Urine salicylic acid mg./100 ml. = (mg. salicylic acid/100 ml. in diluted unknown - mg. salicylic acid/100 ml. in diluted urine blank) $\times$ dilution factor.

RESULTS

Successive c.s.f., plasma, whole blood, serum and urine samples, obtained from patients who were not taking salicylates, were analysed by the proposed method. The results in Table 2 show that the 'blank' values (in mg. salicylic acid/100 ml.), were less than 1.1 for serum, c.s.f., and plasma; less than 2.0 for whole blood and less than 4.5 for urine. Recovery experiments were performed by analysing pooled serum, whole blood and urine, to which known amounts of sodium salicylate had been added. The results given in Table 3 indicate that the recoveries were almost quantitative. The recovery figures were not affected by the addition of 100 mg. of phosphate ion, 20 mg. of bilirubin, 25 mg. of phenol, 10 000 i.u. of heparin, 1000 mg. of glucose or 1000 mg. of urea, per 100 ml. of serum. The addition of 250 mg. of Wintrobe's anticoagulant (150 mg. of ammonium oxalate, $(\text{COONH}_4)_2$, H_2O , plus 100 mg. potassium oxalate, $(\text{COOK})_2$, H_2O /100 ml. of serum) increased the results by 0.3 mg. of salicylic acid/100 ml. The addition of 50 mg. of ethyl acetoacetate/100 ml. of serum, increased the results by 1 mg. of salicylic acid/100 ml. The effect of other keto acids on the recovery figures was not studied.

DISCUSSION

Although a rapid method is worth developing in the interests of economy, the aim should not be to sacrifice accuracy for speed. In the present method a single determination occupies only 5 min., but the method is at least as accurate as other methods. The recovery figures are good and the blank values are low even for whole blood, which cannot be analysed

by other methods. If an instrument, such as the Spekker Absorptiometer, is available, which can give readings with small volumes of fluid, the method can be used on samples of blood obtained by finger prick, using 0.2 ml. of blood and 1 ml. of colour reagent. This modification would be especially useful for the determination of salicylate in the blood of children. The results obtained using urine are not so reliable as those obtained using serum, owing to the somewhat high blank values for normal urine. In practice the concentration of salicylate in urine, even after small doses of salicylates, is so high (100 mg. or more of salicylic acid/100 ml. of urine) that the relative error is small.

SUMMARY

1. A rapid method for the determination of salicylate in biological fluids is presented, based on a reagent containing ferric nitrate, mercuric chloride and hydrochloric acid, which precipitates the proteins and simultaneously reacts with salicylic acid to give a purple colour.
2. The recovery of sodium salicylate added to biological fluids is quantitative.
3. The blank values on normal serum and plasma samples are less than 1.1 mg. of salicylic acid/100 ml.
4. The effect of some possible interfering substances is considered.
5. A single determination occupies 5 min.

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The Inhibition of Trypsin and Chymotrypsin by Certain Organic Phosphorus Esters

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(Received 13 January 1954)

Certain organophosphorus compounds have recently acquired a rapidly increasing importance as insecticides, and as they also show high mammalian toxicity, it appears desirable to have as much information as possible about their mode of action. A full knowledge of this will involve not only a knowledge of which essential enzyme systems are inhibited in living organisms, but also an under-

standing of the chemical process of inhibition. A very powerful anticholinesterase activity is one of the characteristic properties of the phosphorus compounds in this group, but other enzymes, such as human milk and liver esterase, citrus acetyl-esterase, chymotrypsin and trypsin are also inhibited. It is now generally agreed that the toxicity towards vertebrates is closely associated with the

