We wish to thank Mr A. L. Bacharach, Glaxo Laboratories Ltd., for the gift of crystalline ergocalciferol, and the Superintendent of the Low Temperature Station for the facilities provided for drying and storage of material. We are indebted to Mr S. G. Impey, Miss N. Gass and Mr D. R. Ashby for their valuable technical assistance, and to Miss I. K. Allen for computing assistance.

REFERENCE (PART II)

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Pancreatic Trypsin Inhibitor

2. REACTION WITH TRYPSIN

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(*Received* 29 *August* 1952)

The reactions of trypsin with its inhibitors are among the few known cases of specific interaction between two purified proteins (Kunitz & Northrop, 1936; Kunitz, 1947; Fraenkel-Conrat, Bean & Lineweaver, 1949; Fraenkel-Conrat, Bean, Ducay & Olcott, 1952; Schmitz, 1939; Laskowski & Laskowski, 1951). Under favourable conditions, the inhibitor-trypsin compound is formed extremely easily; thus, we have observed that when aqueous suspensions of pancreatic inhibitor crystals and trypsin crystals were allowed to merge on a microscope slide under a cover-slip, the characteristic crystals of inhibitor-trypsin compound formed rapidly in the boundary zone. The course of the reaction in solution can be studied, since the resulting compound is devoid of tryptic activity. This study, besides yielding information on the mode of reaction of trypsin with inhibitor and possibly with substrate, might also yield information on the interaction of proteins in general.

Kunitz & Northrop (1936) found that the pancreatic inhibitor (mol.wt. 6000) combined at pH 7 with about an equimolecular amount of trypsin (mol.wt. 34 000); the resulting compound was completely dissociated at pH 1 and reformed at higher pH. Both combination and dissociation required 'measurable time intervals', but kinetic data were not published. Grob (1949), using 10^{-9} M solutions, found the dissociation constant of the compound to be approximately 6×10^{-10} M.

In the present paper, detailed results are given of an investigation on the nature and course of reaction between trypsin and the crystalline pancreatic inhibitor whose properties and composition were described by Green & Work (1953). A method for following the course of reaction is described and the effect of pH on reaction velocity considered in particular.

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EXPERIMENTAL AND RESULTS

Trypsin, inhibitor and haemoglobin substrate were all crystalline preparations obtained as described by Green & Work (1953). The method of estimation of trypsin activity (adapted from Anson, 1938) was also as described, the degree of digestion of denatured crystalline carboxyhaemoglobin over 5 min. at pH 7·5 and 25° being measured by the optical density at 280 mμ. of the deproteinized solution. Unless otherwise stated, final trypsin concentration in the reaction mixture was 19 μg./ml. (1 trypsin unit (T.U.)/ml.) Measurement of protein concentration and definition of trypsin and inhibitor units (I.U.) were also as described. Since combination of inhibitor with trypsin under the conditions of experiment was stoicheiometric and almost irreversible, the trypsin activity of a mixture of the two compounds represented a measure of the uncombined trypsin.

Dissociation constant

This was calculated by investigating in detail near the equivalence point an inhibition curve (Fig. 1) obtained by adding increasing amounts of inhibitor to a fixed amount of trypsin at a greater dilution (4·75 μg./ml.) than that normally employed, so that dissociation would be more marked. The residual trypsin activity (0·2 unit) in the presence of one equivalent of inhibitor was used to calculate an approximate dissociation constant for the equilibrium, $IT \rightleftharpoons I + T$ (in which I = inhibitor and T = trypsin).

Table 1. *Effect of variation of haemoglobin concentration on inhibitor activity*

(Trypsin concentration 9·2 μg./ml. All haemoglobin solutions contained 32% (w/v) of urea.)

Haemoglobin concentration* (%, w/v)	0·41		0·82		1·65	
Inhibitor added (ml.)	0·15	0·3	0·15	0·3	0·15	0·3
Inhibition (%)	24	65	22	69	22	64

* Concentration of haemoglobin required to saturate trypsin = 1·1%.

Assuming the value 34000 for mol.wt. of trypsin,

$$1 \text{ T.U./ml.} = 5{\cdot}5 \times 10^{-7} \text{ mole/l.};$$

from Fig. 1,

$$[I] = [T] = \frac{0{\cdot}02}{2} \text{ T.U./ml.}$$

(total vol. of digest = 2 ml.)

$$[IT] = \frac{0{\cdot}51 - 0{\cdot}02}{2} = 0{\cdot}245 \text{ T.U./ml.}$$

therefore

$$K = 5{\cdot}5 \times 10^{-7} \times \frac{0{\cdot}01^2}{0{\cdot}245} = 2{\cdot}2 \times 10^{-10} \text{M}.$$

A second experiment gave $K = 3 \times 10^{-10}$ M.

