The Reaction of *p*-Nitrophenyl Esters with Chymotrypsin and Insulin

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In a previous paper (Hartley & Kilby, 1952), the reaction of chymotrypsin with diethyl *p*-nitrophenyl phosphate (E 600, Paraoxon)

$$(EtO)_2PO.O.C_6H_4.NO_2$$

was discussed. This compound is a member of a group of organophosphorus insecticides which are powerful inhibitors of cholinesterases. They will also inhibit chymotrypsin, and it has been shown that this inhibition is due to combination of the compounds with a single active centre in the enzyme (Jansen, Nutting & Balls, 1949; Hartley & Kilby, 1952). We investigated the reaction of chymotrypsin with certain structural analogues of E 600, namely *p*-nitrophenyl ethyl carbonate (NPC)

$$p\text{-}NO_2.C_6H_4.O.CO.OEt$$

and *p*-nitrophenyl acetate (NPA)

$$p\text{-}NO_2.C_6H_4.O.CO.CH_3.$$

These esters were shown to be substrates for chymotrypsin, but the turnover numbers were very low compared with those for synthetic amino acid ester and amide substrates (Kaufman, Neurath & Schwert, 1949). In this connexion we must apologize for errors in the calculation of the turnover numbers given in our previous paper; these should read 0·078 mole NPC/min./mole chymotrypsin and 0·92 mole NPA/min./mole chymotrypsin at 25°, pH 7·6. E 600 inhibited the proteolytic, amino acid esterase and amidase activities of chymotrypsin at the same rate (as typified by the hydrolysis of haemoglobin, L-tyrosine ethyl ester, and *N*-acetyl-L-tyrosinamide, respectively). This rate, moreover, was identical with that of the progressive liberation of nitrophenol resulting from a stoicheiometric reaction between enzyme and E 600. However, the rate of inhibition of the enzymic hydrolysis of NPC was anomalous, in that the percentage inhibition of this activity by E 600 at any time was always less than that of the three other activities. When the enzyme had reacted completely with inhibitor and all the other activities were abolished, it would still liberate nitrophenol from NPC. The reaction of chymotrypsin with *p*-nitrophenyl ethyl carbonate and *p*-nitrophenyl acetate, and the anomalous rate of inhibition of nitrophenyl esterase activity were therefore studied.

EXPERIMENTAL

Materials

Chymotrypsin. Crystalline bovine α-chymotrypsin was used. Sample *B* was a gift from Dr Hurtig of Ottawa; sample *C* was purchased from Armour and Co. Both had been recrystallized from $MgSO_4$ solutions. The percentage purities, calculated as in a previous paper (Hartley & Kilby, 1952) from the specific proteolytic activities were 65 and 48% for samples *B* and *C*, respectively.

Chymotrypsin inhibited by diisopropyl phosphonofluoridate. A gift from Dr E. F. Jansen. It was stated that α-chymotrypsin had been more than 99% inhibited with di*iso*propyl phosphonofluoridate, the protein recrystallized twice, exhaustively dialysed, and lyophilized. The water content was 8%.

Insulin. Two samples were used; an aqueous solution of Boots' '40-Insulin' and a highly purified crystalline product (Boots Pure Drug Co., Ltd.) Batch 9011*G* containing 8% moisture.

Diethyl p-*nitrophenyl phosphate* (*E* 600). B.p. 142° at 6×10^{-3} mm. Solutions in phosphate buffer, pH 7·6, were allowed to stand at room temperature for 24 hr. before use in order to destroy traces of easily hydrolysed impurities, such as tetraethyl pyrophosphate (Aldridge, 1952).

Diisopropyl phosphonofluoridate (*diisopropyl fluorophosphonate, DFP*). B.p. 72–73° at 15 mm. (Saunders, Stacey, Wild & Wilding, 1948).

p-*Nitrophenyl acetate* (*NPA*). M.p. 66·5–67° (Ransom, 1898).

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

Tyrosine derivatives and analogues. L-Tyrosine ethyl ester, m.p. 107° (Fischer, 1901); *N*-acetyl-L-tyrosine ethyl ester, m.p. 80° (Du Vigneaud & Meyer, 1932); DL-phenylalanine ethyl ester hydrochloride, m.p. 122° (Curtius & Müller, 1904); *N*-acetyl-DL-phenylalanine ethyl ester was prepared by acetylating DL-phenylalanine ethyl ester with keten, m.p. 69° (Cherbuliez & Plattner (1929) give m.p. 69°); tyramine hydrochloride (L. Light and Co.); 2-phenylethylamine (British Drug Houses Ltd.); methyl β-(*p*-hydroxyphenyl)propionate, b.p. 108° at 11 mm. (Bowden & Adkins, 1940); methyl β-phenylpropionate, b.p. 108° at 12·5 mm. (Fischer & Nouri, 1917); tyramine methyl ether hydrochloride, a gift from Dr P. Hey.

Methods

p-*Nitrophenol concentration and proteolytic activity.* These were measured by methods described by Hartley & Kilby (1952).

Total nitrogen. This was estimated by a micro-Kjeldahl method.

Amino nitrogen. This was measured by the Van Slyke gasometric method, the temperature being 19° and the reaction time 11 min., with shaking during the last minute.

Total acetyl groups in insulin. Samples of the insulin solutions (5 ml.) containing about 15 mg. protein were incubated at 90° for 2 hr. with 3 ml. 0·1 N-NaOH in 15 ml. centrifuge tubes. The solutions were cooled, and 2 ml. N-$CuSO_4$ were added to each to precipitate the excess of protein together with sulphides which interfere in the final titration. The solutions were centrifuged, the supernatants decanted, and the residues washed with 5 ml. water. The combined supernatants were washed into a Pregl steam distillation apparatus and were followed by 12 ml. saturated $KHSO_4$. Steam was blown through until 100 ml. distillate collected in a cooled receiver. The acetic acid was titrated with 0·008 N-$Ba(OH)_2$ using phenol red as indicator, CO_2 being excluded by passing a stream of CO_2-free air through the solution. Control samples of 5 ml. 0·002 M sodium acetate and blanks of 5 ml. distilled water were analysed in parallel with all estimations. The recovery of acetic acid in the first 100 ml. of distillate was 69±3% over the range 5–20 μmoles acetate. *O*-Acetyl groups would be hydrolysed under the experimental conditions (Gordon, Martin & Synge, 1943), but *N*-acetyl groups are probably stable. No acetic acid was recovered from *N*-acetyltyrosine by this method over a range 5–20 μmoles analysed.

