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SUMMARY

1. A simple method for isolating 2-guanidino-ethy1 2-amin0-2-carboxyethyl hydrogen phosphate (lombricine) from natural sources has been described. The method results in good yields of analytically-pure crystalline material.

2. 2-Aminoethyl 2-amin0-2-carboxyethyl hydrogen phosphate has been detected in earthworm extracts and it is suggested that this compound is the biological precursor of lombricine. The precursor was identified by the preparation of a guanylated derivative, by degradative procedures and by comparison with the synthetic product.

Our thanks are due to Dr D. I. Magrath for a sample of β-chloroethylamine, to Dr Joyce Fildes, Department of Medical Chemistry, for the microanalyses and to Mr B. Thorpe for skilled technical assistance.

REFERENCES


Steps in the Reactions of Chymotrypsin with Tyrosine Derivatives

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Chymotrypsin catalyses the hydrolysis of peptide, amide and ester bonds of many N-acylated amino acids, but it exhibits special specificity for compounds of aromatic amino acids (for a summary of chymotrypsin substrates, see Foster & Niemann, 1955). The esters and amides of other aromatic acids, such as methyl hydrocinnamate (Smoke & Neurath, 1949), are also hydrolysed by this enzyme and its specificity for compounds containing a ring structure is further emphasized by its ability to
bind and hydrolyse aliphatic esters of phenols such as \( p \)-nitrophenyl acetate (Hartley & Kilby, 1954).

We have suggested that the reactions of chymotrypsin with its substrates involve three steps (Hammond & Gutfreund, 1955; Gutfreund & Sturtevant, 1956a, b). The first of these steps is a rapid adsorption of the substrate on to the specificity site of the enzyme, the second involves a chemical reaction resulting in the acylation of the catalytic site of the enzyme and the third step involves the hydrolysis of the acyl-enzyme (see Scheme 1).

\[
E + AB \rightleftharpoons K_1 \rightarrow E\text{-}AB \rightarrow K_2 \rightarrow EA + B \rightarrow K_3 \rightarrow E + A + B
\]

\( k_1 \)

\( k_2 \)

\( k_3 \)

\( k_4 \)

Compound I Compound II

Scheme 1

Both an hydroxy group of serine and an imidazole group of histidine are essential components of the catalytic site of chymotrypsin; the diverse evidence for this has been summarized by Jandorf & Michel (1957). Hartley & Kilby (1954) and Gutfreund & Sturtevant (1956a, b) have shown that, at least in reactions with \( p \)-nitrophenyl acetate, the acylated enzyme is a chemical intermediate with a real existence and that this compound is formed by an imidazole-catalysed acylation of a serine hydroxyl group of the enzyme. The spectroscopic evidence of Dixon & Neurath (1957), which indicated that an imidazole group of the enzyme was acylated during the reaction, was shown to be misleading when Spencer & Sturtevant (1959) demonstrated that the acetylated enzyme studied by Dixon & Neurath (1957) was not on the pathway of the enzyme reaction.

The evidence for the existence of an acyl enzyme intermediate and for the acylation of a serine hydroxyl group depended on the results of studies with \( p \)-nitrophenyl acetate, a rather special substrate which is only hydrolysed slowly by chymotrypsin. It has been suggested by Bernhard & Gutfreund (1958) that different enzyme-substrate intermediates might occur during the reaction of chymotrypsin with substrates which are more like its natural ones, namely compounds of aromatic amino acids. The present study with the nitrophenyl ester of \( N \)-benzoxycarbonyl-L-tyrosine was suggested to us when Martin, Golubow & Axelrod (1959) prepared this compound and used it for the assay of chymotrypsin. The purpose of the experiments reported here was to find whether there is any evidence for the existence of an \( N \)-benzoxycarbonyl-tyrosyl-enzyme intermediate (compound II of Scheme 1) during the reaction of chymotrypsin with \( N \)-carbobenzoxy-L-tyrosine nitrophenylate, and, if such a compound exists, to find which group or groups of the enzyme are involved in its formation. It will be shown that an \( N \)-benzoxycarbonyl-tyrosyl-enzyme intermediate is formed, but its formation is so rapid that its kinetics cannot be analysed and the chemistry of the formation of compound II with this substrate has therefore not been elucidated.

**EXPERIMENTAL**

**Buffers.** Potassium phosphate solutions (0·05 M) containing 20% (v/v) of propan-2-ol, pH 7·2 (as measured with a glass electrode), were used as solvent in all reaction mixtures.