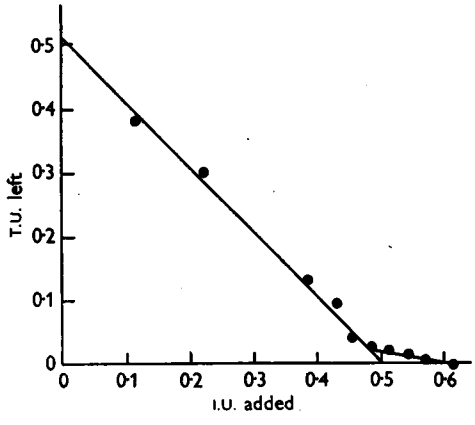

Fig. 1. Inhibition curve, illustrating the dissociation at pH 7·5 of the inhibitor-trypsin compound near the equivalence point. Trypsin concentration in final reaction mixture 4·75 μg./ml.

Variation of substrate concentration

In an attempt to decide whether inhibition of trypsin by the pancreatic inhibitor was competitive or non-competitive, determinations were made of inhibition by two different amounts of inhibitor in the presence of three different substrate concentrations. The concentrations of substrate and urea were maintained constant, and calibration curves of trypsin activity were plotted for each haemoglobin concentration. Table 1 shows that inhibition was independent of substrate concentration over a four-fold variation of concentration. When the haemoglobin concentration was further raised, the results were rendered inconclusive because of partial precipitation of the haemoblobin during the digestion.

Velocity constant

In the usual determination of inhibitor activity by constructing inhibition curves (Green & Work, 1953), inhibitor and trypsin were allowed to react for 5 min. before the addition of substrate. The resulting digestion value was then proportional to the

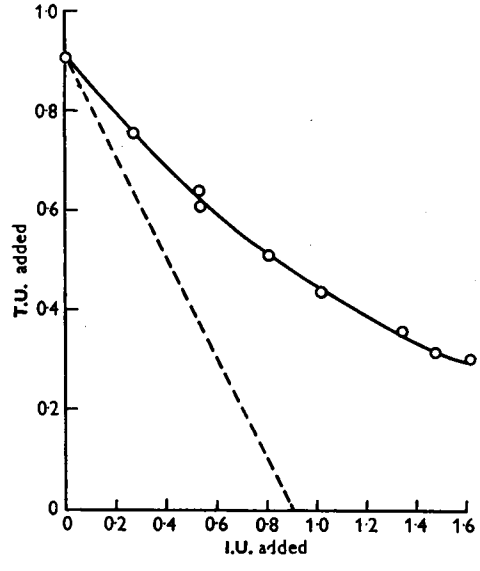

Fig. 2. Effect of order of addition of reactants on inhibition curve. O—O, Trypsin added to mixture of haemoglobin and inhibitor; – – –, standard type of curve which would have resulted if haemoglobin had been added to a mixture of trypsin and inhibitor after 5 min. standing at 25°.

difference between trypsin and inhibitor concentrations ($[T] - [I]$). If, on the other hand, trypsin was added to a mixture of inhibitor and substrate, it was found that the observed inhibition was much lower (Fig. 2). This is readily understood if the reaction between inhibitor and trypsin is a slow one, as indicated by Kunitz & Northrop (1936). The trypsin activity at the beginning of the 5 min. digestion period would then be that of the initial free trypsin, while the activity at the end would be

approximately $[T]-[I]$. The observed digestion value would be a measure of the average trypsin activity during 5 min.; moreover, it would be some function of the initial concentrations of free inhibitor and free trypsin, and hence could be used to determine the extent of reaction in a partially reacted mixture of inhibitor and trypsin. In order to follow the reaction between inhibitor and trypsin, a calibration curve was determined (Fig. 3), showing the degree of digestion of haemoglobin in the presence of known equivalent amounts of trypsin and inhibitor. Digestion flasks were prepared containing substrate and different amounts of inhibitor; trypsin, equivalent in amount to the inhibitor present, was added to each flask and the degree of digestion after 5 min. determined in the usual way.

