the range of 1 mole per mole of ITP, or 1000 moles per mole of myosin (molecular weight, 1 X 10⁶). At higher concentrations of Mg (around 0.03 M) superprecipitation is slower and at lower concentrations (around 0.0002 M) is incomplete.

Calcium chloride in concentrations between 0.02 and 0.001 M with ITP was entirely ineffective.

Mode of Action of ITP—Although spectrophotometric examination failed to indicate contamination of the ITP with adenine, the possibility existed that the ITP was contaminated with adenosinediphosphate (ADP) and the actomyosin with myokinase. The Mg required for superprecipitation
by ITP might then serve to activate a myokinase reaction. As is shown in Fig. 2, ADP and myokinase induce excellent superprecipitation without added Mg, but ADP is ineffective alone. Moreover, ITP added to an actomyosin-salt solution with myokinase failed to cause superprecipitation.

2 This system provides a means for the qualitative or perhaps semiquantitative determination of myokinase activity in tissue extracts.
Hence, the effectiveness of ITP and Mg was not due to contamination of actomyosin with myokinase or of ITP with ADP.

The contraction of actomyosin following the addition of ADP and myokinase was always slower than with ATP but, when finished, appeared to be more complete. It was also apparent in these experiments that ADP is not significantly altered by the actomyosin, for, if myokinase was added an hour or more after the ADP, superprecipitation occurred as usual.

The possibility that the mode of action of ITP is through amination to ATP was tested by utilizing Kleinzeller's finding that myokinase does not cause dismutation of IDP (7). If ATP is formed, the nucleotide in the superprecipitation tests would be converted to ADP. The addition of myokinase would then have two effects: (1) liberation of a second mole of inorganic phosphate; (2) deamination of the adenylic acid with a shift in the absorption maximum toward that of inosine. To investigate amination as a mode of action, superprecipitation was carried out in each of four tubes: the first pair (Tubes 1 and 2) received ATP and the other pair (Tubes 3 and 4) ITP plus Mg. 15 minutes after the precipitate had formed, myokinase was added to Tubes 2 and 4. The reactions were stopped in all tubes 15 minutes later by addition of perchloric acid to a concentration of 2 per cent. Table I shows that the effects anticipated for myokinase when ADP is formed occurred only in the ATP-treated tube; i.e., (1) the inorganic phosphate increased and (2) the absorption maximum shifted toward the maximum for inosinic acid. The fact that the absorption in the tube receiving only ITP and Mg was that of inosine also indicates that amination did not occur. It is evident ATP was not formed from ITP at any time in the superprecipitation reaction.

Table I

<table>
<thead>
<tr>
<th></th>
<th>ATP</th>
<th>ITP + Mg</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Tube 1, ATP</td>
<td>Tube 2, ATP + myokinase</td>
</tr>
<tr>
<td>P, µM per ml.</td>
<td>0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Wave-length, maximum optical density, µμ</td>
<td>258</td>
<td>250</td>
</tr>
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</table>

Role of Mg in Superprecipitation with ATP—Superprecipitation by ATP in the presence of 0.005 M MgCl₂ was delayed about 20 minutes and the
precipitate contracted incompletely and slowly. After 60 minutes the final change in volume of the precipitate was less than half the usual change.

Since Mg might be required in trace amounts for superprecipitation by ATP, an attempt was made to ascertain the amount of Mg available in these reaction mixtures. Since actin is the only source of Mg in the reaction mixtures, the protein was precipitated from 5 ml. of G-actin and the Mg content of the supernatant was estimated by the 8-hydroxyquinoline method (14). The results show that actins which contain as little as 0.1 \( \gamma \) of Mg per ml. of solution (5 to 6 mg., dry weight, per ml.) are quite suitable for superprecipitation even though the concentration of Mg in the reaction mixture did not exceed \( 4 \times 10^{-7} \) M.

**Comparison of Superprecipitation by ITP and ATP**—Superprecipitation of actomyosin by ITP and Mg requires approximately twice the quantity of ITP that is required of ATP, the smallest effective amount in these tests being 0.9 and 0.5 mg., respectively. When the quantity of ITP was reduced below 0.9 mg., the magnitude of the contraction was also reduced. The different requirement for ITP and ATP may be related to the greater affinity of actomyosin for ATP, as is described subsequently.

