# The Inhibition of Chymotrypsin by Diethyl *p*-Nitrophenyl Phosphate

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The alkyl fluorophosphonates were first shown in 1940 (Adrian, Feldberg & Kilby, 1947) to be very powerful inhibitors of cholinesterase, and for a time they were thought to be quite specific for this enzyme. Later, other esterases, such as human milk lipase and liver esterase (Webb, 1948) and citrus acetylesterase (Jansen, Nutting & Balls, 1947) were also shown to be inhibited, although not to the same extent. The discovery that trypsin and chymotrypsin display esterase as well as proteolytic activity (Schwert, Neurath, Kaufman & Snoke, 1948; Kaufman, Schwert & Neurath, 1948) prompted the investigation of the action of fluorophosphonates on these enzymes (Jansen, Nutting, Jang & Balls, 1949), and it was found that both were sensitive to di*iso*propyl fluorophosphonate (DFP) and that the esterase and proteolytic activities were inhibited in parallel. A considerable number of other organic phosphorus compounds have been discovered which resemble the fluorophosphonates in being highly toxic and powerful anti-cholinesterases. Some of these are now being produced commercially as insecticides, *OO*-diethyl *O*-*p*-nitrophenyl thiophosphate (Parathion) being one of the best known. The increasing use of such toxic compounds makes a fuller knowledge of their mode of action desirable. It would also be valuable if one could eventually suggest, on a rational basis, the direction in which a search could most profitably be made for compounds of high insecticidal activity combined with low mammalian toxicity. It appeared to us that a suitable starting point for the investigation of the mode of action of organic phosphorus compounds would be a detailed study of a model system using an inhibitor whose chemistry is straightforward and an enzyme which could be obtained pure and crystalline, preferably of small and known molecular weight. As an inhibitor, we have selected diethyl *p*-nitrophenyl phosphate (E 600, Paraoxon), the oxygen analogue of Parathion, as the chemistry of the latter is complicated by isomerization and really pure material is very difficult to obtain. Chymotrypsin has been found to be a very suitable enzyme, and is interesting in possessing several types of enzymic activity. The enzymic hydrolysis of certain *p*-nitrophenyl esters has been investigated, and the inhibition of the proteolytic, esterase and amidase activities of chymotrypsin by E 600 has been studied.

## EXPERIMENTAL

### *Materials*

*Chymotrypsin.* Two samples of enzyme were used: crystalline bovine α-chymotrypsin supplied by Armour and Company of Chicago (sample *A*) and a similar specimen from Canada of unknown origin (sample *B*). Both had been obtained by recrystallization from magnesium sulphate solution. The proteolytic activities were determined by Anson's haemoglobin method (Anson, 1938), as used by Kunitz & Northrop (1935) and expressed as tyrosine units T.U.$^{Hb}$/mg. protein nitrogen (P.N.), where one T.U. is the amount of enzyme which will liberate the equivalent of 1 mmol. tyrosine/ml./min. at 35·5°. Kunitz & Northrop (1935) report a mean value of 0·039 T.U.$^{Hb}$/mg. P.N. for pure chymotrypsin recrystallized 8–10 times and containing 15·5% N. Owing to the small amount of material available, recrystallization of our samples to constant activity was not attempted, and the amounts of active chymotrypsin in them were calculated using the value given by Kunitz & Northrop for pure enzyme (Table 1).

*Diethyl* p-*nitrophenyl phosphate* (E 600). Diethylchlorophosphonate was added to a suspension of an equimolecular amount of anhydrous sodium *p*-nitrophenate in dry xylene and heated at 100–110° for 1 hr. After removal of salt and solvent, the oil was washed with $Na_2CO_3$, dried and distilled, b.p. 142°/6 × $10^{-3}$ mm. (Schrader, 1946).

*Ethyl* p-*nitrophenyl carbonate.* M.p. 66·5–67° (Ransom, 1898).

p-*Nitrophenyl acetate.* M.p. 79·5–80° (Kaufmann, 1909).

Table 1. *Chymotrypsin content of enzyme samples*

(T.U.$^{Hb}$/mg. P.N. defined above).

| Sample | N analysis* (% in sample) | Protein (% in sample) | Proteolytic activity (T.U.$^{Hb}$/mg. P.N.) | Chymotrypsin in protein (%) | Chymotrypsin in sample (%) |
|---|---|---|---|---|---|
| *A* | 11·5 | 74 | 0·026 | 67 | 49 |
| *B* | 14·1 | 91 | 0·028 | 71 | 65 |

* The non-protein nitrogen was less than 0·1%.