A velocity constant for the reaction between inhibitor and trypsin in the presence of haemoglobin was calculated from the calibration curve. In view of the low dissociation constant of the trypsin-inhibitor complex, the velocity of dissociation of this complex was neglected.

Let a = initial inhibitor concentration
= initial trypsin concentration.
$a-x$ = trypsin activity left after time t.
k = 2nd order velocity constant (ml./T.U./min.).

$$-\frac{dx}{dt}=k(a-x)^2,$$

therefore
$$kt=\frac{1}{a}\frac{x}{a-x}, \qquad (1)$$

$$x=\frac{a^2kt}{1+akt}.$$

Average trypsin activity during t min.

$$=\frac{1}{t}\int_0^t (a-x)\,dt,$$

substituting for x

$$=\frac{a}{t}\int_0^t \frac{1}{1+akt}\,dt$$

$$=\frac{1}{kt}\ln(1+akt). \qquad (2)$$

The dotted line in Fig. 3 was obtained by plotting equation (2) using a value for k of 1·05 ml./T.U./min. ($3{\cdot}1\times10^4$ l./mole/sec.). (k is expressed in two types of units, owing to uncertainty over the mol.wt. of trypsin.)

As there was reasonable agreement between the calculated curve and the observed points, this value of k was used to calculate the time of half reaction ($1/kt$) and gave a value of about 2 min. In contrast, preliminary experiments showed that the inhibitor-trypsin reaction in the presence of buffer at pH 7 was almost complete in 1 min. There was thus a considerable discrepancy between the time of the inhibitor-trypsin reaction in the presence and in the absence of substrate.

Effect of urea. The explanation of this discrepancy could not lie in the effect of substrate on the reaction, since haemoglobin concentration had already been shown to have no effect on inhibition (Table 1). There was the possibility that the high

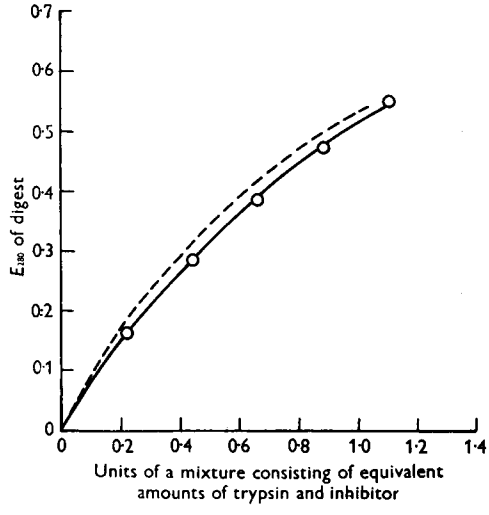

Fig. 3. Calibration curve for determining the amount of free inhibitor present in a partially reacted trypsin-inhibitor mixture. O—O, digestion values obtained from the reaction of equivalent amounts of trypsin and inhibitor for 5 min. in the presence of 1 ml. of substrate solution; – – –, plot of equation (2) using $k=1{\cdot}05$ ml./T.U./min.

urea content of the substrate solution might be affecting reaction velocity. Determination of the effect of 14 % urea on the rate of reaction of trypsin with inhibitor in buffer at pH 7 showed that this was the correct explanation; inhibitor was mixed with buffered trypsin (pH 7) in the presence or absence of urea; at 10 sec. intervals after mixing, 1 ml. portions of these mixtures were added to 1 ml. of substrate solutions so adjusted as to give identical final urea concentrations in the two digests. The digestion value after 5 min. was measured as usual, and the degree of reaction for each point read from the calibration curve (Fig. 3). The velocity constants k for the two reaction mixtures were determined by means of equation (1) from the slope (ka) of the lines obtained by plotting t against $x/(a-x)$ (Fig. 4). The value 0·86 ml./T.U./min. ($2{\cdot}6\times10^4$ l./mole/min.) obtained in the presence of urea was of the same order as that determined by calculation from Fig. 3. In the absence of urea k was 6·9 ml./T.U./min. (21×10^4 l./mole/min.), confirming that urea lowered the velocity of the inhibitor-trypsin reaction.

