A STUDY OF THE EFFECTS OF ETHYLENEDIAMINE-
TETRAACETIC ACID ON MYOSIN
ADENOSINETRIPHOSPHATASE

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The tri- or tetrasodium salt of ethylenediaminetetraacetic acid (EDTA
or Na EDTA) is a strong chelating agent for heavy metals and the alkaline
earth metals, calcium and magnesium. By this action the activity of en-
zymes which are known to be influenced by calcium or magnesium is altered
by the addition of EDTA; e.g., muscle adenylate kinase, which is accel-
erated by magnesium, is completely inactivated by 0.001 M EDTA (1).
Slater and Cleland stabilized α-ketoglutaric oxidase of heart muscle sarco-
somes by additions of EDTA, an action which they attribute to the removal
of deleterious calcium which enters sarcosome suspensions (2). Altman
and Crook found that horse heart succinoxidase is greatly activated by
EDTA, an activation which can also be produced by phosphate and chro-
mate or by purifying the substrate and enzyme (3). Gross, working at low
ionic strength, found that the adenosinetriphosphatase (ATPase) activity
of mouse heart homogenate was strongly inhibited by EDTA at 0.004 to
0.01 M, but at 0.001 to 0.003 M the activity was 150 per cent that of un-
treated homogenates (4). Using myosin B as ATPase, we found that the
rate of dephosphorylation was decreased by EDTA at 0.05 M KCl (5). On
the other hand, Friess observed that EDTA accelerates the ATPase ac-
tivity of myosin B 400 per cent at 0.6 M KCl and pH 7 (6). It has now
been confirmed by Friess and us that these opposite effects of EDTA were
dependent upon the concentration of KCl.

Since this corroboration, we have explored the effect of triNa EDTA
upon the ATPase activity of myosin A and myosin B over a wide range of
concentrations of KCl and have done the other experiments indicated by
the section titles in response to queries which arise from consideration of the
marked acceleration of myosin ATPase by EDTA.

EXPERIMENTAL

Myosin B (natural actomyosin) was prepared from rabbit muscle by
extraction for 24 hours in Weber's solution and purified by three successive

1 In unpublished experiments we also found that EDTA completely inhibits mus-
cle adenylate kinase. In addition, we found that the activity can be completely
restored by passing the enzyme-EDTA mixture over Dowex 1.
precipitations and dissolutions in 0.05 and 0.6 M KCl, respectively. Myosin A (crystalline myosin) was prepared according to Kessler and Spicer (7) and actin according to Feuer et al. (8). The adenosinetriphosphate (ATP) was a commercial product (Sigma). The KCl used was an analyzed salt of reagent grade and, except for one instance, was not further purified.

The chelating agents were supplied by the Bersworth Chemical Company. The ethylenediaminetetraacetic acid was supplied as disodium Versenate which was suspended in water and dissolved by adding NaOH. At pH 9 EDTA is dissolved and is chiefly the trisodium salt.

Dephosphorylation of ATP was done in reaction mixtures composed of the following proportions: 2.5 ml. of 0.02 M Na Veronal-acetate (pH 8) containing KCl of such concentrations as to yield the desired final concentration, 1.5 μmoles of Na ATP added in 0.1 ml. of solution, 0.3 ml. of trisodium EDTA or other chelating agent of such concentration as to yield concentrations ranging from $10^{-5}$ to $2 \times 10^{-2}$ M, and 0.1 ml. of myosin containing 1 to 5 mg. per ml. In control reaction mixtures, water was substituted for the solutions of chelating agent.

To measure quantitative aspects of the acceleration caused by EDTA (e.g. Figs. 1 and 2), the reaction mixtures were made in volumes and amounts 3 times those given above. From such a volume of reaction mixture a blank and samples after both 5 and 10 minutes of incubation were withdrawn for analysis. The latter procedure permitted examination of the data for maintenance of constant rates of reaction and, from the values obtained, rates of dephosphorylation in the linear phase were selected for presentation.