L-*Tyrosine ethyl ester*. M.p. 107° (Fischer, 1901).

*N-acetyl* L-*tyrosine amide*. M.p. 223° (Kaufman & Neurath, 1949).

### *Methods*

*Measurement of proteolytic activity*. This was measured by a haemoglobin digestion method similar to that described by Anson (1938) for the assay of the proteolytic activity of trypsin. A standard solution of denatured haemoglobin was prepared from whipped ox blood. This substrate (5 ml.) was incubated at pH 7·5 and 35·5° with 1 ml. of enzyme solution for exactly 10 min. The reaction was then stopped, and unchanged protein precipitated, by shaking with 10 ml. of 0·3 N-trichloroacetic acid. After standing for 30 min. the reaction products were filtered through Whatman no. 1 paper. 0·5 N-NaOH (10 ml.) and 3 ml. Folin & Ciocalteu's (1927) phenol reagent were added to 5 ml. of filtrate, and the colour which developed was compared within 5–10 min. with that from a standard solution of tyrosine by means of the 'Spekker' photoelectric absorptiometer using orange filters (O.Y. 2). Blanks were prepared for each determination by mixing 1 ml. enzyme solution with 10 ml. 0·3 N-trichloroacetic acid before adding 5 ml. of substrate, and the colour developed and measured as before. A calibration curve for the instrument was plotted, and a linear relationship found to hold between the drum reading and tyrosine concentration over the range 1–8 mmol.-tyrosine/5 ml. solution. There was also a linear relationship between the concentration of enzyme incubated with substrate and the colour subsequently developed in the filtrate over the range 0·01–0·10 mg. enzyme sample/ml. enzyme solution.

During inhibition by diethyl *p*-nitrophenyl phosphate (E 600), nitrophenol is liberated, but experiments showed that at the concentrations observed it has no effect on either the digestion or colour development stage of this determination.

*Assay of* p-*nitrophenol*. The absorption spectrum of nitrophenol in alkaline solution was found to show a peak at 4000 A. due to the nitrophenate ion. The optical density at this wavelength, measured on the Unicam Quartz Spectrophotometer, was used to estimate nitrophenol concentrations. Glass cells 10 × 10 × 40 mm. were used. A calibration curve of optical density against nitrophenol concentration over a range $2 \times 10^{-6}$ to $6 \times 10^{-5}$ M in 0·1 M-phosphate buffer, pH 7·60, and containing 5% *iso*propanol, was plotted and used in subsequent estimations (5% *iso*propanol had no effect on chymotrypsin activity during incubation at 25° for 18 hr.).

When the nitrophenol liberated in solutions containing chymotrypsin and a *p*-nitrophenyl ester was being measured, blank solutions identical except that no enzyme was present were prepared and incubated in parallel.

During runs in which the liberation of nitrophenol was being followed kinetically, the glass cells with lids were kept at the bottom of boiling tubes deeply immersed in a thermostatically controlled bath. The tubes contained a little cotton wool and water such that the level of liquid inside and outside the glass cell was the same. At intervals, the cells were removed from the tubes, dried and the optical densities measured as rapidly as possible.

*Hydrolysis of* L-*tyrosine ethyl ester* (*TEE*). The rate of hydrolysis of TEE was measured by a continuous potentiometric titration method similar to that of Schwert *et al.* (1948), whereby the acid liberated during hydrolysis was continuously titrated with dilute alkali to constant pH as measured on a Marconi pH meter.

The electrodes dipped into 25 ml. of a 0·025 M solution of TEE, pH 6·20, contained in a beaker in a bath at 25° and stirred by a stream of $N_2$. Enzyme solution (1 ml.) was added, and 0·02 N-NaOH was run in in portions of 0·50 ml., the time when the pH meter registered pH 6·20 being noted. The pH varied between 6·15 and 6·30 during the hydrolysis.

Unbuffered solutions were found preferable with this dilute alkali, and the reference pH 6·20 was chosen to give the optimum pH of 6·25 during the hydrolysis as quoted by Jansen *et al.* (1950).

*Hydrolysis of* N-*acetyl* L-*tyrosine amide* (*ATA*). This was followed by a method similar to that of Schwert *et al.* (1948), whereby the $NH_3$ liberated at intervals during the hydrolysis is measured by a modification of the Conway microdiffusion method.