Effect of acid reaction. To test the effect of change in pH in the acid region on the velocity of the inhibitor-trypsin reaction, the time course of reactions at pH 3, 4, 5, 5·9 and 7 were determined by mixing equivalent amounts of trypsin and inhibitor in 0·02 M-citrate-phosphate buffer of the required pH, testing trypsin activity of portions at various time intervals and calculating percentage reaction from Fig. 3. The results (Fig. 5) show that as the pH

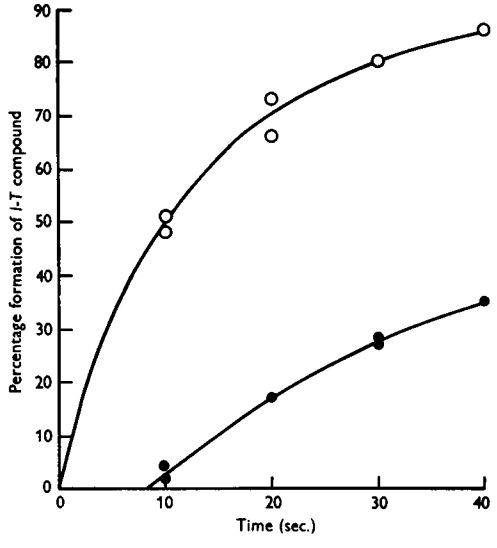

Fig. 4. Effect of urea on rate of reaction between inhibitor and trypsin at pH 7·0. 0·5 ml. trypsin (6 T.U./ml.) added to 2·4 ml. 0·01 M-phosphate buffer pH 7 + 0·55 ml. inhibitor (5·6 I.U./ml.) + 1·55 ml. of either urea (45%, w/v) or water. 1 ml. samples of these reaction mixtures were added at 10 sec. intervals to 1·0 ml. of substrate solution containing 1·2% haemoglobin and either 16 or 30% urea, the final urea concentrations being the same in each case. Resultant degree of reaction obtained from digestion value using calibration curve (Fig. 3). ●—●, reaction in presence of 14% urea; ○—○, normal reaction without urea.

was lowered the velocity dropped rapidly between pH 7 and 5·9; there was little change between pH 5·9 and 5, but at pH 4 the velocity was further reduced. At pH 3 there was no measurable reaction, the resulting digestion values being identical with the control values obtained by adding the same amount of trypsin to a mixture of inhibitor and substrate.

Effect of alkaline reaction. This was less easy to investigate than acid reactions owing to the ready alkaline denaturation of trypsin. It was first necessary to determine if denatured trypsin combined with inhibitor; accordingly, inhibition curves were constructed with trypsin which had been partially denatured by either heat or alkali; Fig. 6 shows the slope of these lines to be the same (within 10%) as that with control untreated trypsin; therefore, denatured trypsin had a negligible effect on the inhibitor-trypsin combination.

The effect of alkaline reaction on the combination was estimated by allowing trypsin to react with different amounts of inhibitor in glycine-sodium hydroxide buffers at pH 12·4, 11·8, 10·5 for 1 min.

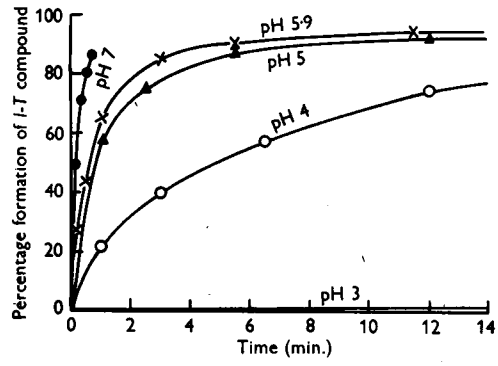

Fig. 5. Effect of acid pH on velocity of inhibitor-trypsin reaction. Equivalent amounts of trypsin and inhibitor reacted in 0·02 M-citrate-phosphate buffer; trypsin activity tested on portions after times shown. Degree of reaction calculated from Fig. 3. Trypsin concentration in inhibitor-trypsin reaction mixture 43 μg./ml., except in case of pH 7 reaction where it was 22 μg./ml.