Product of reaction of p-*nitrophenyl acetate with* DL-*phenylalanine ethyl ester*

A solution of 230 mg. DL-phenylalanine ethyl ester hydrochloride (5×10^{-3} M) and 730 mg. NPA (2×10^{-2} M) in 200 ml. 0·2 M phosphate buffer, pH 7·6, containing 10% *iso*propanol was incubated at 37° for 72 hr. It was then cooled and extracted with four 50 ml. portions of ether. The ether extract was washed with cold 2% (w/v) Na_2CO_3 until no appreciable yellow colour remained in the aqueous phase, then with 50 ml. cold 0·1 N-HCl, and finally with water. After being dried over anhydrous Na_2SO_4, the ether was evaporated. The product was crystallized and recrystallized from a mixture of ether and light petroleum (b.p. 40–60°) and dried *in vacuo*. Yield: 46 mg. (20%), m.p. 68°. The mixed m.p. with *N*-acetyl-DL-phenylalanine ethyl ester was 68°.

Product of reaction of p-*nitrophenyl acetate with insulin*

Two 40 ml. samples of a solution of insulin in 0·1 M phosphate buffer, pH 7·6, were placed in dialysis bags. Each bag was suspended in 500 ml. buffer in a conical flask. NPA (100 ml. 10^{-2} M in *iso*propanol) was added to one flask and 100 ml. *iso*propanol to the other, so that final concentrations were: 0·23 mg. insulin sample/ml., $1{\cdot}5 \times 10^{-3}$ M NPA and 15% *iso*propanol. After being incubated for 100 min. at 37°, the contents of the dialysis bags were transferred to 50 ml. centrifuge tubes. The insulin was precipitated from the control sample by adjusting it to pH 5·0, but the 'acetylated' sample precipitated only when the pH was reduced to 4·2. The precipitates were centrifuged, washed twice with 0·002 M potassium hydrogen phthalate, and dissolved in 10 ml. 0·02 N-H_2SO_4 (sample 1, Table 4). In other experiments the period of incubation of NPA with insulin was altered. Moreover, in samples 2 and 3 the reaction product was purified by dialysis for 12 hr. against water at 0°. The control solution for sample 3 contained nitrophenol and acetic acid at the same concentration as the NPA used in the reaction mixture.

RESULTS

Inhibition of the p-*nitrophenyl esterase activity of chymotrypsin*

The rate of inhibition by 10^{-3} M E 600 of the nitrophenyl esterase activity of chymotrypsin was measured using *p*-nitrophenyl acetate (NPA) or *p*-nitrophenyl ethyl carbonate (NPC) as substrate. Chymotrypsin was incubated at 25° with purified E 600 in 0·066 M phosphate buffer, pH 7·60. The progress of the reaction was observed by measuring the optical density at 4000 Å. The spontaneous hydrolysis of the inhibitor was measured in parallel using control solutions containing no enzyme. The nitrophenol liberated at infinite time (18–20 hr.) in these experiments corresponded to 0·96, 1·00 and 1·04 moles nitrophenol/mole chymotrypsin, assuming a molecular weight of 23000 for chymotrypsin.

The nitrophenyl esterase activity was measured at intervals during the inhibition by incubating suitably diluted samples of the mixture of enzyme and inhibitor with NPA or NPC. The rates of liberation of nitrophenol in excess of that due to spontaneous hydrolysis of the substrates were measured and the percentage inhibition of nitrophenyl esterase activity was calculated by comparison with the activity of control solutions containing uninhibited enzyme.

The results are shown in Fig. 1. The rate of inhibition of enzymic hydrolysis of NPA differs from

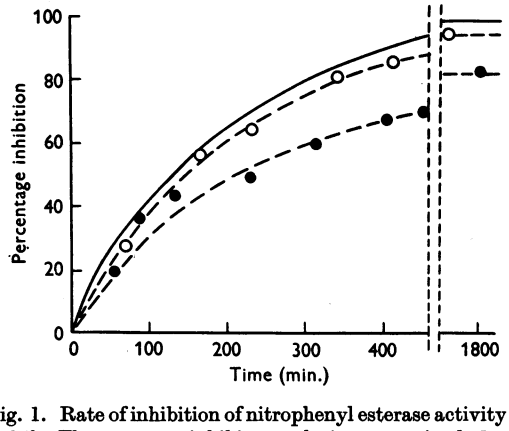


Fig. 1. Rate of inhibition of nitrophenyl esterase activity at 25°. The enzyme:inhibitor solution contained 1 mg. sample *C* chymotrypsin/ml. and 10^{-2} M E 600 in 0·066 M phosphate buffer, pH 7·60. The unbroken curve represents the nitrophenol liberated in this solution expressed as a percentage of its value at infinite time. Activity tests were made at intervals using 5×10^{-4} M NPA (○) or 5×10^{-4} M NPC (●).

that observed when NPC is used as substrate. Both rates of inhibition differ from the rate of liberation of nitrophenol from reaction of enzyme with inhibitor, and the fully dialkyl-phosphorylated enzyme appears to possess a significant nitrophenyl esterase activity.

The percentage residual activity of 'completely inhibited' chymotrypsin was investigated in more detail. The results of experiments using both NPA and NPC as substrates are shown in Table 1. The residual activity appears to depend upon the substrate concentration.

Recrystallizing an enzyme sample by a method similar to that of Butler (1940) did not alter the turnover number for 5×10^{-4}M NPC, which was 0·072 mole nitrophenol/min./mole enzyme. The recrystallized specimen was incubated for 19 hr. at 25°, pH 7·60, with 10^{-3}M E 600. Comparison of the rates of nitrophenol liberation in solutions containing 5×10^{-4}M NPC and inhibited or active enzyme showed that the residual activity was 25 %, which is identical within the experimental error with that of the original enzyme sample. The nitrophenyl esterase activity of inhibited chymotrypsin does not seem to be due to an impurity in the enzyme sample.

