the shoot apex (95 %) and the lowest in the cotyledons (43 %). The cotyledons are no longer the main source of inositol in the plant. In the ungerminated seed 5.2 % of the inositol is in the free state, whereas in the young plant this has risen to 74 %. There has been an over-all increase of 910 % in the free inositol, after taking account of the losses in dry weight on germination, but a loss of 16.2 % in the total inositol. This result is in contrast with that of Cheldelin & Lane (1943), who found that with lima beans germinated for 48 hr. an increase in inositol occurred. Since their method of determining inositol was that of digestion with papain and Taka diastase, their result is open to criticism.

As the leaf develops, the concentration of free and total inositol increases, with a corresponding increase in the absolute amounts (Table 4). The fully open green leaves collected at different times of the day do not show any changes which might be ascribed to a diurnal variation.

SUMMARY

1. A study has been made of the distribution of free and combined inositol in both the ungerminated bean seed and the young bean plant.

2. In the ungerminated seed only 5.2 % of the total inositol is in the free state, and this rises to 74 % in the young plant.

3. A concentration gradient of inositol down the

plant from the shoot apex to the roots has been demonstrated.

4. The concentration of inositol in the leaves increases with progressive stages of growth.

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Kinetics of the Reaction between Trypsin and the Pancreatic Trypsin Inhibitor

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The reversible reactions between trypsin and several protein inhibitors have a number of features in common which suggest that the main forces holding the two molecules together are electrostatic. The most important observations supporting this hypothesis are: (1) the velocity of combination of inhibitor and trypsin has in most cases proved too great to be measured by simple techniques; (2) the inhibitor-trypsin compounds are dissociated at acid pH; (3) free amino groups disappear during the reaction; (4) blocking of amino or carboxyl groups of trypsin or inhibitor often prevents combination between the two proteins. This evidence will not be considered further here since it has recently been

reviewed in some detail (Green & Neurath, 1954; Laskowski & Laskowski, 1954).

The reaction between trypsin and the pancreatic inhibitor is much slower than are the other reactions and the rate can therefore be measured. It was suggested (Green & Work, 1953*b*) that this relatively low rate might be due to electrostatic repulsion between the pancreatic inhibitor and trypsin, since they are both positively charged below pH 9. All the other inhibitors which have been investigated have acid isoelectric points and are therefore negatively charged at neutral pH. Laskowski & Laskowski (1954) questioned this interpretation, mainly on the grounds that 14 % urea, which raises the dielectric constant of the medium and hence reduces electrostatic forces,

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slows the inhibitor-trypsin reaction instead of accelerating it. They did not, however, consider the specific effects of urea on protein molecules, which would almost certainly outweigh any medium effects.

In order to gain a more accurate idea of the role of electrostatic forces in this reaction a detailed investigation has been made of the effect of temperature and ionic strength on the rate. If the low rate were due only to electrostatic repulsion the entropy of activation would be large and negative and the heat of activation would be small. It will be seen below that the experimental values are the reverse of such expectation, but that the original hypothesis is not thereby invalidated.

EXPERIMENTAL

Materials

Trypsin. Twice-crystallized Worthington trypsin was dialysed for 24 hr. in a rocking dialyser against 0.001 M-HCl, and then freeze-dried. Solutions in 0.001 M-HCl were freshly prepared each day and stored in ice-water.

Pancreatic trypsin inhibitor. The twice-recrystallized, salt-free 'preparation (1)' of Green & Work (1953*a*) was used. The solutions in water were kept for 2 or 3 weeks in the refrigerator without loss of activity.

Soya-bean trypsin inhibitor. A salt-free crystalline preparation was kindly supplied by Dr M. Kunitz.

Benzoyl-L-arginine ethyl ester (BAEE). This was prepared by standard methods (Schwert & Eisenberg, 1949).

Methods

Protein concentration. Protein concentration was determined from optical-density measurements at 280 m μ with a Hilger Uvispek spectrophotometer. Since the conversion factor (mg. of protein)/ml. of solution of unit optical density for obtaining trypsin concentration from optical density has been found to vary from one preparation to another (Green, 1953; Laskowski & Laskowski, 1954), it was re-determined on duplicate trypsin samples dried to constant weight at 110°. It was found to be 0.638, 0.642 (mean 0.640), corrected for the presence of 0.2% of ash, obtained on incineration of a trypsin sample. The factor for the pancreatic inhibitor was taken as 1.22 (Green & Work, 1953*a*), and for the soya-bean inhibitor as 1.18 (Kunitz, 1947).

Esterase activity. Trypsin esterase activities were measured by the following modification of the continuous potentiometric titration method (Schwert, Neurath, Kaufman & Snoke, 1948). This method has been used mainly for measuring hydrolysis rates of the order of 0.1–1 m-mole/l./min., in an open beaker, the absorption of atmospheric CO₂ being neglected. In the present experiments it was necessary to follow much lower rates of hydrolysis and so the reactions were carried out in a specially constructed closed glass vessel. The calomel and glass electrodes were fitted with rubber seals and inserted through the top of the vessel, which also had a small inlet for a burette and another for making additions of reactants. Carbon dioxide-free air could be passed in through a small side arm. The vessel required about 10 ml. of solution to cover the electrodes.