A solution of chymotrypsin in 0·1 M-phosphate buffer, pH 7·60, was mixed with a solution of 0·04 M-ATA in buffer containing 10% *iso*propanol and incubated at 25°. Samples (0·2 ml.) were withdrawn at intervals and introduced into the outer rings of Conway plates. Saturated $K_2CO_3$ (1 ml.) was added to the outer rings to volatilize the $NH_3$, and the plates were tipped and mixed, the $NH_3$ being absorbed in the inner chambers in 1% boric acid plus indicator. The plates were left at room temperature overnight before titrating with 0·01 N-HCl from a Conway microburette. This acid had previously been calibrated against standard $(NH_4)_2SO_4$ solutions under the same conditions. The period of digestion was taken as the time from addition of substrate until the mixing of the sample with alkali. Blanks were carried out by placing 0·1 ml. of enzyme solution and 0·1 ml. of ATA solution apart in the outer rings of the plates and mixing only after addition of the saturated $K_2CO_3$. Constant low blanks were obtained. The spontaneous hydrolysis of ATA under these conditions was negligible.

## RESULTS

### *Inhibition of chymotrypsin by E 600*

E 600 inhibits the proteolytic activity of crystalline chymotrypsin. Various concentrations of E 600 in *iso*propanol were added to a solution of chymotrypsin in 0·01 M-phosphate buffer, pH 7·60. The final concentrations were: chymotrypsin (sample *B*), 1 mg./ml.; E 600, $10^{-4}$ to $1{\cdot}5 \times 10^{-3}$ M; *iso*propanol, 5%.

The solutions were incubated for either 254 or 423 min. at 25° and the proteolytic activity then determined by the method given in the experimental section. A number of experiments showed that there was negligible change in activity of control solutions (without inhibitor) incubated under the same conditions for 24 hr. The logarithm of the percentage residual activity was then plotted against the inhibitor concentration (Fig. 1). After 423 min. incubation under the conditions used, a 50% inhibition of enzyme was given by $3 \times 10^{-4}$ M-E 600. Tests were carried out on the reversibility of this inhibition.

The rate of inhibition by $10^{-3}$ M-E 600 of a solution of chymotrypsin at pH 7·60 at 25° was measured in

the presence of a concentration of nitrophenol one hundred times that liberated during the inhibition. Control experiments showed that such concentrations had negligible effect on the estimation of proteolytic activity by Anson's method. No differences in activity were found between samples withdrawn at various times from a solution containing enzyme, inhibitor and nitrophenol, and a control containing only enzyme and inhibitor.

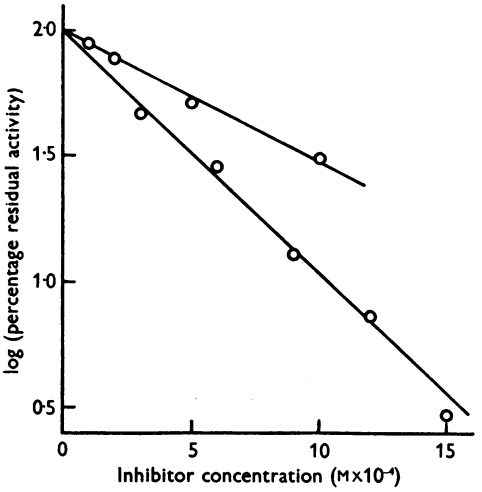


Fig. 1. Inhibition of proteolytic activity of chymotrypsin by E 600 at 25° after incubation for 254 min. (upper line) and 423 min. (lower line). Chymotrypsin concentration, 1 mg./ml. (sample *B*).

A further check on the irreversibility of the inhibition was obtained by testing the activity of a solution of chymotrypsin which had been completely inhibited by reaction with $10^{-3}$ M-E 600 for 24 hr. at 25°, and then dialysed against 0·005 N-sulphuric acid at 5° for 26 hr. The proteolytic activity of this sample, estimated by Anson's method, was compared with that of the original solution of chymotrypsin which had been incubated and dialysed in parallel. The activity of the inhibited sample was 3 % of the control, which is less than the experimental error of the determination.