For some purposes (e.g. Table I) only the qualitative effect of EDTA was ascertained by measuring the orthophosphate liberated in 10 minutes in a

<table>
<thead>
<tr>
<th>Concentration of EDTA</th>
<th>0.02 M KCl</th>
<th>0.07 M KCl</th>
<th>0.12 M KCl</th>
<th>0.17 M KCl</th>
<th>0.24 M KCl</th>
<th>0.62 M KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-5}$</td>
<td>0.02</td>
<td>0.9</td>
<td>1.4</td>
<td>1.9</td>
<td>2.9</td>
<td>1.4</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>0.01</td>
<td>0.7</td>
<td>1.5</td>
<td>1.7</td>
<td>2.4</td>
<td>1.2</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>0.02</td>
<td>0.7</td>
<td>2.2</td>
<td>4.2</td>
<td>9.3</td>
<td>8.0</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>0.05</td>
<td>0.7</td>
<td>1.9</td>
<td>4.0</td>
<td>11.0</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.5</td>
<td>1.3</td>
<td>2.6</td>
<td>6.7</td>
<td>12.2</td>
</tr>
</tbody>
</table>

Uramildiacetic acid was kindly supplied by Dr. Martin Rubin.
single volume containing the quantities listed above. From the values obtained, appropriate blanks were subtracted.

In all experiments the reactions were stopped by the addition of trichloroacetic acid, the mixtures centrifuged to remove the denatured protein, and 2 ml. of the supernatant solution analyzed for orthophosphate by the Fiske-Subbarow method.

Results

Effect of TriNa EDTA on Myosin B As ATPase and Its Association with KCl—The rate of dephosphorylation of ATP by myosin B at pH 8.0 in the presence of varying concentrations of EDTA and at several concentrations of KCl is shown in Fig. 1.
It will be noted that both the accelerating and inhibiting effects of EDTA are intimately associated with the concentration of KCl. At all concentrations of KCl, $10^{-5}$ M EDTA had little or no effect on myosin B, except at $0.02$ M KCl, at which $10^{-5}$ M EDTA caused a rate of splitting above that obtained when no EDTA was included in the reaction mixture. This enhancement is small, but results of experiments not presented show that it is probably part of the association of the EDTA effect with the concentration of KCl.

Myosin A with and without Actin—Experiments similar to those done on the ATPase activity of myosin B were also carried out at pH 8.0 on myosin A. The accelerating effect of EDTA on the ATPase of myosin A (Table I) was of the same general nature as that on the ATPase of myosin B, except

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$^3$ At a recent meeting Dr. John Gergely reported that EDTA accelerates the ATPase activity of both trypsin- and chymotrypsin-digested myosin A. The increases are of about the same magnitude as those occurring with myosin A.
that acceleration occurred at lower concentrations of KCl with myosin A than with myosin B, and at 0.6 M KCl acceleration was greater with myosin A.

The addition of actin to myosin A decreases the accelerative effect of EDTA upon myosin A ATPase (Fig. 2). When the amount of ATP dephosphorylated is calculated per mg. of total protein (myosin plus actin), the ATPase activity is reduced to about the activity of myosin B.4

Influence of pH on EDTA Effect—The effect of pH on the EDTA-KCl action was studied with synthetic actomyosin as ATPase. The results (Fig. 3) show that the maximal EDTA-KCl effect occurs at pH 8 when the concentration of KCl is 0.6 M and that 10^{-3} M EDTA is not accelerative at low concentrations of KCl when the pH value is other than 8.0. The inhibition by EDTA at low concentrations of KCl and pH 8 (Fig. 1) is not overcome by changing the pH of the reaction mixture.

The effect of pH on the increase of ATPase of myosin B was also studied with other chelating agents. Of those studied (see below), the four which have an action comparable to EDTA are more effective at pH 9 than either pH 6, 7, or 8.

Effect of Mg EDTA, Ca EDTA, and K EDTA on Myosin B As ATPase—The effects of magnesium disodium EDTA (Mg EDTA) and calcium disodium EDTA (Ca EDTA) were tested at concentrations of KCl which resulted in inhibition and acceleration in the presence of Na EDTA. In

4 The reduction of dephosphorylation caused by adding actin to myosin A also occurs at high KCl concentration (0.3 M) when no EDTA is present, but at low KCl we found, as did Hasselbach (9), that actin enhances the ATPase activity of myosin A many fold.
several experiments it was found that either Mg EDTA nor Ca EDTA (Table II) accelerates myosin B ATPase activity at 0.6 M KCl as does Na EDTA. Only Mg EDTA inhibits activity at low concentrations of KCl. Ca EDTA at low concentrations of KCl has no effect on activity. This failure of Ca EDTA to inhibit ATPase indicates that the inhibition caused by Mg EDTA and Na EDTA is due to the inactivation of calcium in the reaction mixtures.