The effect of changing the inhibitor was investigated by incubating chymotrypsin with 10^{-4}M DFP at 25° for 45 min. Such incubation gives complete inhibition of proteolytic and amino-acid esterase activities (Jansen, Nutting, Jang & Balls, 1949). Samples of the inhibited enzyme solution or of an uninhibited enzyme control were incubated with NPA or NPC. The percentage residual activity of the inhibited enzyme (Table 1) was the same as that found with E 600 as inhibitor.

Table 1. *Residual nitrophenyl esterase activity of 'completely inhibited' chymotrypsin*

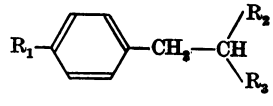
(NP = nitrophenol, NPA = *p*-nitrophenylacetate, DFP = di*iso*propyl phosphonofluoridate, NPC = *p*-nitrophenyl ethyl carbonate, E 600 = diethyl *p*-nitrophenyl phosphate. Conditions similar to those given for Fig. 1; DFP 10^{-4}M.)

Substrate	Inhibitor	Period of inhibition (hr.)	Rate of reaction (10^{-10} mole NP/min./mg. enzyme N)	Residual activity (%)
NPC (5×10^{-4}M)	E 600	25	22	18
	E 600	24	23	17
	DFP	0·8	24	18
NPC (10^{-3}M)	E 600	20	32	26
	E 600	24	29	24
NPA (10^{-3}M)	E 600	22	70	4
	E 600	25	67	5
	E 600	25	62	5
	DFP	0·8	105	5

Table 2. *Reaction of NPA or NPC with tyrosine analogues*

(For explanation of contractions see Table 1. The reactions were conducted with 5×10^{-4}M NPA or NPC, 10^{-3}M tyrosine analogue and 5 % *iso*propanol in 0·066M phosphate buffer, pH 7·60, at 25°.)

The general structure of the tyrosine analogue is:



Compound	R_1	R_2	R_3	Reaction rate (moles NP $\times 10^{-4}$/min./mole derivative)	Ratio (compound *c* as unity)
a	—OH	—NH_2	—CO.OEt	1·33 (2·67 with NPA)	2·3
b	—OH	—NH.CO.CH_3	—CO.OEt	0·63 (1·60 with NPA)	1·1
c	—H	—NH_2	—CO.OEt	0·58	1·0
d	—H	—NH.CO.CH_3	—CO.OEt	0·01	0·0
e	—OH	—NH_2	—H	3·88	6·7
f	—H	—NH_2	—H	3·33	5·7
g	—OH	—H	—CO.OMe	0·54	0·9
h	—H	—H	—CO.OMe	0·01	0·0
i	—OMe	—NH_2	—H	2·92 (5·23 with NPA)	5·0
*j**	—OH	—NH_2	—CO.OH	About 2·2 (about 5·1 with NPA)	About 3

* Because of the limited solubility, the solution of tyrosine was approx. half saturated, i.e. $1{\cdot}4 \times 10^{-3}$M (Hitchcock, 1924), instead of 10^{-2}M.

The reaction of p*-nitrophenyl ethyl carbonate and* p*-nitrophenyl acetate with amino acid derivatives*

A possible explanation of the anomalies in the reaction of NPA and NPC with chymotrypsin was suggested by the discovery that *p*-nitrophenol is liberated rapidly in well-buffered solutions containing these esters and L-tyrosine ethyl ester. It seemed probable that this liberation of nitrophenol might be due to a reaction between NPA or NPC and groups in the tyrosine ethyl ester molecule. The reaction of NPA and NPC with various analogues and derivatives of L-tyrosine was therefore investigated to determine which groups in the tyrosine ethyl ester molecule were involved.

Solutions of these analogues mixed with solutions of NPA or NPC in *iso*propanol were incubated at 25°, and the rates of increase of optical density at 4000 Å were compared with those in appropriate control solutions. The results are shown in Table 2. In the final column of this table the ratio of the rates of hydrolysis is given, taking the rate for compound *c* (DL-phenylalanine ethyl ester) as unity. From these results it appears that free amino and phenolic groups will react with NPC or NPA. The ratio of the rates gives values of approximately 1 for $-NH_2$ groups in amino esters (compounds *a* and *c*), 1 for phenolic groups (compounds *b* and *g*), and 5 for $-NH_2$ groups in true amines (compounds *f* and *i*). Where two functional groups are present, the reaction rates appear to be roughly additive.

The reaction of *p*-nitrophenyl acetate with tyramine methyl ether was followed to completion to test the stoicheiometry. To achieve appropriate concentrations it was necessary to conduct the reaction in buffer containing 50 % *iso*propanol. The results are shown in Fig. 2. The nitrophenol concentration in excess of that due to spontaneous hydrolysis rises to a maximum of $1{\cdot}02 \times 10^{-4}$ M with NPC and $0{\cdot}94 \times 10^{-4}$ M with NPA. Hence, within the experimental error, one mole of nitrophenol is liberated per mole tyramine methyl ether.

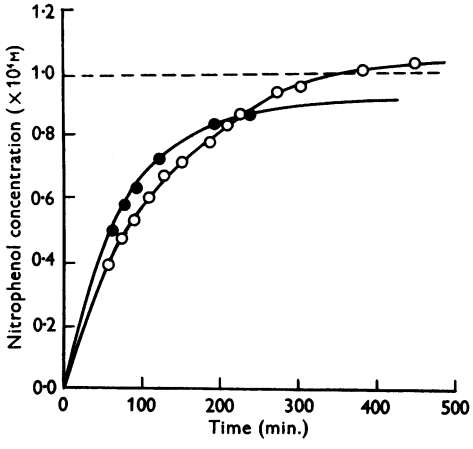


Fig. 2. Nitrophenol liberated by reaction of 10^{-4} M tyramine methyl ether with an excess of NPA or NPC at 25°, in buffer, pH 7·6, containing 50% *iso*propanol. ●, 3×10^{-3} M NPA; ○, 3×10^{-3} M NPC. The broken line represents the theoretical value of nitrophenol for an equimolecular reaction.