**Enzyme.** The chymotrypsin stock solution was prepared by activation of crystalline chymotrypsinogen (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) by the method of Northrop, Kunitz & Herriott (1948). Recrystallized enzyme was stored at 2° in a solution of pH 5·0. It was assayed by measuring the initial rapid liberation of nitrophenol on addition of a portion of the enzyme to a solution (1 mm) of nitrophenyl acetate (Hartley & Kilby, 1954). The data of Biggs (1954) were used for calculation of the concentration of nitrophenol from the extinction at 400 m\( \mu \) and pH. The stock solution was adjusted to be 2 mm with respect to active enzyme.

**Substrates.** \( p \)-Nitrophenyl acetate was kindly supplied by Dr W. N. Aldridge; the \( p \)-nitrophenyl ester of \( N \)-benzoxycarbonyl-L-tyrosine (CTN) was kindly supplied by Dr C. J. Martin (Martin et al. 1959).

**Spectrophotometric techniques.** The results reported in this paper relied on the photometric observation of the rate of appearance of \( p \)-nitrophenol during the hydrolysis of \( p \)-nitrophenyl esters. Observations on a time scale of minutes were carried out in a Unicam spectrophotometer (SP. 500) at 400 m\( \mu \). The rapid-reaction measurements, on a time scale of fractions of a second, were carried out in a stopped-flow machine (Gibson, 1954) as modified by Gutfreund (1955) for the observation of the rate of appearance of \( p \)-nitrophenoxide ions during enzyme reactions. The magnitude of the deflexions of the oscilloscope beam were calibrated against standard solutions of known \( p \)-nitrophenol concentration. It was found that over the concentration range of \( p \)-nitrophenol used (0·50 \( \mu \)M) the instrument gave a linear response to concentration changes, which were calibrated in cm. deflexion per 10 \( \mu \)M-\( p \)-nitrophenol. Since all the solutions used were of the same pH and in the same solvent the proportion of \( p \)-nitrophenol in the ionised, coloured form was constant.

The time base of the ‘solaroscope’ oscilloscope (Solartron Electronic Group Ltd., Thames Ditton, Surrey) was used for the determination of reaction velocities. Photographs of the oscilloscope tracings were projected in an enlarger and drawn on graph paper together with markers which gave a direct measure of the enlargement factor from oscilloscope screen to graph paper.

**RESULTS**

The Michaelis parameters for the reaction of chymotrypsin with CTN have been determined by Martin et al. (1959), who obtained a maximum overall rate at pH 8·0 and 30° of 480 sec.\(^{-1}\) and \( K_m \) 32 \( \mu \)M. From four measurements of the initial
velocity we obtained the range of 100 ± 10 sec.⁻¹ for the steady-state turnover of the enzyme at 25°, pH 7-2 and CTN concentration 40 μM. Correcting for the suboptimum conditions of pH and substrate concentration from the graphs given by Martin et al. (1959) we obtain a maximum turnover of 300 sec.⁻¹ at 25° which is in reasonable agreement with the value of 480 sec.⁻¹ at 30°. Fig. 1A gives the record of one experiment carried out with a reaction mixture of final enzyme and substrate concentrations 0-4 μM and 40 μM respectively; this gives an example of the time course of the chymotrypsin-catalysed hydrolysis at relatively low enzyme concentration, suitable for calculations of the turnover number. Fig. 1B gives the record of an experiment at high enzyme concentration (20 μM); the substrate concentration and the concentration scale is the same as that in Fig. 1A, but the time scales of the figures are different. Comparison of the two records in Figs. 1A and B indicates that in the experiment at high enzyme concentration approximately half the total p-nitrophenol has been liberated by the time observation commenced. This is reminiscent of the observations of Hartley & Kilby (1954), who found on a very much slower time scale that during the relatively slow reaction between chymotrypsin and p-nitrophenyl acetate there occurred an almost instantaneous liberation of 1 mole of p-nitrophenol.

![Fig. 1](image)

Fig. 1. Records of two observations of the time course of the liberation of p-nitrophenol during the reaction between chymotrypsin and the p-nitrophenyl ester of N-benzyloxy carbonyl-L-tyrosine (40 μM) at 25° in 20% (v/v) propan-2-ol, 0-05 M-potassium phosphate, pH 7-2. Chymotrypsin concentrations: (A) 20 μM; (B) 0-4 μM. All concentrations given are those in the final reaction mixture. Note different time scales for (A) and (B).

