THE EFFECTS OF DIVALENT CATIONS ON TRYPsin*

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(Received for publication, April 6, 1953)

Although the activating effect of metal ions, such as Co++ and Mn++, on a variety of aminopeptidases has long been known (see Smith (2)), no requirements of the pancreatic proteinases for these ions have been demonstrated. It has been found, however, that calcium salts improve the yield of trypsin from trypsinogen (3) and that they increase the stability of trypsin at alkaline pH (4, 5). Earlier reports contain conflicting data on the effects of ions on crude pancreatic enzyme preparations and, apart from the work of Sugai (6) on the activation of crude trypsin preparations by some metal ions (Mn++, Co++, Ni++, Fe++, and Fe+++) and the inhibition by others (Ag+, Hg++, Zn++, and Pb++), these data appear to be of questionable significance.

In recent work in this laboratory it was noted that the addition of 0.01 M Ca++ to the reaction mixture increased the zero order rate constant of the tryptic hydrolysis of benzoyl-L-arginine ethyl ester (BAEE) by approximately 25 per cent and at the same time improved the reproducibility of the rate measurements (1). Since, in comparison to typical metal ion activation (2, 7), the increase in activity was small, it was thought to represent an effect of the divalent cation on the stability of the enzyme, similar to that suggested by Gorini (4) and by Bier and Nord (5). In addition to calcium ions, Mn++, Cd++, Co++, and, to a lesser extent, Mg++ and Ba++ were found to increase the activity of trypsin toward BAEE, whereas metal ions such as Hg++, Cu++, Ag+, and, to a lesser extent, Zn++ inhibited trypsin at low concentrations (1). The metal ion-inhibited enzyme could be partially reactivated by calcium and other inorganic salts, as well as by the complex-forming agent Versene (ethylenediaminetetraacetic acid) and by thioglycolate. The results of these studies are the subject of the present paper.

EXPERIMENTAL

Materials and Methods

Twice crystallized trypsin (Worthington), containing approximately 50 per cent magnesium sulfate, was dialyzed against 0.001 N hydrochloric acid.

* A preliminary account of this work has been published (1).
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acid for 16 hours in the cold, with stirring, and then lyophilized. Solutions
were made in cold 0.001 N hydrochloric acid and used on the same day.

*Chymotrypsinogen*, crystallized seven times, was prepared by Mr. J. A.
Gladner according to the method of Kunitz and Northrop (8). Salmine
sulfate was a commercial sample, obtained through the courtesy of Dr.
E. G. Krebs.

The *synthetic substrates*, benzoyl-L-arginine ethyl ester, benzoyl-L-argin-
inamide (BAA), and acetyl-L-tyrosine ethyl ester (ATEE), were prepared
according to methods previously published (9, 10).

*Metal salts* were reagent grade chlorides, except for Mn⁺⁺ and Cd⁺⁺,
which were used as sulfates, and Ag⁺, which was used as nitrate. A stand-
ard calcium chloride solution was prepared by dissolving a weighed quantity
of Iceland spar in the minimal quantity of 6 N hydrochloric acid and dilut-
ing with water to the required volume. Glass-distilled or deionized water
was used throughout.

Protein concentrations were determined at 280 nm in a Beckman model
DU spectrophotometer and checked against nitrogen determinations by
the micro-Kjeldahl method, assuming 15.0 per cent protein nitrogen (11).
The protein concentration of a trypsin solution of unit optical density was
found to be 0.695 mg. per ml., considerably higher than the value 0.585
given by Kunitz (12).

Tryptic esterase activity was determined with 0.005 M BAEE as sub-
strate and 17 γ of trypsin per ml. The reaction was followed by the con-
tinuous potentiometric titration method of Schwert et al. (13) in 0.01 M
borate buffer, pH 7.8. The activities were expressed as zero order velocity
constants (moles per liter per minute) per mg. of trypsin nitrogen. For
experiments in the presence of Ag⁺ ions, BAEE hydrochloride was con-
verted to the nitrate by passage through a Dowex 2 ion exchange column
in the nitrate form, and calcium chloride was replaced by calcium nitrate.
Any variations from these conditions will be given in the text.