### *Hydrolysis of* p-*nitrophenyl carbonate (NPC) and acetate (NPA)*

These substances were investigated as possible substrates for chymotrypsin. Solutions of the substrate were made up in *iso*propanol to give the required final substrate concentrations in 5 % *iso*propanol when added to a solution of the enzyme in 0·1 M-phosphate buffer, pH 7·60. The rate of liberation of nitrophenol was measured by the rate of increase of optical density at 4000 A. Preliminary experiments indicated that the substrates were hydrolysed 200 times more quickly at 25° at pH 7·60 in the presence of 1 mg./ml. chymotrypsin than in its absence. The rate of spontaneous hydrolysis was constant over the observed period and proportional to the substrate concentration. The first-order velocity constants ($k$) were $9{\cdot}2 \times 10^{-4}$ min.$^{-1}$ for the acetate and $3{\cdot}5 \times 10^{-4}$ min.$^{-1}$ for the carbonate. The latter was used in most experiments to reduce corrections for spontaneous hydrolysis.

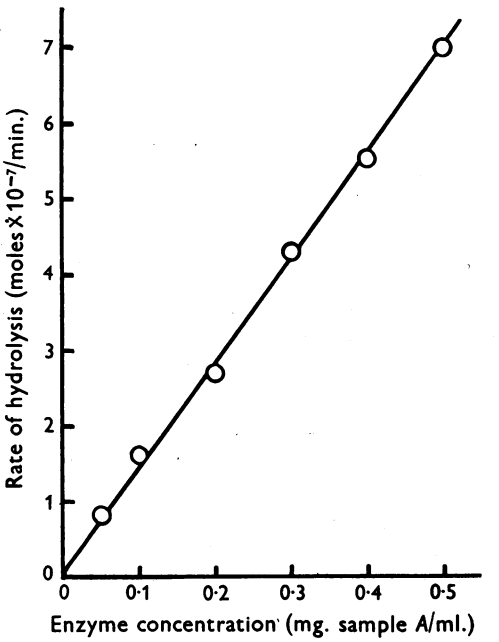


Fig. 2. Relationship between the rate of enzymic hydrolysis of *p*-nitrophenyl ethyl carbonate (at 25° and pH 7·6) and the concentration of chymotrypsin present. (Substrate concentration, $10^{-3}$ M.)

The rate of hydrolysis of NPC was found to be proportional to chymotrypsin concentration (Fig. 2). The concentration of NPC was $10^{-3}$ M. The average activity found was $2{\cdot}86 \times 10^{-6}$ moles/min./mg. pure chymotrypsin giving a turnover number of 78·6 molecules substrate/min./molecule chymotrypsin. The initial rates of enzymic hydrolysis of NPC were independent of substrate concentration over the range $5 \times 10^{-4}$ to $2{\cdot}5 \times 10^{-5}$ M, but at the latter concentration the rate of hydrolysis ceased to be linear after 8 min. and hydrolysis was complete after 15 min. Since the initial velocity of hydrolysis was constant, except at very low substrate concentrations, it was found desirable to use a 'progress curve' method to determine the Michaelis constant ($K_m$). A solution of chymotrypsin in M/15 phosphate buffer, pH 7·60, was diluted with NPC in buffer containing *iso*propanol to final concentrations of chymotrypsin (65% pure), 1 mg./ml.; NPC,

$3 \times 10^{-5}$ M; *iso*propanol, 5 %. The rate of hydrolysis was determined by the optical density method.

Tangents to the hydrolysis curve were constructed where it began to depart from a straight line (Fig. 3), giving the rate of hydrolysis ($V$). The substrate remaining ($S$) was calculated by subtracting the nitrophenol concentration at that time

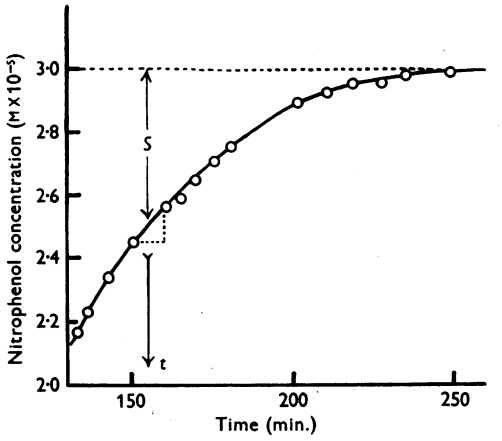


Fig. 3. Later (non-linear) portion of hydrolysis curve of *p*-nitrophenyl ethyl carbonate by chymotrypsin. The tangent to the curve at time $t$ gives the rate of hydrolysis $V$ at substrate concentration $S$. Chymotrypsin concentration, 1 mg./ml. (sample $B$).