The inference that NPA and NPC are acting as acylating agents under these conditions was supported by isolating crystalline *N*-acetyl-DL-phenylalanine ethyl ester from the reaction of NPA with DL-phenylalanine ethyl ester, as described in the Experimental section.

Reaction of the nitrophenyl esters with insulin

Since NPA and NPC will react with derivatives of amino acids to liberate nitrophenol, it seemed possible that the esters would react similarly with proteins. This was found to be the case.

When solutions of insulin or protamine sulphate were incubated with NPC, a liberation of nitrophenol in excess of the spontaneous hydrolysis was observed. The rate was constant over a period of 40 min., being $5{\cdot}2 \times 10^{-10}$ mole nitrophenol/min./mg. insulin and $3{\cdot}3 \times 10^{-10}$ mole nitrophenol/min./mg. protamine sulphate.

The initial rate of the reaction of NPA with insulin at pH 7·60 was investigated as a function of concentration of insulin, of NPA, of *iso*propanol, and of temperature. A solution of crystalline insulin in buffer at pH 7·60 was dialysed overnight at 0°. The insulin concentration of the dialysate was calculated from its nitrogen content. The insulin solution was suitably diluted with buffer solution and incubated with solutions of NPA in *iso*propanol. The increase in nitrophenol concentration over a period of 50 min. was measured. Appropriate spontaneous hydrolysis controls were carried out in all cases.

Fig. 3 shows the effect of insulin concentration on the initial rate. Fig. 4 shows the effect of NPA concentration on the initial rate. At the lower concentrations of insulin, the initial rate of the reaction is directly proportional to both insulin and NPA concentration. Hence

$$v_{ins} = k_{ins}\,[\text{NPA}]\,[\text{Insulin}], \quad \text{and} \quad v_W = k_W[\text{NPA}],$$

from which the velocity constants for the reaction of NPA with insulin (k_{ins}) and for the spontaneous hydrolysis of NPA (k_W) can be calculated. These velocity constants are shown in Table 3 for experiments in which the concentration of *iso*propanol was varied. It can be seen that *iso*propanol does not affect the velocity constant for the reaction with insulin, but depresses the spontaneous hydrolysis slightly.

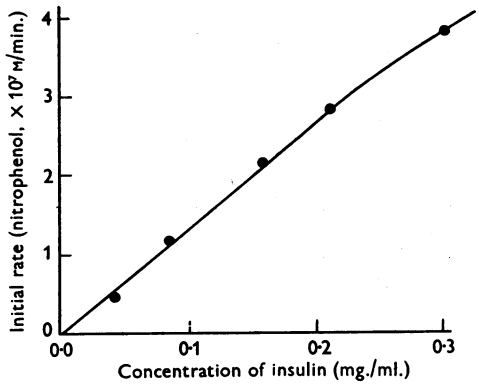


Fig. 3. Rates of reaction of 5×10^{-4} M NPA with various concentrations of insulin. Reaction conducted at 25° in 0·1 M phosphate buffer, pH 7·64, containing 5% *iso*propanol.

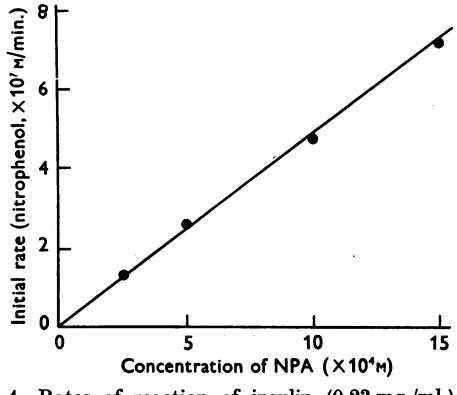


Fig. 4. Rates of reaction of insulin (0·23 mg./ml.) with various concentrations of NPA. Reaction conducted at 25° in 0·1 M phosphate buffer, pH 7·56, containing 5% *iso*propanol.

Table 3. *Effect of* isopropanol *on the reaction of insulin with NPA*

(Reaction mixture: insulin, 0·7 mg./ml.; NPA, 3×10^{-4} M; pH 7·60; incubated at 25°.)

Concentration of *iso*propanol (%)	k_W (min.$^{-1} \times 10^3$)	k_{ins} (moles^{-1} litres^{+1} min.$^{-1}$)
2·4	1·30	34·3
5	1·31	34·9
10	1·26	34·3
15	1·15	35·2
19	1·06	32·1

Fig. 5 shows the Arrhenius plot of these velocity constants over a range of temperatures. The spontaneous hydrolysis has a heat of activation, ΔH, of 18·4 kcal. at this pH, but the velocity constants for the reaction with insulin do not appear to obey the Arrhenius equation.

Further information about the reaction with insulin was obtained by following the liberation of nitrophenol until all the NPA had been hydrolysed. A solution of insulin plus NPA, and a control solution containing only NPA, were incubated at 37·5°. Samples (1 ml.) were withdrawn at intervals and added to 20 ml. buffer for measurement of nitrophenol concentration. The results are plotted in Fig. 6 as $\log a/(a-x)$ against t, where a is the concentration of nitrophenol at infinite time (20 hr.) and x is the concentration at time t.

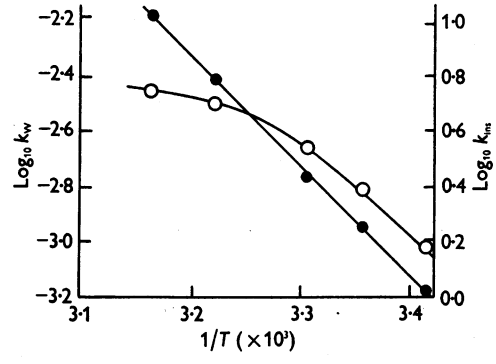


Fig. 5. Arrhenius plot for reaction of NPA with insulin and for spontaneous hydrolysis of NPA at pH 7·6. $\text{Log}_{10} k_{ins}$, ○; where k_{ins} = velocity constant for reaction of NPA with insulin expressed as moles^{-1} litres^{+1} min.$^{-1}$. $\text{Log}_{10} k_W$, ●; where k_W = velocity constant for spontaneous hydrolysis of NPA expressed as min.$^{-1}$. T = temperature in °K. Reactions conducted over range 20 to 43° C with 0·23 mg. insulin/ml., 5×10^{-4} M NPA, and 5% *iso*propanol in 0·1 M phosphate buffer, pH 7·56.