Amidase activity of trypsin was determined with 0.01 M BAA as sub-
strate in 0.05 M borate buffer, pH 7.8 (13).

Proteinase activity of trypsin was determined at 25° with salmine (0.5
per cent) as substrate in 0.1 M borate buffer, pH 7.8. Aliquots containing
0.1 ml. of the incubation mixture were withdrawn and added to 1 ml. of
ninhydrin reagent and analyzed according to the method of Moore and
Stein (14). The trypsic activation of chymotrypsinogen was followed by
mixing a 0.3 per cent solution of the protein in 0.1 M tris(hydroxymethyl)-
aminomethane hydrochloridc (Tham) buffer, pH 7.8, with trypsin at 4°,
and assaying samples for chymotryptic activity, with ATEE as substrate
(9).
Results

Table I shows the magnitude of the effect of 0.001 and 0.01 M divalent cations on the esterase activity of trypsin at pH 7.8. The results are expressed as ratios of zero order reaction constants. In the absence of added divalent cations, a value of $k_0 = 0.28$ was found, in agreement with the values reported by Schwert et al. (13), but addition of 0.001 M calcium raised the value to 0.36. The activating effects of Ca++, Mn++, Co++, and Cd++ were all of the same magnitude, provided that sufficiently high metal ion concentrations were used, and they were the same when the ion was added to the enzyme solution before or after the addition of the substrate. Since at the pH of the reaction several of the metal ions gave precipitates of the corresponding hydroxides, the results for Fe++ and Pb++ are not accurate. Cd++, Co++, and Mn++ gave only a slight initial precipitate, but the addition of base during the reaction caused further precipitation, owing presumably to local excess of base. This led to

<table>
<thead>
<tr>
<th>Cation added</th>
<th>Relative activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.001 M</td>
</tr>
<tr>
<td>None</td>
<td>1.00</td>
</tr>
<tr>
<td>Ca++</td>
<td>1.28</td>
</tr>
<tr>
<td>Mn++</td>
<td>1.34</td>
</tr>
<tr>
<td>Cd++</td>
<td>1.34</td>
</tr>
<tr>
<td>Co++</td>
<td>1.18</td>
</tr>
<tr>
<td>Mg++</td>
<td>1.09</td>
</tr>
<tr>
<td>Ba++</td>
<td>1.08</td>
</tr>
<tr>
<td>Sr++ or Ni++</td>
<td></td>
</tr>
<tr>
<td>Fe++ or Pb++</td>
<td>1.0 Ca.</td>
</tr>
<tr>
<td>Zn++</td>
<td>0.81</td>
</tr>
<tr>
<td>Cu++ (borate buffer)</td>
<td>0.0</td>
</tr>
<tr>
<td>Hg++</td>
<td>0.0</td>
</tr>
<tr>
<td>Ag+ (borate buffer)</td>
<td>0.0</td>
</tr>
<tr>
<td>Versene, 0.01 M</td>
<td></td>
</tr>
<tr>
<td>Co++ + Versene (0.012 M)</td>
<td>1.00</td>
</tr>
<tr>
<td>Ca++ + &quot; (0.012 M)&quot;</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* Activity is expressed as the ratio of zero order reaction constants in the presence of the added cation to that observed in 0.005 M tris(hydroxymethyl)aminomethane hydrochloride buffer, pH 7.8, alone.

† Corrected for the effect of partial precipitation of the metal oxide on alkali consumption during ester hydrolysis.
spuriously high base consumption, which was corrected by subtracting the base equivalent to 100 per cent hydrolyzed substrate from the total base consumed during the reaction. This quantity was small, and, since no deviation from zero order kinetics was observed, it was assumed that its contribution to the rate was uniform.