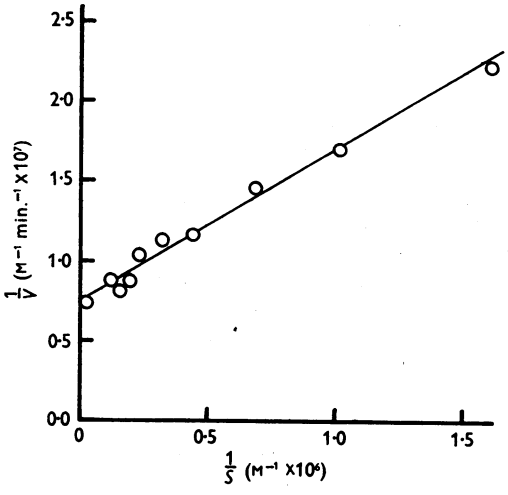


Fig. 4. Velocities of hydrolysis by chymotrypsin ($V$) of *p*-nitrophenyl ethyl carbonate at 25°, pH 7·6, at various substrate concentrations ($S$) plotted as reciprocals to obtain $K_m$.

from the infinity value. A plot of $1/V$ against $1/S$ (Fig. 4) gave a straight line from which $K_m$ was found to be $1{\cdot}3 \times 10^{-6}$ M from the equation

$$\frac{1}{V} = \frac{K_m}{V_0}\frac{1}{S} + \frac{1}{V_0}$$ (Lineweaver & Burk, 1934).

Similar measurements were made with NPA and the approximate values of $K_m$ and turnover number were $3 \times 10^{-3}$ M and 920 molecules/min./molecule chymotrypsin respectively.

*Amount of nitrophenol liberated after complete inhibition of chymotrypsin* (ChTr) *by E* 600

Table 2 gives the results of a series of experiments in which the concentration of nitrophenol was measured by the optical density method after the proteolytic activity of the enzyme had been completely inhibited. The 'infinity' value was measured after 18–27 hr. incubation. It remained constant for several hours.

Table 2. *Observed and calculated concentrations of nitrophenol after complete inhibition of chymotrypsin by E* 600

(Nitrophenol observed = 96·7 % of theoretical (mean of 13). The theoretical nitrophenol concentrations were calculated assuming Eqn. 1, enzyme purities of 49 and 65 % for samples $A$ and $B$ respectively, and a mol.wt. for chymotrypsin of 27,000.)

| Concn. of ChTr (mg./ml.) | Concn. of E 600 (M x $10^{-4}$) | Observed nitrophenol (M x $10^{-5}$) | Theoretical nitrophenol (M x $10^{-5}$) |
|---|---|---|---|
| 1·0 | 10 | 1·64 (mean of 5) | 1·82 |
| (sample $A$) | 5 | 1·75 | 1·82 |
| 1·5 | 15 | 3·62 (mean of 2) | 3·61 |
| (sample $B$) | 10 | 3·54 (mean of 3) | 3·61 |
| | 7·5 | 3·70 (mean of 2) | 3·61 |

The purity of the enzyme was calculated by comparing the proteolytic activity at 35·5° with that given by Northrop for the pure enzyme. The last column gives the theoretical concentration of nitrophenol calculated from the equation

$$\underset{(a-x)}{\text{ChTr—H}} + \underset{\text{(I)}}{\text{NO}_2.\text{C}_6\text{H}_4.\text{O.PO.(OEt)}_2} \xrightarrow[k_i]{} \underset{(x)}{\text{ChTr—PO.(OEt)}_2} + \underset{(x)}{\text{NO}_2.\text{C}_6\text{H}_4.\text{OH}}, \quad (1)$$

assuming a molecular weight of 27,000 for the enzyme (Jansen, Nutting & Balls, 1949). Denatured chymotrypsin does not react with E 600 according to Eqn. 1. A solution of chymotrypsin in 0·0025 N-hydrochloric acid was divided into two; one portion was partly denatured by heating at 100° for 15 min. Samples of both solutions were incubated at 25° and pH 7·60 with $10^{-3}$ M-E 600, and the nitrophenol liberated after 22 hr. was measured. The proteolytic activities of the denatured and control enzyme solutions were measured after incubating overnight at 25°. The denatured enzyme showed a fall in proteolytic activity to 72 % of that of the control, while the nitrophenol which it liberated from E 600 was 68 % of that liberated by the control. Thus the 28 % of denatured protein formed does not react with E 600 to liberate nitrophenol.