Superprecipitation by ITP plus Mg takes about 4 times longer to occur than superprecipitation by ATP. It seemed possible that the additional time with ITP was due to failure to carry out the test at optimal pH. However, such is not the case, for a plug did not form in less than 2 minutes with ITP at any pH value between 5.9 and 6.9. The optimal pH for contraction of the ATP-induced precipitate lies between 6.5 and 6.9, whereas the optimum for contraction with ITP is at pH 6.2 to 6.4.

**Observations on Other Forms of Actomyosin**—ATP added to glycerol-treated muscle fibers causes the fibers to contract drastically (3). Fibers prepared for these experiments shortened from 5.0 to 1.6 cm. When ITP was added, only slight or no shortening occurred, but when the ITP was followed by Mg (final concentration 0.02 M), the fibers contracted to the same extent as with ATP. The Mg has the same effect if added either before or after ITP.

Buchthal and coworkers (15) state that ITP, unlike ATP (4), does not cause contraction of myosin B threads, but does enhance the effect of subsequently applied ATP. In the present studies threads made by extruding myosin B in 0.6 M KCl into 0.04 M KCl from a capillary tip were measured in a depression slide on a microscope stage. ATP caused contraction to 20 per cent of the initial length of the threads. ITP alone caused no shortening, but when ITP was accompanied by Mg, the threads shortened to 25 to 50 per cent of the initial length.

We have found in agreement with Needham et al. (5) that ITP without
Mg reduces flow birefringence, but only this qualitative observation has been made.

The effect of both ITP and ATP on the viscosity of actomyosin in solution has been studied. The experiments were done with 6.6 mg. of actin and 10 mg. of myosin dissolved in 8.0 ml. of 0.5 M KCl buffered to pH 6.9 with barbital acetate. Mg is required for a reduction in viscosity equal to that following the addition of ATP (Fig. 3). The more rapid recovery of $\eta_{sp}$ after ITP with Mg than following ATP with or without Mg is associated with a more rapid rate of phosphorylysis (Table II).

Enzymatic Phosphorylysis of ATP and ITP—Myosin and actomyosin are well known for ATPase and ITPase activity (7). Possibly the hydrolysis of ATP or ITP is related to superprecipitation. Mg is known to inhibit the ATPase activity of myosin (4, 16) and has been found to inhibit superprecipitation of actomyosin with ATP. Since Mg is required for superprecipitation by ITP, it was thought of interest to ascertain the effect of Mg on ITPase activity.

The enzymatic activity was determined by measuring the increase of

![Figure 3. The effect of ITP and ATP, with and without added Mg, on the apparent specific viscosity ($\eta_{sp}$) of synthetic actomyosin. The viscosities were determined with a Cannon-Fenske viscometer. Curve 1, 1.0 mg. of ITP and $6 \times 10^{-3}$ M MgCl$_2$ added; Curve 2, 1.0 mg. of ITP and $4.5 \times 10^{-3}$ M MgCl$_2$ added; Curve 3, 1.0 mg. of ITP and no added MgCl$_2$; Curve 4, 0.5 mg. of ATP and $6 \times 10^{-3}$ M MgCl$_2$ added; Curve 5, 0.5 mg. of ATP and no added MgCl$_2$. A, $\eta_{sp}$ before MgCl$_2$; B, $\eta_{sp}$ after MgCl$_2$; C, $\eta_{sp}$ after ITP or ATP.](http://www.jbc.org/)

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inorganic phosphate during a 3 to 5 minute incubation of myosin and nucleotide at 38°. The Fiske-Subbarow method for inorganic phosphate was used. The tests were made in the range in which hydrolysis is proportional to time and to amount of enzyme, a minimal amount of myosin being added to 2 ml. of solution containing 1 to 2 \( \mu \text{M} \) of ATP or ITP as potassium or sodium salt, and 0.06 M histidine buffer of the desired pH. The reaction was stopped by adding trichloroacetic acid to a concentration of 4 per cent. The results are expressed as \( Q_p \), which represents the microliters of a hypothetical gas (\( \text{H}_3\text{PO}_4 \)) equivalent to the micrograms of P liberated per mg. of protein per hour (17). Protein concentration was determined by the Kjeldahl procedure. Experiments were run at the high KCl concentration (0.55 M), at which optimal activity is said to occur, and at the low concentration (0.15 M), at which superprecipitation occurs. The 0.06 M histidine buffer, through more effective binding of heavy metals or some unknown mechanism, gave on the average 30 per cent greater