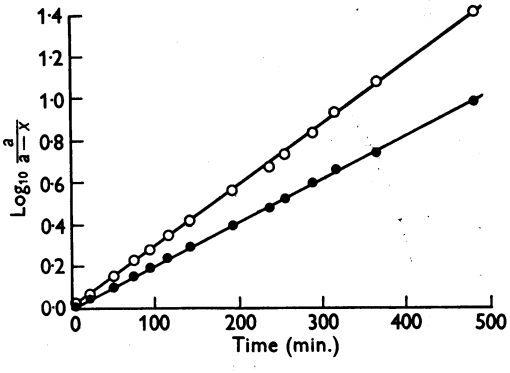


Fig. 6. Rate of reaction of $1{\cdot}5 \times 10^{-3}$ M NPA with insulin (0·34 mg./ml.) at 37·5°, pH 7·6, in presence of 5% *iso*propanol. x is the concentration of nitrophenol in solution at time t, and a is the concentration at infinite time (20 hr.). ○, reaction mixture; ●, spontaneous hydrolysis control.

Let us assume two independent reactions in the solution containing insulin:

$$\underset{I}{\text{Ins—H}} + \underset{a-(y+z)}{\text{NO}_2.\text{C}_6\text{H}_4.\text{O.CO.CH}_3} \xrightarrow{k_{ins}} \text{Ins—CO.CH}_3 + \underset{y}{\text{NO}_2.\text{C}_6\text{H}_4.\text{OH}}$$

$$\text{Ins—CO.CH}_3 \xrightarrow[k_3]{\text{H}_2\text{O}} \text{Ins—H} + \text{CH}_3.\text{COOH}$$

and

$$\underset{a-(y+z)}{\text{NO}_2.\text{C}_6\text{H}_4.\text{O.CO.CH}_3} \xrightarrow[\text{H}_2\text{O}]{k_w} \underset{z}{\text{NO}_2.\text{C}_6\text{H}_4.\text{OH}} + \text{CH}_3.\text{COOH}.$$

We have already shown that under the experimental conditions the initial rate of reaction is proportional to the concentration of NPA and insulin. Hence k_{ins} must be the rate-governing velocity constant for the first reaction, and

$$\frac{\mathrm{d}(y+z)}{\mathrm{d}t} = k_{ins}I[a-(y+z)] + k_w[a-(y+z)],$$

i.e.

$$t = \frac{2{\cdot}3}{k_{ins}I + k_w} \log \frac{a}{a-(y+z)} = \frac{2{\cdot}3}{k_{ins}I + k_w} \log \frac{a}{a-x}. \quad (1)$$

The straight line of Fig. 6 suggests that the concentration of reactive groups in insulin is unchanged throughout the experiment, since the insulin concentration ($2{\cdot}8 \times 10^{-5}$ M) is much less than the concentration of NPA ($1{\cdot}5 \times 10^{-3}$ M). In the spontaneous hydrolysis control we have

$$t = \frac{2{\cdot}3}{k_w} \log \frac{a}{a-x}. \quad (2)$$

From the slopes of these lines k_{ins} was found to be 67 moles^{-1} litre^{+1} min.$^{-1}$ and k_w to be $4{\cdot}7 \times 10^{-3}$ min.$^{-1}$.

The hypothesis that the reaction between insulin and NPA is mainly a catalysed hydrolysis of the ester is supported by measurements of the degree of acetylation of the protein before and after reaction. Insulin was incubated with excess of NPA for various periods, and the 'acetylated' sample was purified by dialysis or isoelectric precipitation as described in the Experimental section. Table 4 shows the results of total nitrogen, amino nitrogen, and *O*-acetyl analyses, performed on these samples. It can be seen that less than two acetyl groups are introduced into the molecule of insulin, although between 15 and 20 moles nitrophenol/mole insulin are liberated under the same conditions. The increase in acetyl content is not due to adsorption of acetic acid, since the inclusion of nitrophenol and acetic acid in the control caused no increase in acetyl content. However, there appears to be a slight acetylation of the protein which might account for the change in isoelectric point and the fact that the liberation of nitrophenol in the early stages of the reaction is rather more rapid than expected.

The kinetics of nitrophenyl esterase activity

In all measurements of the hydrolysis of NPA or NPC by chymotrypsin, it was observed that the extrapolated linear hydrolysis plot did not pass through the origin at zero time (Fig. 7). This suggested that the slow linear hydrolysis was preceded by a rapid initial reaction. The variation of this intercept with enzyme and substrate concentration was therefore investigated. Enzyme and substrate solutions were mixed in the cells of the Unicam spectrophotometer at room temperature to facilitate rapid measurement. Spontaneous hydrolysis controls were mixed simultaneously. Frequent readings of optical density at 4000 Å were made over a period of 10 min. Typical hydrolysis curves are shown in Fig. 7. The linear portion of these curves gave rates proportional to enzyme concentration, corresponding to $0{\cdot}074 \pm 0{\cdot}003$ mole nitrophenol/min./mole chymotrypsin with NPC at 21°, and $0{\cdot}68 \pm 0{\cdot}01$ mole nitrophenol/min./mole chymotrypsin with NPA at 22°. In Fig. 8, the intercepts obtained by extrapolating the linear hydrolysis to zero time are plotted against enzyme concentration. The intercept is proportional to enzyme concentration, and has the value 1·20 mole nitrophenol/mole

Table 4. *Acetyl content of insulin after reaction with NPA at* pH 7·6

(The effective concentrations of reactants in all cases were approx. 0·2 mg. insulin/ml., $1{\cdot}5 \times 10^{-3}$ M NPA and 10–15% *iso*propanol. In Expts. 1 and 2, the control solution contained only insulin and *iso*propanol. In Expt. 3 the control was insulin + nitrophenol + acetic acid + *iso*propanol. The reaction conditions were: Expt. 1, 100 min. at 37°, followed by isoelectric precipitation; Expt. 2, 18 hr. at 37°, followed by dialysis at 0°; Expt. 3, 24 hr. at 25°, followed by dialysis at 0°. For details see Experimental section.)