The accelerating effects of $10^{-4}$ and $10^{-3}$ M K EDTA upon myosin B ATPase are identical to those of Na EDTA. Both require high concentration (0.3 to 0.6 M) of KCl to produce enhancement of the activity. This result is significant in that the substitution of K EDTA for Na EDTA does not eliminate the necessity of KCl in order to enhance the activity of ATPase.

Effect of EDTA in Presence of Other Salts—When NaCl is substituted for KCl in experiments like those reported in Figs. 1 and 2 and Table I, EDTA does not enhance myosin B and myosin A ATPase. The results shown (Table III) are for a concentration of EDTA (0.001 M) which accelerated dephosphorylation of ATP with KCl and for four concentrations of each of NaCl and KCl. Concentrations of EDTA ranging from $10^{-5}$ to $10^{-2}$ M were also tested at each of those of NaCl shown in Table III. None of them accelerated ATPase and all inhibited in a manner similar to that caused by EDTA at low concentrations of KCl.

The effect of EDTA on myosin B ATPase in the presence of 0.6 M KNO$_3$, KHCO$_3$, and K$_2$SO$_4$ was tested. All were as effective as KCl in causing acceleration, except K$_2$SO$_4$ which caused inhibition.
ate was no more effective than NaCl. Lithium chloride enhanced activity slightly.

**Chelating Compounds Other Than EDTA**—The accelerating effect of several chelating compounds upon myosin B ATPase was ascertained qualitatively in solutions to which the addition of EDTA caused acceleration. They were tested at each of four pH values. Uramildiacetic acid, N-hydroxyethylethlenediaminetriacetic acid, 1,2-propylenediaminetetra-acetic acid, and nitrilotriacetic acid caused accelerations comparable to those caused by EDTA, but at pH 9 rather than at pH 8. N-hydroxy-

### Table III

**Comparison of Effect of NaCl and KCl on Myosins A and B As ATPase in Presence of 10^{-8} \text{M} \text{EDTA}**

The values are in micrograms of P split per mg. of myosin per 10 minutes. pH 8.0.

<table>
<thead>
<tr>
<th>Molarity of salt</th>
<th>0.15</th>
<th>0.3</th>
<th>0.6</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Myosin B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>1.4</td>
<td>40</td>
<td>135</td>
<td>148</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.0</td>
<td>0.19</td>
<td>0.00</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Myosin A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>40</td>
<td>131</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>2.0</td>
<td>0.4</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

ethyliminodiacetic acid, L-histidine hydrochloride, and ethylenediamine caused little or no acceleration at any of the pH values. L-Histidine hydrochloride and ethylenediamine are equal to or greater than EDTA as chelating agents of copper, a heavy metal which strongly inhibits ATPase. Of the four chelating agents which cause acceleration, each binds alkaline earth metals effectively, as does EDTA. Histidine and ethylenediamine do not chelate alkaline earth metals.

**ITPase, UTPase, and Potato Apyrase**—EDTA does not cause acceleration of the dephosphorylation of inosinetriphosphate (ITP) and of uridine triphosphate (UTP).\(^5\) The reaction mixtures in which ITP was included were 0.02 and 0.6 \text{m} KCl and at pH 8. Of these concentrations of KCl, dephosphorylation of ITP occurred only at 0.6 \text{m} KCl and that was still further inhibited by 10^{-2} to 10^{-4} \text{m} EDTA. UTP was also studied at pH

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\(^5\) UTP was kindly supplied by Dr. H. M. Kalckar.
8 at concentrations of KCl ranging from 0.02 to 0.6 M and, at each, 10^{-2} M EDTA caused inhibition.

Potato apyrase is a potent ATPase, but EDTA caused only inhibition of its splitting ability. These tests were done at pH 8 and 0.06 and 0.6 M KCl in the presence of 10^{-5} to 10^{-2} M EDTA. Concentrations of EDTA ranging from 10^{-2} to 10^{-4} M caused practically complete inhibition.