These observations show that at pH 8 the concentration of divalent cation in solution is often much less than that of the added metal salt. As an extreme example we may calculate from data in the literature (15, 16) that a $10^{-4}$ M solution of HgCl$_2$, in the presence of 0.01 M Cl$^-$ at pH 8, contains $10^{-14}$ M Hg$^{2+}$, $2.4 \times 10^{-6}$ M HgCl$_2$, and $7.6 \times 10^{-4}$ M Hg(OH)$_2$. The use of symbols such as Cu$^{2+}$ or Hg$^{2+}$, when referring to the metal salts, must, therefore, be regarded as a convenient shorthand without chemical significance, except for the alkaline earth cations and magnesium, which are not appreciably hydrolyzed under these conditions.

Effects of Dialysis and Versene—Prolonged dialysis of trypsin for 3 days in the cold against 0.001 N HCl did not lower $k_0$ below 0.28. Versene ($10^{-4}$ M), which binds divalent cations strongly, lowered $k_0$ to 0.255. Versene also reverses the action due to added metal ions, but the activity can be restored by the further addition of metal salts. These activations and inactivations are practically instantaneous, the slope of the rate curve changing sharply at the point of the addition of the reagent. This is in marked contrast to the slow activation of the aminopeptidases (2).

Effect of Calcium—The effect of calcium ion concentration on the esterase activity of trypsin is shown in Fig. 1. The experimental points follow approximately the continuous curve calculated on the basis of reversible combination of a single calcium ion with trypsin according to the law of mass action, with a dissociation constant of $4 \times 10^{-6}$ M. The assumptions involved in deriving this curve are discussed below. There is, however, considerable departure of the experimental points from the curve at higher Ca$^{2+}$ concentrations, which remains unexplained. The effect of Ca$^{2+}$ on the amidase activity of trypsin (BAA as substrate) was found to be similar to that on esterase activity. The pseudo first order velocity constant per mg. of trypsin nitrogen per ml., at pH 7.8, was 0.058 in the absence, and 0.071 in the presence, of 0.01 M Ca$^{2+}$. However, no significant effect of Ca$^{2+}$ could be found when the proteinase activity of trypsin was measured with chymotrypsinogen and salmine, respectively, as substrates. The initial rate of tryptic activation of chymotrypsinogen to $\alpha$-chymo-

1 In higher concentrations Versene is less effective, and, in fact, in concentrations of $10^{-2}$ M a slight increase in $k_0$ is observed. It is probable that Versene has a dual effect, one inhibitory, owing to the binding of metal ions, and the other one activating, shown in higher concentrations, which is probably similar to the effect produced by other organic compounds such as alcohol (17) or isobutyric acid (unpublished experiments).
trypsin was the same in the presence and absence of 0.1 M Ca++, but after 24 hours incubation a slight enhancing effect of Ca++ on the final activity was noted. Similarly, the tryptic digestion of salmine sulfate was unaffected by the presence of 0.02 M Ca++, nor did the addition of Versene reduce the rate. These findings are in contradistinction to the reported activation of tryptic digestion of hemoglobin by Ca++ (18).

Exhaustively dialyzed trypsin (against 0.001 M HCl) contains only traces of calcium, as measured by the intensity of the 646.6 m\(\mu\) line in a Weichselbaum-Varney flame spectrophotometer. In comparison to a standard solution of Iceland spar, the calcium content of a 1 per cent trypsin solution was found to correspond to 0.1 mole per mole of trypsin (molecular weight 24,000 (11)).

Inhibition by Metal Ions—It can be seen from Table I that trypsin is strongly inhibited by Cu++, Hg++, and Ag+ ions and, to a lesser extent, by

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Footnote 2: We are indebted to Dr. Robert D. Ray of the Department of Surgery for use of this flame spectrophotometer.
Zn\textsuperscript{++} ions. The effect of metal ion concentration on the inhibition by Cu\textsuperscript{++}, Hg\textsuperscript{++}, and Ag\textsuperscript{+} ions is shown in Figs. 2, 3, and 4. In all cases, the inhibiting ion was added after trypsin and substrate (BAEE) had been mixed. It will be noted that all three ions are about equally effective inhibitors and that Ca\textsuperscript{++}, when added before the inhibiting ion, raises the metal ion concentration required to produce a given percentage of inhibition.