inhibition of cholinesterases *in vivo*, but the situation in insects is less certain owing to lack of information about the relative importance of cholinesterases and other esterases in the insect economy. As the organophosphorus compounds appear to be specific inhibitors of enzymes showing carboxylic esterase activity, it is possible that there is a common chemical basis to the inhibition process, and this is the aspect of the mode of action which has been our particular interest. A choice of both enzyme and inhibitor is available, and chymotrypsin and diethyl *p*-nitrophenyl phosphate (E. 600) were selected for study first (Hartley & Kilby, 1952) because chymotrypsin was available in crystalline form and had a known and conveniently small molecular weight, and the inhibitor was active, readily purified and of unambiguous structure. The results were consistent with the hypothesis that inhibition is due to the reaction of one molecule of inhibitor with one molecule of enzyme to give a phosphorylated inactive enzyme and the elimination of a molecule HX, which was *p*-nitrophenol in the example cited. It seemed desirable that these studies should be extended to see whether or not a similar mechanism was operative with a different enzyme and another inhibitor. For this purpose trypsin was selected, as it was also available in the crystalline state and measurements on the molecular weight had been published. It was known to be inhibited by diisopropylphosphorofluoridate (Jansen, Nutting, Jang & Balls, 1949), and investigation soon showed that it was also inhibited by the phosphorus compounds we wished to employ, namely E. 600 and *OS*-diethyl *O*-*p*-nitrophenyl phosphorothiolate ('*S*-ethyl compound'). The stoichiometry of the inhibition of trypsin has been studied, and velocity constants at different temperatures for the inhibition of both trypsin and chymotrypsin by the two compounds have been measured. Some of the results have been briefly indicated in a preliminary communication (Kilby & Youatt, 1952).

EXPERIMENTAL

Materials

Enzymes. Two samples of crystalline bovine trypsin as supplied by Armour and Company, of Chicago, were used. As sample *A* gave cloudy solutions which interfered with the optical density measurements, all solutions prepared from it were centrifuged before use, and the clear solutions assayed for the amount of active enzyme present. Sample *B* gave no trouble in this respect but the same technique was followed, and the solutions were assayed before use. The crystalline chymotrypsin used was also an Armour product and was the same as sample *A* of Hartley & Kilby (1952). The proteolytic activities were determined by Anson's haemoglobin method (Anson, 1938) and expressed as tyrosine units, T.U. Hb/mg. protein nitrogen (P.N.), where one T.U. is

Table 1. *Activities of Enzyme samples*

Sample	Protein-N in solid sample (%)	Proteolytic activity at 35.5° (T.U. Hb/ mg. P.N.)	Active protein (as % of total)
Chymotrypsin <i>A</i>	11.5	0.026	67
Trypsin <i>A</i> *	—	0.062	37
Trypsin <i>B</i>	4.9	0.041	24

* Determinations on trypsin *A* were made on the clear solution after removal of insoluble material by centrifuging.

the amount of enzyme which will liberate the equivalent of one millimole tyrosine per ml. per min. at 35.5°. Kunitz, Northrop & Herriot (1948) report mean values of 0.17 T.U. Hb/mg. P.N. for pure trypsin containing 15% N, and 0.039 T.U. Hb/mg. P.N. for pure chymotrypsin containing 15.5% N. Owing to the small amounts of material available, recrystallization of our samples to constant activity was not attempted, and the amounts of active enzyme in them were calculated by comparison with the values given by Kunitz *et al.* for the pure enzymes.

Reagents. Diethyl *p*-nitrophenyl phosphate, (E. 600, Paraoxon.) b.p. 142°/6 × 10⁻³ mm. (Hartley & Kilby, 1952). *OO*-Diethyl *O*-*p*-nitrophenyl phosphorothionate, (C₂H₅O)₂. PS.OC₆H₄NO₂ (E. 605, Parathion), and *OS*-diethyl *O*-*p*-nitrophenyl phosphorothiolate, (C₂H₅O)(C₂H₅S)PO.OC₆H₄NO₂, ('*S*-ethyl compound'), were pure samples supplied by Albright & Wilson Ltd., and were used as received. α -Toluene-*p*-sulphonyl-L-arginine methyl ester hydrochloride. M.p. 146°, and α -toluene-*p*-sulphonyl-L-arginine amide. M.p. 183.5–184° (Bergmann, Fruton & Pollok, 1939). Ethyl *p*-nitrophenyl carbonate. M.p. 66.5–67° (Ransom, 1898). *p*-Nitrophenyl acetate. M.p. 79.5–80° (Kaufmann, 1909).

METHODS

Measurement of proteolytic activity. The haemoglobin digestion method of Anson (1938) was used. The final colour which developed was read against a reagent blank on a Unicam D.G. Spectrophotometer at 6900 Å. It was shown that 15% isopropanol did not affect the enzyme activity.