DISCUSSION

The inhibiting and accelerating actions of EDTA on myosin ATPase at low and high concentrations of KCl, respectively, are quite probably due to entirely different phenomena. The inhibiting action may be due to the removal of a divalent cation, such as calcium, which is considered necessary for the action of myosin as ATPase. If this is true, then it is to be expected that a low concentration of NaEDTA would remove what calcium is carried into the reaction mixtures as a contaminant. That this is done is indicated by the inhibiting effect of 10^{-5} M EDTA at nearly all the concentrations of KCl tested on myosin A ATPase (Table I). The results of experiments with myosin B (Fig. 1) also support this explanation, except for those with 0.02 M KCl at which concentration 10^{-5} M EDTA caused acceleration. (This acceleration was discussed above in the section dealing with Fig. 1.) The failure of Ca EDTA and the ability of Mg EDTA to inhibit dephosphorylation at a low concentration of KCl also support this explanation of the inhibition of myosin ATPase by EDTA because calcium will replace magnesium in Mg EDTA and, therefore, would be withdrawn from action as it is by Na EDTA, but Ca EDTA would have no such effect.

Concentrations of EDTA higher than 10^{-5} M in the presence of sufficient KCl also remove calcium, but in addition have some as yet unexplained accelerative action on myosin ATPase of great magnitude. The experimental results presented above serve to characterize this reaction and present data to be considered in discussion of possible explanations of this phenomenon. The approaches to an explanation have been reduced to two for the purposes of the present discussion. One is that the effective chelating agent removes inhibiting heavy metal carried into the reaction mixtures by one of the reagents. The other is that the chelating compounds, together with potassium, aid in substrate-enzyme complex formation or in the breakdown of this complex into orthophosphate, adenosinediphosphate, and free enzyme.

Until Friess' description (6) of the action of EDTA and myosin with 0.6 M KCl, the effects of chelating compounds were generally attributed to the removal of some deleterious cation, usually that of a heavy metal. Gross' findings (4) with heart homogenate possibly exemplify an instance
of this nature. It is likewise possible that the moderate accelerative action of ethylenediamine and histidine is attributable to the removal of some inhibiting ion. There are several objections, however, which make this explanation unacceptable at this time for the great accelerative effects produced by some chelating agents at increased concentrations of KCl. Among these objections are (1) the requirement of adequate KCl and the close association of the acceleration with the concentration of KCl; (2) the failure of $10^{-3}$ M EDTA to cause acceleration at 0.25 and 0.32 M KCl of the same magnitude as at $10^{-4}$ M; (3) the fact that KCl and NaCl at 0.6 M have diametrically opposite effects, yet both contain about the same impurities both quantitatively and qualitatively, and both have the same dissolving property for myosin; (4) the failure of Mg EDTA and Ca EDTA to cause acceleration because heavy metal ions can replace Ca or Mg in a molecule such as EDTA; and (5) the fact that histidine and ethylenediamine are strong binders of heavy metal but still do not cause acceleration of myosin ATPase.

In addition to the above considerations of the hypothesis of removal of heavy metal, the possibility exists that a heavy metal is bound to myosin which is removed or inactivated by certain chelating agents only when ATP is present. We tested this possibility by letting myosin dephosphorylate ATP in the presence of $10^{-3}$ M EDTA and 0.6 M KCl. After 10 minutes of reaction, the protein was precipitated by diluting the reaction mixture. The concentration of EDTA in the suspension of precipitated myosin was reduced by washing it two times. Then the myosin was used again as an enzyme in a reaction mixture containing the same amount of ATP and 0.6 M KCl, but the KCl used in the second reaction had been crystallized once from a solution containing $10^{-2}$ M EDTA and twice from water. Before this treatment, 1 mg. of this myosin split 20 $\gamma$ of orthophosphate without EDTA and 60 $\gamma$ with $10^{-3}$ M EDTA at 0.6 M KCl and pH 7 to 7.5. After the treatment, 1 mg. split 21 and 18 $\gamma$ without EDTA and 60 $\gamma$ with EDTA in each of two experiments, respectively.