![Graphs showing inhibition of trypsin activity by Hg\textsubscript{2}Cl\textsubscript{2} and Ag\textsuperscript{+}](image)

**Fig. 3.** Inhibition of trypsin activity by Hg\textsubscript{2}Cl\textsubscript{2}. ○, without Ca\textsuperscript{++}; ●, in the presence of 0.001 M Ca\textsuperscript{++}; ■, in the presence of 0.01 M Ca\textsuperscript{++}.

**Fig. 4.** Inhibition of trypsin activity by Ag\textsuperscript{+}. ○, without Ca\textsuperscript{++}; ●, in the presence of 0.001 M Ca\textsuperscript{++}; □, in the presence of 0.01 M Ca\textsuperscript{++}.

The observation that the curves obtained in the presence of Ag\textsuperscript{+} and Hg\textsuperscript{++} ions, respectively, are not sigmoid shows that more is involved in the inhibition than reversible combination at a single site. This is also borne out by the reactivation studies described below. The different shape of the curves obtained in the presence of Cu\textsuperscript{++} ions is probably the result of precipitation of cupric hydroxide which occurs when the Cu\textsuperscript{++} ion concentration exceeds $5 \times 10^{-4}$ M. The sigmoid shape of these curves is thus an artifact and does not indicate simple reversible combination.

The investigation of the inhibition by Cu\textsuperscript{++} ions is also complicated by...
the formation of complexes with BAEE and its hydrolysis product, benzoyl-L-arginine. This leads to an increase in trypsin activity as hydrolysis proceeds and in an apparent departure from zero order kinetics. Complex formation was demonstrated spectrophotometrically, the visible absorption of Cu$^{+}$ (800 mp) being shifted to shorter wave-lengths and raised in intensity by BAEE, and to a greater extent by benzoyl-L-arginine. The complexes were not sufficiently stable to prevent precipitation of cupric hydroxide by alkali.

Reversal of Metal Ion Inhibition—The inhibition by Cu$^{+}$, Hg$^{++}$, and Ag$^{+}$ (10$^{-4}$ M) was completely reversed by thioglycolic acid (10$^{-3}$ M), which itself had no effect on trypsin activity at this concentration. This shows that the metal ions do not cause any irreversible change in the enzyme protein.

Metal ion inhibition can be partially reversed by Versene and by Ca$^{++}$, maximal reactivation being about 80 per cent. The interaction of trypsin with Hg$^{++}$, Ca$^{++}$, and Versene is shown in Fig. 5, in which the sequence of the addition of reagents, given along the abscissa, is indicated by the arrows and the corresponding zero order velocity constant for the tryptic hydrolysis of BAEE, $k_0$, is plotted along the ordinate. It will be noted that a lower $k_0$ resulted if Hg$^{++}$ and trypsin were left in contact before the addition of substrate. The subsequent reactivation by Ca$^{++}$ was likewise decreased. Similar effects were noted with Cu$^{++}$ as the inactivating ion. Silver ions behaved differently in that (1) inhibition was independent of the time of incubation with trypsin prior to the addition of substrate and (2) the reactivation by Ca$^{++}$ was independent of Ca$^{++}$ concentration (0.001 to 0.1 M) and reached only 70 per cent of the original activity.

Effect of Ionic Strength on Inhibition—In order to test the specificity of Ca$^{++}$ in preventing the inhibition of trypsin by the above metal ions, various salts, which alone have no effect on the activity of this enzyme, were added to trypsin and BAEE prior to the addition of Hg$^{++}$. The results are shown in Fig. 6 in which the resulting $k_0$, obtained in the presence of 5 X 10$^{-5}$ M mercuric chloride, is plotted against the ionic strength of the added salt. It is apparent that sodium chloride, strontium chloride, and ammonium sulfate are equally effective in reducing the inhibition by Hg$^{++}$ at the same ionic strength. Furthermore, comparison of these results with those shown in Fig. 3 demonstrates that, if allowance is made for the specific activating effect of Ca$^{++}$, calcium chloride is not more effective than the above salts.