Assay of *p*-nitrophenol. This was carried out in exactly the same manner as described by Hartley & Kilby (1952).

Hydrolysis of toluene-*p*-sulphonylarginine methyl ester (TSAME). The rate of hydrolysis of TSAME was measured by a continuous potentiometric titration method similar to that of Schwert, Neurath, Kaufmann & Snoke (1948), whereby the acid liberated during hydrolysis was continuously titrated with dilute alkali to constant pH as measured on a Cambridge pH-meter.

The electrodes dipped into 15 ml. of a 0.0026 M solution of TSAME adjusted to pH 8 and contained in a beaker, at 37°, and stirred mechanically. Enzyme solution (0.2 ml.) was added and 0.2 N-NaOH was introduced in portions of 0.01 ml. from a micro-syringe, the successive time intervals required for the pH to return to 8 being noted. Unbuffered solutions were found to be preferable, and the reference point of pH 8 was chosen to lie close to the optimum of pH 8–9 quoted by Schwert *et al.* (1948).

Hydrolysis of toluene-*p*-sulphonylarginine amide (TSAA). This was followed by a method identical with that used by Hartley & Kilby (1952).

RESULTS

Effect of calcium ions on the stability of trypsin solutions

Alkaline solutions of trypsin are unstable, the enzyme undergoing a progressive inactivation with time (Kunitz *et al.* 1948). At first, attempts were made to correct for this effect in the kinetic studies but the procedure was not satisfactory. Following a personal communication from Dr Gorini (*cf.* also

Gorini, 1951), that calcium ions have a stabilizing effect on trypsin against this inactivation, experiments were carried out to examine the use of this effect as a possible means of obtaining a stable enzyme solution.

A solution of trypsin of known initial activity in 0.05M borate buffer was incubated at 37° in the presence and absence of calcium chloride, and the proteolytic activity measured at intervals. The results (Table 2) show that in the presence of 0.025M calcium chloride there is only a slight loss of activity over a period of 12 hr. As a result all further experiments were carried out in 0.05M borate buffer made 0.025M with respect to calcium chloride. This will subsequently be referred to as the borate:calcium buffer.

Table 2. *Effect of calcium ions on the stability of trypsin solutions*

Time of incubation (hr.)	Activity retained (%)		
	No CaCl ₂	0.025 M-CaCl ₂	0.0025 M-CaCl ₂
0.0	100	—	—
2.5	22	—	—
5.0	12	—	—
6.0	—	103	94
12.0	—	90	82

Inhibition of trypsin by E. 600

E. 600 inhibits the proteolytic activity of trypsin with simultaneous liberation of nitrophenol. Various concentrations of E. 600 in isopropanol were added to a solution of trypsin in borate:calcium buffer pH 7.6, the final concentration of isopropanol being 15 %. The residual activities were determined after 120 min. incubation. Results are given in Fig. 1.

Effect of calcium ions upon nitrophenol liberation from reaction of trypsin with E. 600

Trypsin (1.16 x 10⁻⁴ T.U. Hb./ml.) was incubated at 25° in 0.05M borate buffer, pH 7.6 containing 15 % isopropanol with E. 600 (0.01M). At this high concentration of E. 600, reaction with trypsin was rapid compared with the autolysis of trypsin. The optical density difference at 4000Å between this solution and a similar one incubated in parallel but lacking enzyme, gave a measure of the nitrophenol liberated, corrected for that due to spontaneous hydrolysis of E. 600. Various concentrations of calcium chloride were added, and the results are given in Table 3. Under the conditions of the experiment, the added calcium chloride did not affect the rate or extent of nitrophenol liberation.

Amount of nitrophenol liberated after complete inhibition of trypsin by E. 600

Table 4 gives the results of a series of experiments in which the concentration of nitrophenol liberated from E. 600 or the S-ethyl compound was measured after the proteolytic activity of trypsin had been completely inhibited. The third column of the table gives the amount of nitrophenol to be expected if one molecule of enzyme, of molecular weight 36 500 (Kunitz & Northrop, 1936), reacts with one molecule of inhibitor liberating one molecule of nitrophenol.