	Total N (mg./ml.)	Amino N (mg./ml.)	*O*-Acetyl (μmoles/ml.)	Moles amino N/ mole insulin	Moles *O*-acetyl/ mole insulin
Sample 1	1·68	0·19	1·6	7·6	1·8
Control 1	1·90	0·23	1·3	8·1	1·3
Sample 2	0·33	—	0·58	—	3·2
Control 2	0·41	—	0·28	—	1·3
Sample 3	0·27	—	0·38	—	2·6
Control 3	0·16	—	0·06	—	0·7

enzyme with 5×10^{-4}M NPC at 21°, and 1·12 moles nitrophenol/mole enzyme with 5×10^{-4}M NPA at 22°. Such values are consistent, within experimental error, with a rapid initial reaction liberating 1 mole nitrophenol/mole enzyme.

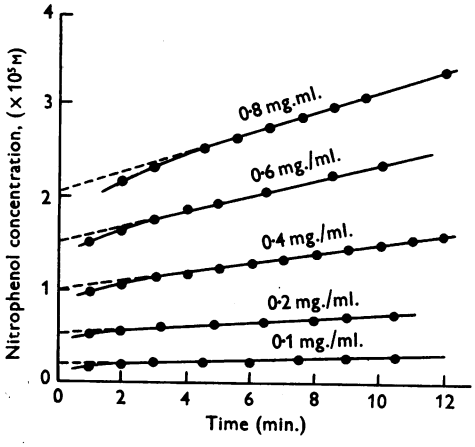


Fig. 7. Reaction of various concentrations of chymotrypsin (sample *C*) with 5×10^{-4}M NPC. Reaction conducted at 20° in 0·066M phosphate buffer, pH 7·60, containing 5% *iso*propanol.

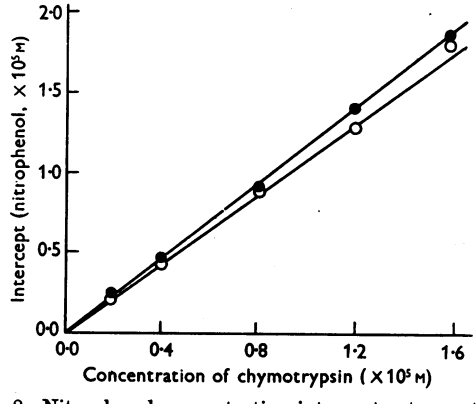


Fig. 8. Nitrophenol concentration intercepts at zero time for the reaction of nitrophenyl esters with various concentrations of chymotrypsin at pH 7·6. ●, 5×10^{-4}M NPC at 21°; ○, 5×10^{-4}M NPA at 22°.

Table 5. *Reaction of NPC with chymotrypsin*

(Reaction conducted with 0·6 mg. chymotrypsin sample *C*/ml. at 21°, pH 7·6.)

Concentration of NPC ($\times 10^4$M)	Intercept (Nitrophenol $\times 10^5$M)	Rate of linear hydrolysis (Nitrophenol $\times 10^7$M/min.)
6	1·34	8·0
4	1·34	8·3
2	1·34	8·7
1	1·26	8·9
0·5	1·24	8·9

Similar experiments were conducted to investigate the variation of initial intercept with substrate concentration. The results are shown in Table 5. Neither the initial intercept nor the slope of the linear portion of the hydrolysis curve are appreciably affected by changes in substrate concentration.

The reaction of the nitrophenyl esters with inhibited chymotrypsin

The kinetics of the reaction between NPA or NPC and inhibited chymotrypsin were studied. A solution containing 3·0 mg. sample *C* chymotrypsin/ml., 10^{-3}M DFP, and 5% *iso*propanol in 0·066M buffer, pH 7·60; and a corresponding control solution lacking chymotrypsin, were incubated at 25° for 4 hr. Both solutions were then diluted with an equal volume of 10^{-3}M NPC (or NPA) in buffer containing 5% *iso*propanol. The progress of the

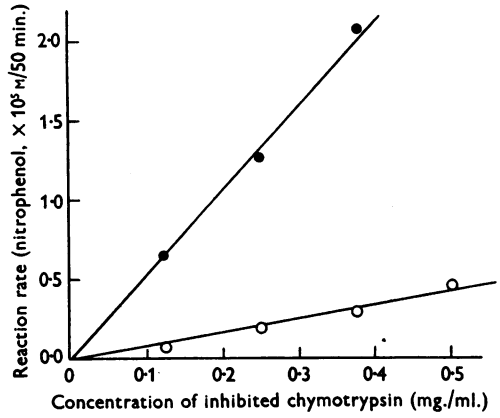


Fig. 9. Reaction rates of NPA and NPC with various concentrations of crystalline DFP-inhibited chymotrypsin at 25°, pH 7·6. ●, 10^{-3}M NPA; ○, 5×10^{-4}M NPC.

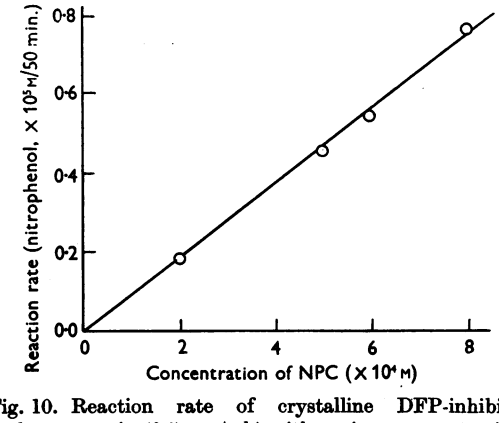


Fig. 10. Reaction rate of crystalline DFP-inhibited chymotrypsin (0·5 mg./ml.) with various concentrations of NPC at 25°, pH 7·6.

reaction at room temperature (21°) was observed by measuring the optical densities at 4000Å. Nitrophenol was liberated in the presence of inhibited chymotrypsin at a constant rate, in excess of the spontaneous hydrolysis, of 0·011 mole nitrophenol/min./mole enzyme with 5×10^{-4}M NPC and 0·031 mole nitrophenol/min./mole enzyme with 5×10^{-4}M NPA. On extrapolating the linear hydrolysis plots, the intercepts at the time of mixing were 0·02 mole nitrophenol/mole inhibited chymotrypsin with NPC, and 0·00 mole nitrophenol/mole inhibited chymotrypsin with NPA. Hence, with completely inhibited enzyme there is no rapid initial reaction such as is observed when the active enzyme reacts with these esters.