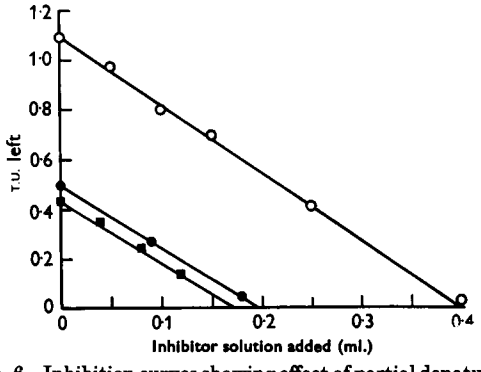

Fig. 6. Inhibition curves showing effect of partial denaturation of trypsin on its combination with inhibitor. ○—○, native trypsin; ●—●, trypsin heated at 50° at pH 7 for 5 min.; ■—■, trypsin exposed to pH 12 for 1 min.

before addition of substrate. With the more alkaline buffers, it was necessary to add a predetermined amount of acid to the substrate to bring the pH of the final digestion mixture to 7·5. Owing to alkaline inactivation of trypsin, no determinations of velocity could be made; the results presented in Fig. 7 show inhibition curves for the various reactions moved vertically so that they start from the same ordinate to facilitate comparison. The circles on the upper and lower curves are control points

showing respectively the effect of no combination (trypsin added to haemoglobin and inhibitor) and of almost complete combination at pH 7. In order to relate the results to those obtained at acid reaction, a similar inhibition curve was plotted at pH 4; this curve and that obtained at pH 12·4 were almost identical with the control 'no reaction' curve showing that there was very little reaction at

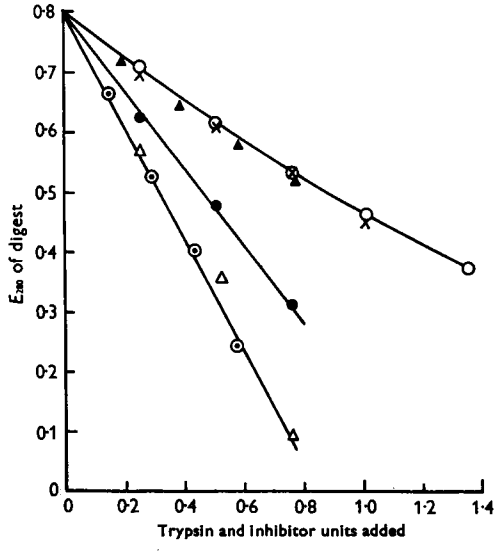

Fig. 7. Effect of alkaline pH on inhibitor-trypsin reaction. Scales adjusted to give common origin. Trypsin (15 μg./ml.) reacted with equivalent amounts of inhibitor in glycine-NaOH buffer for 1 min. before addition of substrate at pH 7·5. Usual 5 min. digestion period. ○, no reaction, control, trypsin added to inhibitor and haemoglobin; ×, pH 4; ▲, pH 12·4; ●, pH 11·8; △, pH 10·5; ⊙, pH 7, complete reaction.

pH 12·4 in 1 min. The curve obtained at pH 10·5 was almost identical with that at pH 7, while at pH 11·8 the degree of reaction was intermediate between the two controls.

DISCUSSION

The value of 2×10^{-10} M for the dissociation constant at pH 7 of the inhibitor-trypsin compound is probably correct only within an order of ten on account of the very small measured value from which it was calculated (residual trypsin activity in the presence of one equivalent of inhibitor, Fig. 1). However, Grob (1949), working at lower enzyme concentration (10^{-9} M as compared with $1{\cdot}4 \times 10^{-7}$ M) and correspondingly higher degrees of dissociation, found $k = 6 \times 10^{-10}$ M, in reasonable agreement with our value. He also showed that trypsin inhibitors from ovomucoid and lima bean formed trypsin complexes which dissociated to similar extents, while the trypsin compound with soya inhibitor showed no dissociation.

It has not been possible to decide whether inhibition is competitive or non-competitive. The dissociation constant of the compound is very low compared to the known Michaelis constants for trypsin with a protein substrate (about 10^{-2} M, Fraenkel-Conrat *et al.* 1949) and if inhibition were competitive, it can be calculated that increase in haemoglobin concentration from 1 to 3% would only increase trypsin activity by 0·015 unit near the equivalence point, which would be scarcely detectable under the present experimental conditions. Also the conditions of test (incubation of enzyme with inhibitor before addition of substrate and short reaction time) are those under which even a true competitive inhibition would appear to be non-competitive (Wilson, 1951). It was decided to leave further work on this point until later, when ester substrates with low Michaelis constants (10^{-5} M, Schwert, Neurath, Kaufman & Snoke, 1948) will be investigated under different experimental conditions. (Preliminary work (Green, unpublished) has shown that, using ester substrates, competitive inhibition occurred with both pancreatic and soya-bean inhibitors.) A further disadvantage of the haemoglobin digestion method of measurement of trypsin activity, is that a strict kinetic interpretation cannot be applied to the results, owing to uncertainty over the value of S, and hence of K_m. The method is however useful in that the presence of urea slows the rate of reaction of enzyme and inhibitor so that the reaction velocity can be measured.