Fig. 1. Inhibition of proteolytic activity of trypsin produced by incubation with various concentrations of E. 600 at 37° and pH 7.6 for 120 min.

Table 3. *Effect of calcium ions upon the liberation of nitrophenol from trypsin and E. 600*

An optical density of 1.0 is equivalent to 6.8 x 10⁻⁵M nitrophenol.

Time (min.)	Nitrophenol liberation (optical density at 4000Å)			
	No CaCl ₂	0.04 M-CaCl ₂	0.02 M-CaCl ₂	0.01 M-CaCl ₂
20	0.066	0.069	0.068	0.067
51	0.109	0.114	0.115	0.115
80	0.137	0.139	0.142	0.144
119	0.162	0.163	0.165	0.168
159	0.176	0.172	0.181	0.186
220	0.194	0.191	0.194	0.198

Table 4. *Observed and calculated nitrophenol liberation resulting from complete inhibition of trypsin by E. 600 and 'S-ethyl compound'*

The calculated nitrophenol is that expected if one molecule is liberated for each molecule of trypsin inhibited, assuming a molecular weight of 36500 for trypsin.

No. of detns.	Activity of trypsin solution (T.U. Hb/ml. $\times 10^{-4}$)	Concentration of inhibitor ($M \times 10^{-3}$)	Nitrophenol		
			Observed ($M \times 10^{-5}$)	Calculated ($M \times 10^{-5}$)	Ratio, obs./calc.
(a) E. 600 as inhibitor					
1	1.73	1.0	1.85	0.93	1.99
4	1.52	7.5	1.77	0.82	2.16
4	1.52	5.0	1.76	0.82	2.14
4	0.77	7.5	0.92	0.42	2.19
4	0.77	5.0	0.90	0.42	2.15
4	0.77	2.5	0.84	0.42	2.00
4	0.57	10.0	0.59	0.27	2.17
Mean					2.13
					(S.D. ± 0.07)
(b) OS-Diethyl O-p-nitrophenyl phosphorothiolate as inhibitor					
4	2.30	1.0	2.53	1.24	2.04
3	2.30	0.75	2.50	1.24	2.02
3	2.30	0.50	2.31	1.24	1.86
1	2.20	0.50	2.34	1.18	1.98
1	2.01	1.0	2.19	1.07	2.05
3	1.73	1.0	2.01	0.93	2.16
4	1.73	0.75	1.87	0.93	2.01
1	1.73	0.75	1.96	0.93	2.10
Mean					2.02
					(S.D. ± 0.05)

Kinetics of liberation of nitrophenol

The kinetics of the inhibition reaction were studied by following the rate of liberation of nitrophenol by measurements of the optical density changes. Enzyme solutions were in borate:calcium buffer pH 7.6 and of activities approximately 1.5×10^{-4} T.U. Hb/ml. for trypsin and 3×10^{-3} T.U. Hb/ml. for chymotrypsin. The trypsin and chymotrypsin solutions contained 15 and 5% isopropanol, respectively. Various concentrations of E. 600 were added and the experiments were carried out over a range of temperatures. Nitrophenol liberated in excess of that due to the spontaneous hydrolysis of the inhibitor was measured.

If the inhibitor concentration (I) is large compared with that of the enzyme (a), then the concentration of nitrophenol (x) observed at time t is given by the equation

$$\frac{dx}{dt} = k_0(a - x),$$

which if integrated gives

$$t = \frac{2.303}{k_0} \log_{10} \frac{a}{(a-x)}.$$

After complete inhibition the nitrophenol concentration is a . In Fig. 2, $\log_{10} \frac{100(a-x)}{a}$ is plotted against t for various inhibitor concentrations at 25°.

Fig. 2. Liberation of nitrophenol from reaction of trypsin and E. 600 at 25° and pH 7.6. The measured nitrophenol concentration is plotted as $\log_{10} \frac{100(a-x)}{a}$, where a is the total nitrophenol concentration observed at completion of reaction, and x the amount of nitrophenol at time t . Concentrations of E. 600 used: (A) $10^{-2}M$, (B) $8.0 \times 10^{-3}M$, (C) $6.0 \times 10^{-3}M$, (D) $4.0 \times 10^{-3}M$, (E) $2.0 \times 10^{-3}M$.