In addition to the experimental results cited above there are also those of Friess (6) which militate against the removal of inhibiting heavy metal as the cause of the acceleration by EDTA and other chelating compounds. Her experiments indicate, as do ours, that the removal of an inhibiting cation is not the cause of the unexpected accelerative ability of EDTA.

Further experiments by us have revealed still more about copper as an inhibiting ion. Analyses of two preparations of myosin B precipitated three times indicated that 1 gm. of myosin contains 4 to 6 $\gamma$ of copper (of the other possible heavy metals, iron and lead were excluded by our method). Then copper as CuSO$_4$ was added to the reaction mixtures. Not until 1.6 $\gamma$ of copper per mg. of myosin were added was inhibition detectable...
(80 per cent of control) and this amount of inhibition did not increase when the CuSO\textsubscript{4} and myosin were allowed to incubate for as long as 1 hour.

The inclusion of 33 μ of copper per mg. of myosin in the reaction mixture completely inhibited dephosphorylation. The addition of either Na EDTA, Ca EDTA, or ethylenediamine to such copper-containing reaction mixtures before the myosin was added prevented the inhibition, but addition of any one of these three chelating agents after the myosin had no effect upon the inhibition.

It is still possible that a metallic ion supplied by myosin is involved in this acceleration, but that it is involved in a complex with myosin and chelating agent with or without ATP. This hypothesis is related to the second approach mentioned above. There is little evidence for this type of explanation with myosin as the protein and enzyme; however, Klotz and Loh Ming (10) have shown that heavy metals act as mediators in complexes of an aniline dye and proteins such as pepsin and serum albumin. Electrophoresis\textsuperscript{6} of myosin A in 0.3 M KCl with and without EDTA showed no differences which would indicate formation of a complex. Ultrafiltration of mixtures of myosin B and 10\textsuperscript{-2} M EDTA in 0.6 M KCl and at pH 8.0 indicated no binding of EDTA when ATP was included in the mixture and when it was excluded.

**SUMMARY**

1. Ethylenediaminetetraacetic acid (EDTA) depresses the ATPase activity of myosin B at concentrations of KCl below 0.17 M,\textsuperscript{7} but, above 0.17 M, EDTA accelerates the ATPase activity (first shown by Friess at 0.6 M KCl) with its optimal concentration being closely associated with the concentration of KCl (Fig. 1).

2. The greater enhancement of ATPase of myosin A by EDTA than that of myosin B is produced by lower concentrations of KCl and at 0.6 M KCl is reduced to that of myosin B by the addition of actin (Fig. 2).

3. The increase of the ATPase activity of myosin in the presence of EDTA at pH 8 and 0.6 or 1.0 M KCl is 15- to 30-fold that occurring in the absence of EDTA.

\textsuperscript{6} Performed by E. R. Mitchell.

\textsuperscript{7} The depression caused by EDTA at low KCl concentration is made more drastic because of the sodium added to the reaction mixtures with the buffer and the ATP. In experiments, done subsequent to submission of this report, in which sodium was excluded from the reaction mixtures, K EDTA caused depression of the enzyme, but not almost total obliteration of activity as is shown in Fig. 1 at 0.02 and 0.17 M KCl. These experiments indicate that the combination of low concentrations of sodium and of EDTA causes greater inhibition than either alone. Removal of calcium remains a likely explanation of the depression by EDTA, but, if it is the cause, there is still activity in the absence of calcium.
4. EDTA caused inhibition only of the ATPase activity of potato apyrase and of the ITPase and UTPase activities of myosin B.

5. EDTA caused enhancement of myosin B ATPase in the presence of KHCO$_3$ and KNO$_3$ equal to or better than that with KCl, but there was no enhancement in the presence of NaCl, NaHCO$_3$, and K$_2$SO$_4$.

6. Neither calcium nor magnesium disodium EDTA causes enhancement of myosin B ATPase and only Mg EDTA causes inhibition (Table II).

7. The optimal pH value for the activity of actomyosin as ATPase with 0.001 M EDTA and 0.6 M KCl is 8 (Fig. 3).

8. Of the other chelating agents tested acceleration of the ATPase activity of myosin B is restricted to those which bind alkaline earth metals effectively.

9. Possible explanations of these effects of EDTA are discussed.

BIBLIOGRAPHY

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