Further evidence was obtained from experiments using a sample of crystalline DFP-inhibited chymotrypsin. The rate of reaction was proportional to the concentration of DFP-inhibited chymotrypsin as shown in Fig. 9. When the linear hydrolysis plots were extrapolated to zero time, they passed through the origin, indicating that no rapid initial reaction precedes the linear hydrolysis.

In another series of experiments, crystalline DFP-inhibited chymotrypsin was allowed to react with various concentrations of NPC. The rate of reaction was found to be directly proportional to the concentration of NPC as shown in Fig. 10.

DISCUSSION

Completely inhibited chymotrypsin, which had no proteolytic, amino acid esterase or amidase activity, could still react with *p*-nitrophenyl acetate (NPA) or *p*-nitrophenyl ethyl carbonate (NPC) to liberate nitrophenol. This apparent residual activity was the same for enzyme samples of different history. Recrystallizing an enzyme sample did not alter the ratio between initial proteolytic activity and nitrophenyl esterase activity after complete reaction with E 600. Hence the apparent residual nitrophenyl esterase activity does not seem to be due to impurities in the enzyme. The reaction of inhibited chymotrypsin with NPA or NPC was unaffected by using a different inhibitor (DFP) and prolonged incubation with inhibitor did not alter the final residual activity. Further anomalies were that the rate of inhibition and final nitrophenyl esterase activity appeared to depend on the particular substrate used and on the substrate concentration.

An explanation was suggested by the discovery that NPA and NPC will react with certain derivatives of tyrosine. The reactive groups appear to be amino or phenolic groups. Alcoholic hydroxyl groups probably do not react, since *iso*propanol does not react with NPA. With the amino group in tyramine methyl ether, a stoicheiometric liberation of 1 mole nitrophenol/mole tyramine methyl ether was observed when the reaction was allowed to go to completion in the presence of an excess of nitrophenyl ester. This suggests that the reaction might be an acylation of the amino group. This was supported by isolating crystalline *N*-acetyl-DL-phenylalanine ethyl ester from the reaction of NPA with DL-phenylalanine ethyl ester at pH 7·6. The reaction of the esters with phenolic groups probably follows the same lines, but there may be appreciable hydrolysis of the *O*-acyl products at this pH.

It seemed possible that the reaction of nitrophenyl esters with inhibited chymotrypsin might be due to some similar mechanism. The esters would react with protamine sulphate or insulin to liberate nitrophenol. With insulin, the initial rate of the reaction was proportional to the concentration of both ester and insulin, and was not affected by the *iso*propanol used to effect solution of the ester. The progress curve of the reaction obeyed kinetics which suggest the mechanism:

$$\text{insulin} + \text{NPA} \xrightarrow{k_{ins}} \text{acetylated insulin} + \text{nitrophenol}$$
$$\text{acetylated insulin} \xrightarrow[k_3]{H_2O} \text{insulin} + \text{acetic acid}$$

where k_{ins} is rate-limiting. Hence the overall reaction at pH 7·6 is a catalysed hydrolysis of the ester.

Two discrepancies were observed. At the higher concentration of insulin, the rate was no longer directly proportional to insulin concentration, and the effect of temperature on the reaction was not explained by the Arrhenius equation. These anomalies might be due to association of insulin molecules at higher concentrations, and to dissociation of the polymers with increasing temperature.

It is possible that, in addition to the reaction discussed above, there is some permanent acetylation of groups in insulin by reaction with NPA. This is suggested by the decrease in the isoelectric point after reaction, and by a slight increase in nitrophenol liberation in the early stages of the reaction above that expected from equation (1). It is unlikely that more than two groups in the molecule (mol.wt. 12 000) react in this fashion, since the acetyl content after reaction lies between one and two groups per molecule, and the discrepancy in the kinetics corresponds to about two moles nitrophenol/mole insulin.

A reaction between these esters and groups in crystalline DFP-inhibited chymotrypsin is supported by kinetic studies. The rates of reaction were similar to those observed with E 600-inhibited chymotrypsin, and the initial rates were proportional to the concentration of nitrophenyl ester. This is in contrast to the reaction with active chymotrypsin, where the rate of catalysed hydro-

lysis is independent of substrate concentration over a wide range. The discovery of this 'protein' reaction of NPA and NPC invalidates previous figures for the Michaelis constant and turnover number of their catalysed hydrolysis by chymotrypsin (Hartley & Kilby, 1952). The Michaelis constant can be considered as only approximate, and the turnover number as a maximum value, when applied to the catalysed hydrolysis at the active centre of the enzyme.

Hydrolysis of NPA and NPC by active chymotrypsin appears to take place in a two-stage reaction. An initial, rapid liberation of nitrophenol is followed by a slower, linear hydrolysis. The intercept obtained by extrapolating the slow, linear hydrolysis to zero time was proportional to the enzyme concentration, and had the value 1·1–1·2 moles nitrophenol/mole enzyme. The magnitude of this intercept was independent of substrate concentration over a wide range. It seems probable that the initial rapid liberation of nitrophenol is a function of the active centre of the enzyme, since no intercept at zero time was observed when the esters were allowed to react with inhibited chymotrypsin (or with insulin).