Table 5. *Velocity constants for reaction of trypsin with E. 600 at 25°, pH 7.6*

Observed k_0	Concentration (I) of E. 600 ($\times 10^{-3}M$)	$k_0/(I)$	$k_0/(I)^2$
0.0148	10	1.48	148
0.0104	8	1.30	162.5
0.0081	6	1.35	225
0.00544	4	1.36	340
0.00246	2	1.23	515

Table 6. *Velocity constants (k_b) for enzyme-inhibitor reactions*

Temp. (°)	Trypsin + E. 600	Trypsin + 'S-ethyl compound'	Chymotrypsin + E. 600	Chymotrypsin + 'S-ethyl compound'
40.1	3.36	—	—	—
37.5	3.11	—	—	—
35.1	2.37	—	—	—
30.5	2.01	61.2	9.66	425
25.8	—	—	6.71	—
25.0	1.31	42.1	5.14*	311
20.4	0.98	—	2.80	—
16.2	—	20.8	—	166
ΔE kcal./mole	15.5	13.0	21.6	11.3

* Hartley & Kilby (1952).

When plotted in this form a series of straight lines is obtained from which the value of k_b can be found. If the reaction between enzyme and inhibitor is bimolecular then k_b will be equal to $k_b(I)$, but if the reaction is termolecular, then k_b will be equal to $k_t(I)^2$, where k_b and k_t are the bimolecular and termolecular velocity constants respectively. The values given in Table 5 support a bimolecular mechanism.

Activation energies for inhibition reactions

A series of measurements at different inhibitor concentrations, similar to those shown in Fig. 2, were made at a number of different temperatures for inhibition of both trypsin and chymotrypsin by the two inhibitors. When $\log k_b$ was plotted against $1/T$, satisfactory straight lines were obtained from which the activation energies (ΔE) were calculated, using the integrated form of the Arrhenius equation $\log k = -\Delta E/RT + \text{const.}$ The results are summarized in Table 6.

Action of E. 605 on trypsin and chymotrypsin

Solutions of trypsin and chymotrypsin were incubated at 25° with 10^{-4} M E. 605 in buffer solution containing 15% isopropanol. After 6.5 hr. no inhibition or liberation of nitrophenol could be detected. In another experiment chymotrypsin was incubated for 2 days with 10^{-4} M E. 605, but no nitrophenol was observed.

Rate of inhibition of different enzymic activities of trypsin

Proteolytic activity. Simultaneous estimations of the rate of inhibition of proteolytic activity and the rate of liberation of nitrophenol were made. Trypsin solutions in 0.05 M borate:calcium buffer (pH 7.6) were incubated at 37° with E. 600. Final concentrations were: trypsin corresponding to an activity of approximately 1.5×10^{-4} r.u. Hb/ml., the exact activity being determined at the beginning of each experiment; E. 600, 3×10^{-3} M; isopropanol, 15%. The proteolytic activity was measured at intervals and the liberation of nitrophenol by the optical density.

Table 7. *Values of bimolecular velocity constant for reaction between trypsin and E. 600 obtained by different methods*

Rate measured	k_b (mole ⁻¹ min. ⁻¹)
Inhibition of proteolytic activity	3.33
Inhibition of esterase activity	3.07
Inhibition of amidase activity	3.28
Liberation of nitrophenol	3.11

Esterase activity. The rate of inhibition of the enzymic hydrolysis of TSAME was measured. A trypsin solution of pH 7.6 and activity approximately 3×10^{-3} r.u. Hb/ml. was incubated at 37° with 3×10^{-3} M E. 600 and the rate of inhibition measured by withdrawing samples at various intervals and measuring the rates of hydrolysis of 0.0026 M TSAME by the potentiometric technique after suitable dilution of the enzyme sample.

Amidase activity. Solutions of trypsin of approximate activity 0.9×10^{-4} r.u. Hb/ml. were incubated at 37° with 3×10^{-3} M E. 600. At intervals samples were withdrawn and diluted with equal volumes of 0.1 M TSAA in buffer containing 15% isopropanol. The rate of liberation of ammonia at 37° was followed by the Conway microdiffusion method. Table 7 shows that the rate of liberation of nitrophenol is the same as the rate of inhibition of each of the three enzymic activities.