![Fig. 2](image)

Fig. 2. Plot of the chymotrypsin concentration against the quantities of p-nitrophenol liberated before observation (3 msec.) during reactions between chymotrypsin and the p-nitrophenyl ester of N-benzyloxy carbonyl-L-tyrosine (40 μM) at 25° in 20% (v/v) propan-2-ol, 0-05 M-potassium phosphate, pH 7-2. All concentrations given are those in the final reaction mixture.

Table 1. Kinetic constants for the chymotrypsin-catalysed hydrolysis of three tyrosine derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>(Kₘ)ₜ (M)</th>
<th>k₂</th>
<th>k₃</th>
<th>k₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Benzoyl-L-tyrosine amide</td>
<td>2-5 x 10⁻⁴</td>
<td>0-24</td>
<td>300</td>
<td>0-24</td>
</tr>
<tr>
<td>N-Benzoyl-L-tyrosine ethyl ester</td>
<td>1-2 x 10⁻⁴</td>
<td>600</td>
<td>300</td>
<td>200</td>
</tr>
<tr>
<td>N-Benzoyloxy carbonyl-L-tyrosine</td>
<td>3-2 x 10⁻⁴</td>
<td>&gt;1000</td>
<td>300</td>
<td>300</td>
</tr>
</tbody>
</table>
kinetics is observed as in our enzyme experiments. From studies of this model system under conditions when reaction half-times from 1 to 10 msec. were observed we could draw the conclusion that for reactions with half-times of 10 msec. or more at least 90% of the course of the reaction can be observed. However, for reactions with half-times of 1 msec. only about 10% of the reaction could be observed, which means that three half-times must have passed between mixing and start of observation.

DISCUSSION
The results presented in Figs. 1 and 2 show clearly that during the reaction of chymotrypsin with CTN there occurs an initial rapid liberation of 1 mole of p-nitrophenol/mole of chymotrypsin and a slower subsequent steady-state liberation of p-nitrophenol. This sequence of events is similar to that observed for the chymotrypsin-catalysed hydrolysis of p-nitrophenyl acetate, except that both the steps characterized by the rate constants $k_2$ and $k_3$ (see Scheme 1) are faster by a factor of at least 10$^4$ for the tyrosine derivative. It is shown that within the time resolution of the method used the initial formation of compound II with CTN is instantaneous. Tests with model systems of the stopped-flow arrangement used here for mixing and observing reactants have shown that the reaction mixture in the observation chamber is about 3 msec. old. The formation of compound II is essentially complete within that time interval and it can be stated therefore that for the chymotrypsin–CTN reaction $k_2 > 1000$ sec.$^{-1}$. The rate constant $k_2$ of the decomposition of the N-benzylxoycarbonyl-L-tyrosine–chymotrypsin compound is given by the steady-state rate $k_0$ of the overall enzyme reaction for which it is rate-determining. It has been shown that under optimum conditions at 25° $k_0 = 300$ sec.$^{-1}$. This value can be compared directly with the turnover number for chymotrypsin with benzoyl L-tyrosine amide $k_0 = 0.24$ sec.$^{-1}$ (MacAllister, 1949) and benzoyl L-tyrosine ethyl ester $k_0 = 200$ (Gutfreund & Sturtevant, 1956b). We have suggested (Gutfreund & Sturtevant, 1956b) that for the hydrolysis of the amide $k_2$ is rate-determining, whereas for the hydrolysis of the ethyl ester the two steps characterized by $k_2$ and $k_3$ jointly control the overall rate. It can be shown that a comparison of the reactions of the three substrates mentioned above (benzoyl L-tyrosine ethyl ester, benzoyl L-tyrosine amide and CTN) is fully consistent with and gives support to Scheme 1 as a description of chymotrypsin-catalysed hydrolysis reactions. The major difference in the reactions of chymotrypsin with the three substrates is in the rate of the second step, which involves a nucleophilic attack by the catalytic groups of the enzyme on the carbonyl group of the substrate. A comparison of the non-enzymic hydrolysis by nucleophilic reagents shows that aliphatic esters of carboxylic acids are much more rapidly hydrolysed than amides and that nitrophenyl esters are much more rapidly hydrolysed than aliphatic esters (Bender, Ginger & Kemp, 1954; Bender & Turnquest, 1955, 1957a, b).

Our conclusion about the relative rates for the three substrates of the enzyme step characterized by $k_2$ (see Table 1) is in accord with the expected chemical reactivities of the different tyrosine compounds. The final step of the enzyme-catalysed hydrolysis reaction, which is characterized by $k_3$, must be nearly identical for the three tyrosine substrates, since compound II is nearly identical in the three cases.