*Rate of inhibition of chymotrypsin with various substrates*

Simultaneous estimations of the rate of inhibition of proteolytic activity and the rate of liberation of nitrophenol were made as in a previous paper (Hartley & Kilby, 1950). Chymotrypsin solutions in 0·1 M-phosphate buffer, pH 7·60, were incubated at 25° with E 600, the final concentrations being: ChTr, 1·0 mg./ml.; E 600, $10^{-3}$ M; *iso*propanol, 5 %. The proteolytic activity was measured at various times by the haemoglobin method and the rate of liberation of nitrophenol by the optical density method. The results of a number of such experiments are indicated in Fig. 5. It can be seen that the proteolytic activity is inhibited at the same rate as nitrophenol is liberated.

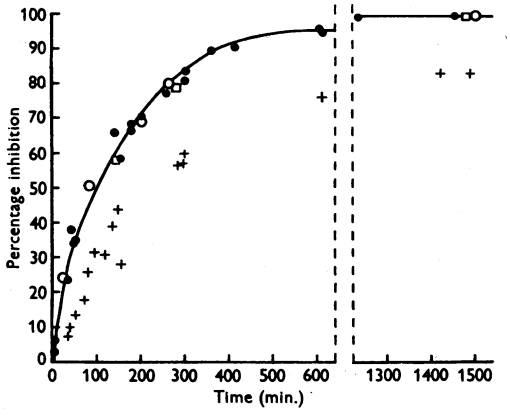


Fig. 5. Rate of inhibition of various enzymic activities of chymotrypsin by $10^{-3}$ M-E 600 at 25°, pH 7·6. Percentage inhibition of proteolytic activity, ● (Hb); of amidase activity, ○ (ATA); of amino-acid esterase activity, □ (TEE); and of nitrophenyl esterase activity, + (NPC). The curve is the rate of liberation of nitrophenol (as percentage of 'theoretical'), calculated from Eqn. 1, using the observed velocity constant, $k_i = 5{\cdot}3$.

The rate of inhibition of the enzymic hydrolysis of TEE was compared with the rate of liberation of nitrophenol. A solution of chymotrypsin at pH 7·60 was incubated at 25° with $10^{-3}$ M-E 600 and the rate of inhibition estimated by withdrawing samples at various intervals and measuring the rate of hydrolysis of 0·025 M-TEE by the potentiometric titration method after suitable dilution of the inhibited enzyme. The results obtained, plotted in Fig. 5, indicate that the hydrolysis of TEE by ChTr is inhibited at the same rate as the proteolytic activity and bears the same relation to the rate of liberation of nitrophenol. From the initial rate of hydrolysis, the turnover number was found to be 1500 molecules substrate/min./molecule enzyme.

Chymotrypsin was inhibited by $10^{-3}$ M-E 600 under the same conditions as before. Samples were withdrawn at intervals and after suitable dilution were incubated at 25° with $10^{-3}$ M-NPC in 0·1 M-phosphate buffer, pH 7·60, containing 5 % *iso*propanol. The rate of hydrolysis was measured by the change in optical density at 4000 A. The rate of inhibition of this esterase activity is shown in Fig. 5. The rate of inhibition of hydrolysis of NPC does not coincide with the inhibition of other activities of chymotrypsin.

Solutions of chymotrypsin in 0·1 M-phosphate buffer, pH 7·60, were incubated at 25° in the presence of E 600 and *iso*propanol, final concentrations being: ChTr (43 % pure), 3·00 mg./ml.; E 600, $10^{-3}$ M; *iso*propanol, 5 %. At intervals, samples were withdrawn and diluted with equal volumes of a solution of 0·04 M-ATA in buffer containing 5 % *iso*propanol.

The rate of liberation of ammonia at 25° in these mixtures was followed by the Conway micro-diffusion technique described above. Control experiments carried out in parallel indicated that the rate of hydrolysis of ATA by chymotrypsin under these conditions was linear, corresponding to a turnover number of 4·8 molecules ATA/min./molecule pure ChTr. The rate of liberation of ammonia in the various inhibited samples compared to that of the control gave the percentage inhibition. The rate of inhibition of amidase activity is shown in Fig. 5. The amidase activity of chymotrypsin is inhibited at the same rate as the proteolytic and amino-acid esterase activity.