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

p-Nitrophenyl carbonate and acetate were examined as possible substrates for trypsin. Solutions of the substrate were made up in isopropanol to give the required final substrate concentration in 5% isopropanol when added to a solution of the enzyme in borate:calcium buffer. The rate of liberation of nitrophenol was measured by the rate of increase of optical density at 4000 Å. The rates of spontaneous hydrolysis of these esters were constant over the observed period and proportional to the ester concentration. The first-order velocity constants (k) at 20° were 2.54×10^{-4} min.⁻¹ for the carbonate and 7.4×10^{-4} min.⁻¹ for the acetate. The

Fig. 3. Relationship between rate of hydrolysis of nitrophenyl esters and trypsin concentration (20° , pH 7.6).

carbonate was used in most of the experiments to diminish corrections for the spontaneous hydrolysis.

The rates of hydrolysis of both NPC and NPA were found to be proportional to the trypsin concentration (Fig. 3). The concentration of nitrophenyl ester was 5×10^{-4} M. The average esterase activities were found to be 5.3×10^{-4} mole/min./mg. P.N. for the carbonate and 11.1×10^{-4} mole/min./mg. P.N. for the acetate.

DISCUSSION

The present work has shown that when trypsin is inhibited with diethyl *p*-nitrophenyl phosphate (E. 600, Paraoxon), the rate of liberation of nitrophenol is the same as the rates of inhibition of the proteolytic, amino acid esterase and amidase activities of the enzyme. This is consistent with the view that the same site on the enzyme surface is concerned in the four processes. The same behaviour has previously been demonstrated for the inhibition of chymotrypsin by E. 600 (Hartley & Kilby, 1952). It is known that trypsin which has been inhibited by diisopropylphosphorofluoridate (DFP) contains phosphorus derived from the inhibitor (Jansen & Balls, 1952; Cunningham, Tietze, Green & Neurath, 1953), while our work has shown that during the inhibition process, a portion of the inhibitor is released (nitrophenol from E. 600 or its *S*-ethyl analogue). These observations suggest that the process of inhibition of trypsin is one of phosphorylation; e.g. with E. 600:

and is thus the same process as the inhibition of chymotrypsin by organophosphorus compounds.

In this latter case, additional evidence is available as it has been shown that DFP-inhibited chymotrypsin contains two isopropyl groups derived from the inhibitor/molecule of enzyme (Jansen, Nutting, Jang & Balls, 1950).

When chymotrypsin is inhibited with E. 600, one molecule of nitrophenol is liberated for each molecule of enzyme inhibited. Calculation of the stoichiometry of the trypsin-inhibitor reactions with the two compounds used involves an assumption as to the molecular weight of trypsin, and the results just presented were calculated on the basis of the original value of 36 500 found by osmotic pressure measurements (Kunitz & Northrop, 1936). This led to a value close to two for the number of molecules of nitrophenol liberated when one molecule of enzyme is inhibited. Our value for the 'equivalent weight' of trypsin (i.e. that weight which reacts with the molecular weight of a simple inhibitor as DFP or E. 600) is 17 000–18 000, while the phosphorus analysis of DFP-inhibited trypsin made by Jansen & Balls (1952) indicate an equivalent weight of 20 700. If the molecular weight of trypsin is twice the equivalent weight (as Kunitz & Northrop's figure would indicate), then reaction of trypsin with inhibitor might show the kinetics of a termolecular reaction. The velocity constants were measured when the inhibition reaction was carried out in the presence of different large excesses of inhibitor, so that the reaction followed first-order kinetics. These velocity constants were found to be proportional to the inhibitor concentration and not to its square, indicating that the inhibition reaction was behaving as a second-order and not a third-order one. This could be explained by suggesting that the enzyme molecule contains two active centres which are independent and that the inhibition of one does not affect, in a measurable manner, the enzymic activity or rate of reaction with inhibitor of the other centre; or that there is a single active centre which reacts initially with one molecule of inhibitor in a slow, rate-controlling reaction, followed by a fast reaction with a second molecule of inhibitor. Another possibility is that the molecular weight of trypsin is half the original value proposed and that there is a single active centre in the molecule, as in the closely parallel case of chymotrypsin. Recently a number of estimations of the molecular weight of trypsin or related compounds, such as trypsinogen or DFP-inhibited trypsin, have been made and values around 20 000 have been reported. Bergold (1946) found a value of 15 100 for trypsin from a single sedimentation analysis. The molecular weight of the complex of soya bean inhibitor with trypsin was found by McLaren (1952) to be 41 000 which corresponds to a molecular weight of 17 000 for trypsin on the basis of a one-to-one combination of enzyme and