The marked effect of pH on the reaction between inhibitor and trypsin suggests that certain ionizing groups must be charged for the reaction to occur. Combination commences in the acid range between pH 3 and 4; the only ionizing groups in the inhibitor with pK in this range are the free carboxyl groups of aspartic and glutamic acids. In the alkaline pH range, ϵ-amino groups (pK 9·5–10·5) or guanidine groups (pK 11·5–12·5) might be playing a part in the reaction. In view of the fact that the inhibitor-trypsin compound is almost undissociated at pH 10·5 and only partially dissociated at pH 11·8, it appears that the dissociation of guanidine, rather than of ϵ-amino groups, is influencing the reaction. Alternatively, this pH effect might be due to the fact that in solution active trypsin is in equilibrium with a reversibly denatured form of trypsin; the extent of denaturation is dependent on pH and is a minimum at pH 5 (Kunitz & Northrop, 1934; Anson & Mirsky, 1934). If the inhibitor failed to combine with this reversibly denatured trypsin, the apparent degree of inhibition of trypsin would be dependent on pH. However, the extent of reversible denaturation at acid reactions is not sufficient to account for the observed retardation in rate of

formation of inhibitor-trypsin compound. In the alkaline region such an explanation in terms of denaturation would be more plausible.

The effect of pH on the combination of trypsin with other inhibitors has only been investigated for soya-bean inhibitor, here the compound dissociated below pH 2·9 but not between pH 3·6 and 10·4 (Sheppard, Maclaren & Johnson, 1950). It seems probable that most of the inhibitor-trypsin reactions are combinations between oppositely charged protein ions. Free amino and carboxyl groups have been shown to be essential for combination between trypsin and either ovomucoid or lima-bean inhibitors; when either of these inhibitors, or soya-bean inhibitor, combined with trypsin, there was a decrease in titratable amino nitrogen (Fraenkel-Conrat *et al.* 1949, 1952; Kunitz, 1947). With the exception of pancreatic inhibitor, all other inhibitors have been shown to react instantaneously with trypsin; pancreatic inhibitor takes a measurable time to react with trypsin in acid solution; it is also the only inhibitor known to have an isoelectric point in the alkaline region (Green & Work, 1953). Therefore, below pH 10, both trypsin (isoelectric point 10·8, Bier & Nord, 1951) and inhibitor will be positively charged, and so the combination between oppositely charged carboxyl and guanidine groups would be opposed by the net positive charge on the two molecules, and the reaction might be a relatively slow one. The reaction velocities have not yet been measured above pH 7, so that final proof of this explanation cannot be yet advanced.

The molar combining ratios with trypsin of the various types of inhibitors have not yet been fully worked out because of the present uncertainty as to the molecular weight of trypsin. Using ester substrates, Green (unpublished) has shown that 1 g. of a certain preparation of pancreatic inhibitor (activity 160) and 1 g. of soya-bean inhibitor were equivalent to 3·3 and 1·2 g. respectively of trypsin. These figures are in the right ratio for inhibitors of molecular weights 9000 and 24 000 respectively (Green & Work, 1953), and the pancreatic inhibitor figure is the same as the value found by Fraenkel-Conrat *et al.* (1952) for the lima-bean inhibitor which also has a molecular weight of about 9000.

SUMMARY

1. The combination of pancreatic trypsin inhibitor with trypsin was investigated. The dissociation constant of the inhibitor-trypsin compound was determined, using haemoglobin substrate.

2. The degree of inhibition of trypsin was unaffected by fourfold variation of substrate concentration.

3. A method was developed for determining the extent of reaction in an inhibitor-trypsin mixture. The velocity constants of the reaction in the presence and absence of substrate were determined.

4. The inhibitor-trypsin combination was considerably retarded by the presence of urea.

5. The presence of denatured trypsin was without effect on the reaction.

6. The effect of pH on reaction rates was investigated. At pH's 4 and 12·4 the degree of combination after 1 min. was negligible, at pH 3 no measurable reaction was found to occur even after several hours, while at pH's 10·5 and 7 combination was almost complete in 1 min.

7. An explanation of the slow rate of reaction and its pH dependence is advanced in terms of ionic combination, considering that both proteins have alkaline isoelectric points.

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