In view of these findings it is surprising that the addition of base during the esterase determinations has apparently no effect on the reaction rate. Except for a reduction in rate during the late stage of the reaction, when the Hg$^{+}$-BAEE ratio becomes high, the reaction remains zero order. This result may be explained by the consideration of the over-all reaction in which BAEE hydrochloride, a unimi-
Effect of Substrate Concentration on Metal Ion Inhibition—Increasing concentration of BAEE progressively reduced the inhibition of trypsin by Cu\textsuperscript{2+}, Hg\textsuperscript{2+}, and Ag\textsuperscript{+} ions. This effect was only partly due to the contribution of the substrate to the total ionic strength, for, if the latter was held constant, the phenomenon was still evident.

The data did not follow the linear relation of Lineweaver and Burk (19) for simple competitive inhibition, nor could the results be quantitatively accounted for by relations developed on the assumption that the concentration of free Hg\textsuperscript{2+} was reduced by complex formation with BAEE. However, the Lineweaver-Burk theory assumes reversible combination of the inhibitor at a single site, which is apparently not valid for this system (see Fig. 3). Competitive inhibition might still account for part of the effect of Hg\textsuperscript{2+}.

Valent electrolyte, is replaced by benzoyl-L-arginine, a dipolar ion, and sodium chloride. Since the dipolar ion contributes relatively little to the ionic strength, it might have little effect on the trypsin-Hg\textsuperscript{2+} combination.
DISCUSSION

Since trypsin retains about 80 per cent of its maximal activity in the absence of any added metal ion, or in the presence of Versene, metal ions do not appear to be essential for the activity of this enzyme. Since the activation by calcium ions and its reversal by Versene occur equally well in the presence or absence of the substrate BAEE, it is unlikely that the metal ions are directly involved in enzyme-substrate combination.

Several independent investigations appear to offer some clue to the rôle of the activating metal ions in this system. From studies of the stability of trypsin at alkaline pH, Kunitz and Northrop (20) concluded that "active native trypsin" was in equilibrium with a "reversibly denatured" form. It was further suggested that the progressive loss of activity in this pH region was the result of digestion of inactive trypsin (T_i) by active trypsin (T_a), a process which may be formulated as follows:

\[ T_a \rightleftharpoons T_i \]  
\[ T_a + T_i \rightarrow T_a + \text{peptides} \]

The subsequent findings of Bier and Nord (5) and Gorini (4) that calcium salts greatly retarded the autolysis of trypsin at alkaline pH led Gorini (4) to suggest that only the active form of trypsin contained bound calcium and that the action of this ion was to shift equilibrium reaction (1) toward the active form. Since, however, calcium is not obligatory for trypsic activity, Gorini's hypothesis requires modification, for example as indicated in equations (3) to (5).

\[ T_a + \text{Ca}^{++} \rightleftharpoons K_2 \rightarrow T_{Ca} \]  
\[ K_d \rightarrow T_i + \text{Ca}^{++} \rightleftharpoons K_2 \rightarrow T_{Ca} \]  
\[ T_a + \text{Ca}^{++} \rightarrow T_a + \text{peptides} \]

The symbol \( K \) denotes the respective equilibrium constant, assuming that only a single \( \text{Ca}^{++} \) is involved. \( K_1 \) may be calculated if it is assumed that \( T_a \) and \( T_{Ca} \) have the same specific activity (\( K_1 = T_i/T_a = (\text{relative activity} - 1) = 0.29 \)). According to this scheme, \( \text{Ca}^{++} \) could reduce autolysis by combining with trypsin, as shown by equation (3) or (4) or both, whereas in the absence of \( \text{Ca}^{++} \) autolysis would occur according to equation (5). Since the shape of the theoretical curve relating the concentration of \( \text{Ca}^{++} \) to trypsic activity is very similar whether calculated