inhibitor. Crystalline DFP-inhibited trypsin was found by Cunningham *et al.* (1953) to have a molecular weight of 24 000 from sedimentation and diffusion rate measurements, and an equivalent weight of 24 800 from the phosphorus content. Crystalline trypsinogen was found by Tietze (1953) to give a value of 23 700, while Davie & Neurath (1953) have shown that a peptide of minimum molecular weight of about 1000 is split off from trypsinogen during autocatalytic activation to trypsin. The rather high values of 36 000 and 34 000 have been found by deuteron bombardment of crystalline trypsin and electron bombardment of amorphous trypsin, respectively (Pollard, Buzzell, Jeffreys & Forro, 1951). Measurements of the film molecular weight of trypsin spread on 35% ammonium sulphate gave a value of 41 000, considered by the authors (Mishuck & Eirich, 1951) to represent a dimer. It would appear that the divergencies in the values are probably due to varying degrees of association under the different conditions employed. Cunningham *et al.* (1953) consider that when trypsin is stable and active (e.g. in the presence of calcium ions at the optimum pH of 7.8) or stable and inactive (e.g. at pH 3.0, or as DFP-trypsin at any pH) then it exhibits the sedimentation behaviour of a monodisperse solution; on the other hand, when trypsin is under conditions when it is active but unstable (e.g. at a pH favourable for enzymic activity, but with no stabilizing calcium ions), then it exists in a monomer-polymer equilibrium which is dependent upon the age of the solution and the protein concentration. Our measurements were made under conditions when trypsin is active and stable (pH 7.6 in the presence of 0.025 M calcium chloride) and so should be present as a monomer of molecular weight around 20 000. Our results are consistent with this behaviour.

Parathion was originally considered to be a powerful anticholinesterase *in vitro*, but recent work (Diggle & Gage, 1951) has shown that this was due to contamination with the 'S-ethyl isomer', and that very pure Parathion has low *in vitro* activity. The anticholinesterase effects *in vivo* have been shown by Gage (1953) to be due to oxidation to E. 600, a powerful inhibitor *in vivo* and *in vitro*. The behaviour of these three phosphorus compounds towards trypsin *in vitro* follows a similar pattern, Parathion showing no detectable inhibitory action in contrast to the high activity of the other two. Trypsin, like chymotrypsin, was found to be able to hydrolyse enzymically *p*-nitrophenyl acetate and *p*-nitrophenyl ethyl carbonate, but the activities are of a low order.

SUMMARY

1. The effect of calcium ions in stabilizing trypsin against autolysis has been confirmed.

2. The proteolytic activity of trypsin is inhibited by diethyl *p*-nitrophenyl phosphate (E. 600) and by *OS*-diethyl *O*-*p*-nitrophenyl phosphorothiolate, but not by diethyl *p*-nitrophenyl phosphorothionate (parathion).

3. *p*-Nitrophenol is liberated during the inhibition of trypsin by the two active inhibitors. The molar concentration of nitrophenol liberated after complete inhibition is equivalent to the molar concentration of the enzyme, assuming a molecular weight of 17 000–18 000 for trypsin.

4. The rates of inhibition of the proteolytic, amidase and amino acid esterase activities are equal and exactly correspond to the rate of liberation of nitrophenol.

5. Velocity constants for the inhibition of trypsin and chymotrypsin by the two inhibitors have been measured at different temperatures and activation energies calculated.

6. The results support the hypothesis of a single active centre in trypsin, and this is discussed in relation to published estimates of its molecular weight.

The authors are indebted to the Agricultural Research Council for a grant which made this work possible. They also thank Dr Gorini for information concerning the effect of calcium upon trypsin before this was published.

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