*Kinetics of liberation of nitrophenol*

The kinetics of the reaction between chymotrypsin and E 600 were studied by following the rate of liberation of nitrophenol by measurements of optical density changes. Solutions in buffer, pH 7·6, containing 0·5–0·7 mg. pure enzyme/ml., 5 % *iso*propanol and various concentrations of E 600 were incubated at 25°. If the inhibitor concentration ($I$) is large compared with that of the enzyme ($a$) and a reaction of the form of Eqn. 1 is assumed, then

$$\frac{dx}{dt} = k_i I (a - x),$$

i.e.

$$t = \frac{2{\cdot}303}{Ik_i} \log \frac{a}{(a-x)}. \qquad (2)$$

The concentration of nitrophenol observed at time $t$ is $x$, while that after complete inhibition (about 18 hr.) is $a$. In Fig. 6, $\log 100\,(a-x)/a$ is plotted against $t$, for various inhibitor concentrations. The straight line plots support a reaction of the form postulated above, and the velocity constant of inhibition ($k_i$) can be calculated from their slopes. Values of $k_i$ obtained in this way from the data of Fig. 6 are given in Table 3.

*Kinetics of inhibition of proteolytic activity*

A check on the identity of the rate of inhibition of proteolytic activity and the rate of liberation of nitrophenol is given by the results plotted in Fig. 1.

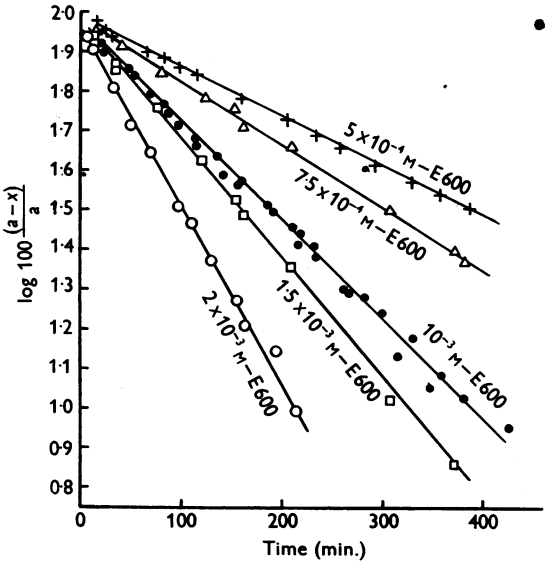


Fig. 6. Rate of liberation of nitrophenol from chymotrypsin and various concentrations of E 600 at 25°, pH 7·6.

Table 3. *Velocity constant for reaction of chymotrypsin and E* 600

| Concn. of E 600 ($M \times 10^{-4}$) | $\frac{d}{dt} \log \frac{100\,(a-x)}{a}$ | $k_i$ (litres moles$^{-1}$ min.$^{-1}$) |
|---|---|---|
| 5 | 0·124 | 5·7 |
| 7·5 | 0·158 | 4·9 |
| 10 | 0·246 | 5·7 |
| 15 | 0·299 | 4·6 |
| 20 | 0·457 | 5·3 |

Since $100\,\frac{(a-x)}{a}$ is the percentage residual proteolytic activity, the velocity constant of inhibition ($k_i$) can be calculated from the slopes of the two lines using Eqn. 2. Values of 5·0 and 5·3 for $k_i$ are obtained from the 245 and 423 min. incubation curves respectively, in satisfactory agreement with the values of $k_i$ in Table 3, which are derived from nitrophenol liberation measurements.

## DISCUSSION

There is an increasing amount of evidence to show that chymotrypsin contains only a single active centre, and that this is responsible for the various different types of enzymic activity shown by the enzyme.

Di*iso*propyl fluorophosphonate (DFP) inhibits chymotrypsin in such low concentration that the enzyme and inhibitor must be present in about equimolecular amounts. It has been shown (Jansen, Nutting & Balls, 1949; Jansen *et al.* 1950) that when chymotrypsin is completely inhibited by DFP containing $^{32}P$, the inactivated enzyme can be obtained crystalline and contains about one atom of phosphorus and two *iso*propoxy groups per molecule; there is some evidence that the fluorine atom of the inhibitor is eliminated as hydrogen fluoride. No combination between inhibitor and protein could be demonstrated when DFP was incubated with chymotrypsinogen, the precursor of the enzyme, in which the 'active centre' is presumably 'masked'.