### Table II

<table>
<thead>
<tr>
<th></th>
<th>ATP</th>
<th>ITP</th>
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<tbody>
<tr>
<td>No Mg</td>
<td>420</td>
<td>110</td>
</tr>
<tr>
<td>0.005 M Mg</td>
<td>170</td>
<td>830</td>
</tr>
</tbody>
</table>

rates than 0.1 M glycine buffer. Actomyosin was prepared by adding actin to myosin in slight excess of the amount required to give the stoichiometric proportions (10).

Whereas Mg inhibits the ATPase activity of actomyosin, it stimulates the ITPase activity under conditions in which superprecipitation occurs (Table II).

In the absence of Ca or Mg the rate of phosphorylysis is greater for ATP than for ITP; however, according to Kleinzeller, ITP is split more rapidly than ATP with Ca present. In consequence of these differences an extended study of the hydrolysis of ATP and ITP by myosin and actomyosin was undertaken. As is shown in Fig. 4, ITP is split more rapidly than ATP, with Ca added, at all pH values from 6 to 9; but the effect of pH on the rate of substrate splitting by myosin differs for ATP and ITP. The latter lacks the area of depressed activity around pH 7 which characterizes the hydrolysis of ATP (18, 19). The ratio of the rates of
ITP to ATP splitting thus varies with pH, being greatest at pH 7.5. This difference between the substrates may serve to explain the anomalous behavior of myosin in having two pH optima of enzymatic activity (18, 19). It seems likely that only the maximum at pH 9 represents the range of optimal activity for the protein. The greater activity at around pH 6, which appears to be an increase in enzymatic activity, may actually be the result of increased availability of ATP. In any event ATP, with \(-\text{NH}_2\) and \(-\text{OH}\) dissociable groups, unlike ITP with only the \(-\text{OH}\) groups, shows a region of low activity between pH 6.5 and 8. Possibly an isoelectric point around neutrality results in altered affinity of ATP for myosin, with a resultant decrease in rate of phosphorylysis. It has not been possible to determine the effect of pH on the affinity of myosin for ATP, for the Michaelis constant is below the range of accuracy of the method; i.e., less than 0.05 mM per liter.

Table III summarizes several experiments in which the effect of Mg and Ca on the enzymatic activity of myosin and actomyosin was studied at three significant pH values. The results presented are averages of two to five determinations on a single batch of myosin and actin.

Mg antagonizes the Ca activation of the enzyme, causing about 70 per cent inhibition of the rapid ITPase and 90 per cent inhibition of the relatively slower ATPase activity. From a comparison of Tables II and

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**Fig. 4.** Effect of pH on ATPase and ITPase activity of myosin. The solutions contained, in addition to myosin and excess substrate, 0.06 M histidine buffer brought to appropriate pH with KOH, KCl to make a final K concentration of 0.15 M, and 0.01 M CaCl$_2$. When $Q_p$ values greater than 4000 were anticipated, 0.02 mg. of protein was used; otherwise 0.04 mg. was used.
III, it appears that with Mg in the system the rate is the same whether Ca is added or not.

The observed Mg inhibition of synthetic actomyosin is in accord with the results of Mommaerts and Seraidarian (19), who obtained inhibition of actomyosin by Mg in the presence of Ca at pH 7. However, the data reported here are not in agreement with the results of Szent-Györgyi (4) on natural actomyosin or those of Kielley and Meyerhof (20) on synthetic actomyosin in which Mg by itself was stimulatory. In experiments to be reported in detail elsewhere, it has been found that Mg activates synthetic actomyosin in the absence of Ca at pH 7.7 (but not at pH 6 and 9) at K concentrations less than 0.03 M. Mg neither inhibits nor activates natural actomyosin at pH 7.1 at K concentrations as high as 0.12 M.

Certain other points of interest are evident in Table III. The phosphorylytic activity in our tests was found to be greater at the low than at the high salt concentration. Actomyosin prepared from this batch of myosin repeatedly gave higher ITPase values than the myosin alone; however, other preparations of the proteins have failed to show this difference. It is known that Ca accelerates the ATPase activity of myosin (21, 22). Tables II and III show that Ca produces a great increase of ITPase as well as ATPase activity.