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\footnote{The possibility that trypsin contains firmly bound cations other than calcium which are essential for activity is not excluded by the present data.}
according to equation (3) or (4), it does not seem possible to differentiate between these schemes experimentally. The curve in Fig. 1 was calculated for the simultaneous occurrence of both reaction equations, with $K_2 = K_3$. This represents the simplest hypothesis to account for the effect of $Ca^{++}$ on the autolysis and the activity of trypsin, but it should be noted that the supporting evidence is indirect.

Preliminary experiments on the effect of calcium ions on the pH maximum for tryptic esterase activity showed below pH 11 a negligible effect of pH on the percentage activation due to calcium. These results, which are in essential agreement with similar measurements of Duke, Bier, and Nord (18) involving hemoglobin as substrate, are surprising, since equilibria such as those shown above would have been expected to involve hydrogen ions.

The failure of calcium to activate tryptic digestion of salmine and the activation of chymotrypsinogen are not readily explicable. The essential difference between these substrates and the synthetic substrates is one of size and number of polar groups. If the function of the calcium ions is to maintain a specific configuration of the peptide chains in trypsin, it is possible that a protein substrate might replace calcium in this respect, while a small molecule could not. It is apparent, however, that the complex protein substrates do not respond uniformly in this respect, since it has been reported that calcium activates the proteinase activity of trypsin and hemoglobin (18) and that calcium stabilizes serum albumin against tryptic digestion (21).

In view of the lack of data on the amino acid composition of trypsin, and the multiple affinities of the inhibiting metal ions for the enzyme, the substrate, and the hydrolysis products, respectively, the mode of action of Cu$^{++}$, Hg$^{++}$, and Ag$^+$ in causing enzyme inhibition is highly conjectural. It is also uncertain whether these ions combine with the enzyme at the same site as the substrate, since the dependence of metal ion inhibition on substrate concentration is not accounted for by simple relations for competitive inhibition. This question will be further discussed in a future publication. The relation between the sites of combination of activating and inhibiting ions is similarly indefinite, since the reactivation of inhibited trypsin by calcium ions is primarily an effect of ionic strength. It may be significant, however, that activating and inhibiting ions, synthetic substrates, and the pancreatic trypsin inhibitor (22) are all positively charged when they combine with this enzyme.

Exploratory experiments on the absorption spectra of copper-trypsin complexes revealed a resemblance in the visible region and over a wide pH range (pH 3 to 8) to the copper-serum albumin complexes of Klotz et al. (23). In the ultraviolet region a band at 250 m$\mu$ was apparent, in con-
trast to the absorption of the complex of several amino acids with copper studied by Spies (24), which absorb at 230 mμ, and the copper-imidazole complexes of Gurd et al. (25) at 280 mμ. In further contrast to the complexes between copper and the histidine groups of serum albumin described by Tanford (26) and by Gurd and Goodman (27), those between copper and trypsin are formed at much lower copper concentrations, the apparent dissociation constants being of the order of $10^{-5}$ for the latter and $10^{-2.7}$ for the former. The site of interaction remains to be elucidated.

This investigation was supported by a grant from the Rockefeller Foundation.

SUMMARY

Kinetic studies on the effects of several divalent ions on the enzymatic activity of trypsin have been carried out. Ca++, Co++, Cd++, and Mn++ increase the esterase and amidase activity of trypsin maximally by 25 per cent, whereas Cu++, Hg++ and Ag+, are inhibitory. The activating effect can be fully reversed by Versene, and the inhibiting effects partly so. Inhibition by metal ions can be reversed by thioglycolate and reduced by calcium ions, as well as by neutral salts which alone have no effect on tryptic activity. The nature of the interactions among enzyme, substrate, activating, and inhibiting ions has been considered.

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