A comparison of the $K_m$ values for the three types of substrates is also instructive. Gutfreund & Sturtevant (1956b) have pointed out that $K_m$ for the overall reaction, i.e. $(K_m)_2$, is in the following relation to $(K_m)_1$:

$$(K_m)_2 = (K_m)_1 \frac{k_3}{k_1 + k_2},$$

where

$$(K_m)_1 = \frac{k_1 + k_2}{k_1}.$$  

$(K_m)_1$ determines the concentration of compound I and $(K_m)_2$ determines the concentration of compound II. As can be seen in Table 1, the faster the rate of formation of compound II, the smaller the value for $K_m$ for the overall reaction.

In this way we have been able to show that tyrosine substrates of chymotrypsin form distinct intermediate compounds with the enzyme. It is only to be regretted that the formation of this compound is so rapid that it has so far not been possible to study the effect of hydrogen ions and changes of other conditions on the rate of its formation. We cannot therefore give a definite answer to the question whether a serine hydroxyl group is acylated by the tyrosyl residue. It is, however, very suggestive that an imidazole-catalysed acylation of the enzyme as well as an imidazole-catalysed hydrolysis of an acyl–enzyme compound occurs in the manner suggested by Gutfreund & Sturtevant (1956b) for the hydrolysis of p-nitrophenyl acetate. Martin et al. (1959) have shown that the overall rate of chymotrypsin-catalysed hydrolysis of CTN has a pH profile corresponding to the involvement of a histidine imidazole residue in the reaction. It is clear from our results that this corresponds to the catalytic role of a free imidazole group in the decomposition of the acyl–enzyme compound and is evidence for a group other than the imidazole group of the catalytic site being responsible for combining the tyrosyl group with the enzyme.

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SUMMARY

1. The reactions between chymotrypsin and the p-nitrophenyl ester of N-benzoyloxy carbonyl L-tyrosine have been studied by a fast-reaction photometric method.

2. It has been shown that an N-benzoxycarbonyl-L-tyrosine-chymotrypsin compound is formed as an intermediate in the enzymic hydrolysis of this substrate. The formation of this intermediate compound is so fast (k > 1000 sec.\(^{-1}\)) that its kinetics could not be studied.

3. A comparison of the reaction parameters of the chymotrypsin-catalysed hydrolysis of the amide, ethyl ester and p-nitrophenyl ester of tyrosine gives further support to a three-step reaction scheme for enzymic hydrolysis, which has been previously proposed.

We are most grateful to Dr C. J. Martin for a sample of the p-nitrophenyl ester of N-benzoxycarbonyl-L-tyrosine as well as for some helpful advice.

REFERENCES


Oxidation of Serotonin and 5-Hydroxyindoles during the Denaturation of Oxyhaemoglobin

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Stacey (1956) reported that serotonin (5-hydroxytryptamine) disappears when it is incubated with washed erythrocytes. In a study of the transport of serotonin into erythrocytes we found no serotonin inside the cells, and it seemed possible that the serotonin was being destroyed by a factor in the erythrocytes. This paper explains why serotonin appeared to be destroyed by erythrocytes. A preliminary report of this work has already appeared (Ling & Blum, 1958).

EXPERIMENTAL AND RESULTS

Blood (about 15 ml.) was obtained from two rats lightly anaesthetized with ether which were bled from the neck into a polyethylene beaker containing 10 ml. of 0·85% NaCl with either 0·1 g. of ethylenediaminetetra-acetic acid or 6 mg. of heparin. The erythrocytes were then centrifuged for 3 min. at 2000 g and the supernatant fluid was decanted. The cells were resuspended in 47–50 ml. of 0·12 M NaCl–0·04 M Na,HPO\(_4\) buffer, pH 7·4. The cells were washed three times in about 40 ml. of this buffer. Blood obtained from other species was treated in the same way. When necessary, the washed erythrocytes were haemolsed by adding 2–5 vol. of water. Crystalline haemoglobin was prepared from dog blood by the procedure of Drabkin (1946).

The creatinine sulphate salt of serotonin, bufotenin (NN-dimethylserotonin) mono-oxalate, tryptamine hydrochloride, indol-3-ylacetic acid and 5-hydroxyindol-3-ylacetic acid (5-HIAA) were purchased from the California Biochemical Foundation. Crystalline egg albumin, serum globulin, cytochrome c, glutathione and 5-hydroxytryptophan were purchased from the Nutritional Biochemical Corp. Catalase was purchased from Worthington Biochemicals Inc., haemin from Eastman Organic Chemicals.

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