The results obtained from our studies on the inhibition of chymotrypsin by E 600 support the hypothesis that this enzyme contains one grouping only which can combine with the phosphorus group of inhibitors. The amount of nitrophenol liberated when chymotrypsin has reacted completely with E 600 is shown in Table 2 and the close correspondence between the observed concentrations and those calculated for a reaction such as

$$\text{ChTr—H} + (\text{EtO})_2\text{PO}.\text{C}_6\text{H}_5.\text{NO}_2 = \text{ChTr—PO—}(\text{OEt})_2 + \text{NO}_2.\text{C}_6\text{H}_4.\text{OH}$$

supports this type of reaction.

The rate of appearance of nitrophenol in a solution containing an excess of inhibitor satisfies first-order kinetics as would be expected from the above reaction; the first-order velocity constant $k_i$ which was obtained from nitrophenol measurements also satisfied the kinetics of proteolytic inhibition (Fig. 5).

The inhibition cannot be reversed by the addition of excess nitrophenol during the reaction. Dialysis of inhibited enzyme failed to regenerate significant activity. It may be concluded that the inhibition is irreversible, in the sense that the reaction with inhibitor proceeds only to the right, although a slow reactivation by hydrolysis of the —O.PO(OEt)$_2$ group attached to the enzyme (such as that reported by Wilson (1951) in the inhibition of cholinesterase by tetraethyl pyrophosphate) cannot be totally excluded.

Many workers believe that the inhibitory power of the organic phosphorus group of compounds depends on the presence of an easily hydrolysed P—X bond in molecules such as $(RO)_2POX$ or $(NMe_2)_2POX$, combined with an electrophilic phosphorus atom. A type of phosphorylation mechanism, as in Eqn. 1, is in keeping with the nature of the inhibitors. The single centre appears to be responsible for the various enzymic activities of chymotrypsin. The proteolytic activity (measured by the haemoglobin method), the amino-acid

esterase and amidase activities, as exemplified by the hydrolysis of L-tyrosine ethyl ester and acetyl L-tyrosinamide respectively, were all found to be inhibited at exactly the same rate as nitrophenol was liberated. A very close correspondence between the centre involved and the site of attack of the inhibitor is indicated. Very stringent structural requirements for the specific substrates for chymotrypsin have been postulated by Schwert & Neurath (1950). The requirements for ester, amide and peptide substrates are very similar, again suggesting reaction at a common active centre. The organic phosphorus inhibitors do not conform to these structural requirements but do show a great affinity for the enzyme, and if, as we believe, they react at the active centre, it appears possible that non-phosphorus analogues might behave as substrates rather than as inhibitors. A carbon analogue of E 600, *p*-nitrophenyl ethyl carbonate, was tried and found to be non-inhibitory, and to be enzymically hydrolysed, although the turnover number was rather lower than that for L-tyrosine ethyl ester. *p*-Nitrophenyl acetate was also a substrate, and was hydrolysed rather more readily.

The inhibition of the *p*-nitrophenyl esterase activity was anomalous, in that it did not follow the same curve as those for the other enzymic activities (Fig. 5), and when the latter had been completely inhibited, about 20% of the nitrophenyl esterase activity remained. The reason for this difference is not clear at present. This anomalous behaviour is being investigated further.

## SUMMARY

1. The proteolytic activity of chymotrypsin is inhibited by diethyl *p*-nitrophenyl phosphate (E 600). 50% inhibition is produced by $3 \times 10^{-4}$ M-E 600 after 423 min. incubation at pH 7·6 at 25°. The inhibition is irreversible.

2. *p*-Nitrophenyl ethyl carbonate and *p*-nitrophenyl acetate are substrates for chymotrypsin. Values of $K_m$ and turnover number are obtained.

3. *p*-Nitrophenol is liberated during inhibition of chymotrypsin by E 600. The molar concentration of nitrophenol liberated after complete inhibition is equivalent to the molar concentration of the enzyme.

4. The rates of inhibition by E 600 of the proteolytic, amidase and amino-acid esterase are equal and exactly correspond to the rate of liberation of nitrophenol during the inhibition.

5. The kinetics of liberation of nitrophenol are explained on the basis of a bimolecular reaction between enzyme and inhibitor. The velocity constant obtained satisfies the kinetics of inhibition of proteolytic activity.

6. The results support the hypothesis of a single active centre in chymotrypsin.

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