An explanation of the kinetics is suggested by the following reaction scheme:

$$\text{ChTr-H} + \text{NO}_2.\text{C}_6\text{H}_4.\text{O.CO.CH}_3 \xrightarrow{(1)} \text{ChTr-CO.CH}_3 + \text{NO}_2.\text{C}_6\text{H}_4.\text{OH}.$$

$$\text{ChTr-CO.CH}_3 \xrightarrow[(2)]{+\text{H}_2\text{O}} \text{ChTr-H} + \text{CH}_3\text{COOH}$$

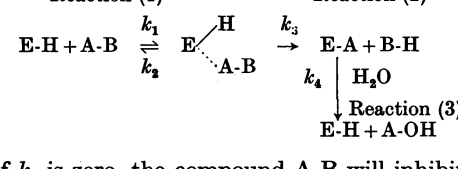
If, in this series of reactions, reaction (1) is very rapid, while reaction (2) is slow, the active centre of the enzyme will be rapidly acetylated, liberating one mole nitrophenol/mole enzyme. Thereafter, the rate-governing step of the series will be the hydrolysis of the acetylated active centre, reaction (2). The rate of this hydrolysis, and hence of the overall reaction, will be proportional to the initial enzyme concentration, but independent of substrate concentration.

It is interesting to compare the velocity constants corresponding to k_1 for the reaction of NPA at 25°, pH 7·60, with acetyltyrosine ethyl ester, with insulin, with DFP-inhibited chymotrypsin, or with active chymotrypsin. These are respectively 0·32, 35, 28 and >2000 mole^{-1} litre^{+1} min.$^{-1}$. The value with active chymotrypsin is only approximate, since the initial rate is very rapid.

The catalytic action of insulin and chymotrypsin on the nitrophenyl esters can be compared. The reaction with chymotrypsin occurs at the active centre of the enzyme, which is a unique structure in the molecule, and the kinetics approximate more closely to those of a typical enzyme:substrate reaction. The reaction with insulin seems to be common to other proteins, and the kinetics differ both in degree and in kind from those with active chymotrypsin. In this 'protein' reaction the reactive sites are possibly amino acid side chains, since reaction will occur with such groups as phenolic or amino groups in simple amino acid derivatives.

In the reaction of the nitrophenyl esters with chymotrypsin the observed intercepts 1·1–1·2 moles nitrophenol/mole chymotrypsin are higher than would be expected from a molecular weight of 27 000. They correspond closely with the ratio of 1·1 atoms phosphorus/molecule DFP-inhibited chymotrypsin found by Jansen *et al.* (1949) using the same molecular weight. Recent figures of 21 000–23 000 for the molecular weight (Schwert & Kaufman, 1951; Smith, Brown & Laskowski, 1951) give values close to 1·0 for both intercept and phosphorus ratio. The present determinations of the nitrophenol liberated from inhibition of chymotrypsin by E 600 (purified as indicated in the Experimental section) give a mean value of 1·01 moles nitrophenol/mole chymotrypsin, taking the molecular weight as 23 000. Previous measurements (Hartley & Kilby, 1952) give a value of 0·85 mole nitrophenol/mole chymotrypsin when recalculated using this molecular weight. This low value may be due to traces of a more powerful inhibitor in the E 600 used in these early experiments, which does not liberate nitrophenol, e.g. tetraethyl pyrophosphate. Aldridge & Barnes (1952) have shown that this is a common impurity in E 600 samples. Such an impurity would not affect the kinetics described in our previous paper, since these were based upon nitrophenol concentration measurements.

Mechanisms have been proposed to explain the inhibition of both chymotrypsin and cholinesterase by organophosphorus inhibitors (Hartley & Kilby, 1952; Aldridge, 1950). Such mechanisms can be extended to explain the hydrolysis of NPA and NPC by chymotrypsin. The main differences between inhibitors and substrates can be qualitatively expressed in terms of the velocity constants in the following series of reactions, where E-H is the enzyme and A-B the reactant:



If k_4 is zero, the compound A-B will inhibit the enzyme. If k_3 is also zero, A-B will act as a competitive inhibitor, e.g. eserine for cholinesterase. If reaction (1) is the rate-governing step, the inhibition will be progressive, irreversible, and of first order with respect to the inhibitor. Such kinetics are shown in the inhibition of chymotrypsin by E 600

(Hartley & Kilby, 1952) or cholinesterase by DFP (Aldridge, 1950). If reaction (2) is the rate-governing step, the inhibition will be gradual, partly reversible, and of zero order with respect to inhibitor.

When k_4 is not zero, A-B is a substrate for the enzyme. However, if reaction (3) is the rate-governing step, the kinetics will be similar to those observed in the reaction of NPA and NPC with chymotrypsin. These esters might, therefore, be looked upon as intermediate between substrates and inhibitors of the enzyme, and their structural analogy with the inhibitor E 600 is reflected in the kinetics of their reaction with chymotrypsin.

SUMMARY

1. The rate of inhibition of the nitrophenyl esterase activity of chymotrypsin by diethyl *p*-nitrophenyl phosphate (E 600) does not correspond with the rate of reaction of enzyme with inhibitor. After complete reaction with E 600, chymotrypsin still reacts with *p*-nitrophenyl acetate (NPA) and *p*-nitrophenyl ethyl carbonate (NPC) to liberate nitrophenol.

2. NPA and NPC react with amino and phenolic groups in derivatives and analogues of tyrosine.

3. NPA and NPC also react with insulin and protamine to liberate nitrophenol. The reaction with insulin is a catalysed hydrolysis of the esters. A mechanism is proposed.

4. A similar reaction of NPA or NPC in proteins would explain the observed reaction with inhibited chymotrypsin.

5. Chymotrypsin catalyses the hydrolysis of these esters at the same active centre as is responsible for the other enzymic activities. The kinetics of this reaction are discussed, and a mechanism is proposed.

6. The catalytic activities of insulin and chymotrypsin are compared.

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In vitro Demonstration of Metabolic Changes During Heat Regulation in Rats

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In a preliminary note (Wertheimer & Bentor, 1950) we described the fact that the diaphragms of rats maintained for 5 hr. at 5–7° required more glucose and synthesized greater quantities of glycogen from glucose when incubated in their own serum than did the diaphragms of rats maintained for the same period at 28–30°. These changes in organ metabolism were already demonstrable at about 30 min. after the start of cooling, and disappeared a few hours after the temperature stimulus had been discontinued. If such diaphragms were incubated in Krebs-Ringer solution, rather than in homologous serum, the increased glucose requirement of the 'cold' diaphragms could no longer be demonstrated, and the glycogen synthesis was only slightly augmented. These experiments were repeated at