The more rapid hydrolysis of ITP than of ATP with Ca present is associated with a lesser affinity of ITP for the enzyme. Thus, the rate of inorganic phosphate liberation in a solution containing ATP and ITP equally activates natural actomyosin at pH 7.1 at K concentrations as high as 0.12 M.

### Table III

**Effect of Mg on Calcium-Activated Hydrolysis of ATP and ITP by Myosin and Actomyosin**

The values are expressed as $Q_p$. The tests were made where the rate of hydrolysis was linear, with 0.02 to 0.05 mg. of myosin, depending on the anticipated rate, and 1 to 2 $\mu$M of ATP or ITP in 2 ml. of 0.06 M histidine buffer. The concentration was 0.01 M for CaCl$_2$ and 0.005 M for MgCl$_2$.

<table>
<thead>
<tr>
<th>pH</th>
<th>ATP</th>
<th>ITP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.15 M KCl</td>
<td>0.55 M KCl</td>
</tr>
<tr>
<td></td>
<td>Ca</td>
<td>Ca + Mg</td>
</tr>
<tr>
<td>Myosin</td>
<td>6.0</td>
<td>1820</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>980</td>
</tr>
<tr>
<td></td>
<td>9.2</td>
<td>4740</td>
</tr>
<tr>
<td>Actomyosin</td>
<td>6.0</td>
<td>1530</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>1020</td>
</tr>
<tr>
<td></td>
<td>9.2</td>
<td>5210</td>
</tr>
</tbody>
</table>
INOSINE- AND ADENOSINETRIPHOSPHATES

in excess is that of the ATP (Table IV). The affinity is also greater for ATP than for ITP in the presence of Mg, with or without added Ca. These results indicate also that a single enzyme reacts with both substrates.

Since Mg activates the enzymatic cleavage of ITP by actomyosin and Mg is required for the physiological reactions of the muscle proteins with ITP, it appears that this cation is needed for the nucleotide to combine with actomyosin. Certainly hydrolysis of high energy phosphate, such as occurs in the presence of Ca, is not alone sufficient for superprecipitation to occur, but other conditions, satisfied for ITP by Mg, must be met. A possible mechanism for the Mg effect may be that a Mg-ITP complex is formed, perhaps through substitution for the missing NH₂ group of ATP, which possesses the proper affinity to actomyosin for superprecipitation to occur.

TABLE IV

Hydrolysis of Mixture of ATP and ITP

The tests contained 0.15 M KCl, 0.06 M histidine, pH 9.2, actomyosin, and excess of ATP and ITP in equal concentration. The concentration was 0.01 M for CaCl₂ and 0.005 M for MgCl₂. The results are expressed as Q₁₂.

<table>
<thead>
<tr>
<th>Addition</th>
<th>ITP</th>
<th>ATP + ITP</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>6830</td>
<td>2930</td>
<td>3150</td>
</tr>
<tr>
<td>&quot; + Mg</td>
<td>1240</td>
<td>360</td>
<td>240</td>
</tr>
<tr>
<td>Mg</td>
<td>1030</td>
<td>50</td>
<td>50</td>
</tr>
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</table>

The requirement of a 1:1 molar ratio of ITP and Mg is in harmony with the thesis of complex formation.

SUMMARY

1. Inosinotriphosphate causes superprecipitation of actomyosin if magnesium is added in a quantity approximately equimolar with the nucleotide. ITP with Mg causes contraction of myosin B threads and glycerol-treated muscle fibers. Formerly adenosinotriphosphate was the only compound known to cause these phenomena. Mg is required for ITP to cause a reduction in viscosity of actomyosin equal to that caused by ATP.

2. If Mg is required for superprecipitation by ATP, the amount is 2000 times less than that required by ITP.

3. Amination of ITP to ATP is eliminated as the mode of action of ITP.

4. At the salt concentration and pH producing superprecipitation, Mg activates the ITPase and inhibits the ATPase activity of actomyosin. The Ca activation of the hydrolysis of both ITP and ATP is inhibited by
Mg. The ITPase activity of actomyosin with Ca present is optimum at but one pH, in contrast to the two optima for ATPase activity.

5. Actomyosin has a greater affinity for ATP than for ITP.

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