It was held in a constant-temperature water bath ($\pm 0.02^\circ$) with its top just above the surface of the water. The top was covered with aluminium foil, cemented in place and earthed by a phosphor-bronze spring to the copper gauze that screened the glass electrode, in order to eliminate electrical interference.

The reaction mixture was stirred magnetically with a small bar magnet attached to a pulley driven from the main stirrer of the water bath. The thermometer in the bath was checked against an N.P.L. standardized thermometer.

The pH was measured on a Pye direct-reading pH and millivoltmeter. It was maintained constant ($\pm$ pH 0.05) during the ester hydrolysis by addition of NaOH (0.1 or 0.02 M) from 0.5 ml. or 0.1 ml. burettes.

The sensitivity of the method was increased in two ways. First, the buffer concentration was lowered from 10^{-2} M to 10^{-5} M, so that much smaller additions of base would give the same pH change. It was not practicable to use lower concentrations than this since the glass electrode became very sluggish and took a minute or more to come to equilibrium with added base. The second modification was to increase the sensitivity of the pH meter by inserting the variable potential source (compensator; see Fig. 1) in series with the voltmeter of the instrument, the jack plug provided being used. The function of this compensator was to oppose the e.m.f. from the amplifying system by an adjustable e.m.f. from a battery circuit. Small changes in pH thus produced changes in the small differential e.m.f., which were registered on the galvanometer. With the circuit illustrated a tenfold magnification of the scale was obtained and a change in pH of one unit produced full-scale deflection of the galvanometer. Any unit of pH could be selected by adjusting the variable resistances. The sensitivity was of the same order as that of the apparatus described by Burch (1954).

All experiments were carried out in the presence of 0.002 M-CaCl₂ to prevent autolysis of the trypsin. 2-Amino-2-hydroxymethylpropane-1,3-diol hydrochloride buffer (tris) was used, unless otherwise stated, and the ionic strength was adjusted to the required value with KCl. When low activities were to be measured reaction mixtures containing 10^{-5} M-tris, pH 7.8, and either trypsin or BAEE, were prepared in the vessel described above. The electrodes were inserted and the vessel was placed in the bath to equilibrate. A 0.1 ml. burette containing 0.02 M-NaOH was fixed in position with its tip beneath the surface of the

Fig. 1. Compensating device for increasing the sensitivity of the pH meter. The battery was a 1.5 v dry cell, the resistances (given in ohms) were ordinary radio components and the galvanometer was a Cambridge mirror galvanometer, internal resistance 20 Ω , full-scale deflection 5 μ A.

reaction mixture, an air-tight seal being made with a short length of rubber tubing. A current of CO_2 -free air, saturated with water vapour at the temperature of the water bath, was passed in through the side arm. The compensator was set to give a galvanometer reading of zero at the pH at which the reaction was to be carried out (7.8), and the pH was raised to 7.9 with alkali from the burette. The enzyme or substrate was then added and the vessel sealed with a small rubber stopper. The time was taken when the galvanometer reading fell to zero, more alkali was added and the time noted when the galvanometer again returned to zero, as in the method of Schwert *et al.* (1948). The slope of the graph of base consumed against time gave the rate of hydrolysis of the substrate. Readings were usually taken at approx. 20 sec. intervals until ten points had been obtained. It was possible to take readings every 10 sec. when necessary.

A slow fall in pH was observed even in the absence of enzyme and substrate, probably due to small amounts of CO_2 , and 1.5×10^{-3} moles of base/min. were required to maintain constant pH. Because of this the lower limit of activity that could be measured with an accuracy approaching that for higher activities was about 2×10^{-8} moles of base/min., corresponding to a trypsin concentration of 2×10^{-9} M. The blank due to CO_2 absorption could probably have been eliminated by more efficient sealing of the apparatus. However, further modification to this end would have made the apparatus less easy to assemble and to clean out, and this was undesirable owing to the large number of measurements that were to be made.

Measurement of higher esterase activities was carried out in the same way, higher buffer concentrations (up to 0.005 M), 0.1 M-NaOH and a 0.5 ml. burette being used. When reaction rates above 10^{-6} mole of base/min. were being measured precautions to exclude atmospheric CO_2 were unnecessary. A linear relation between trypsin concentration and esterase activity was observed from 2×10^{-9} M to 10^{-6} M, showing that there was no effect of changing buffer concentration on the activity.

After each estimation it was necessary to soak the electrodes in 10% KCl, adjusted to pH 2-3 with HCl, for about 1 min., in order to remove trypsin adsorbed on the glass electrode.

Velocity of the inhibitor-trypsin reaction. The reaction was stopped after measured time intervals by addition of the substrate (BAEE). This immediately combined with the free trypsin, whose concentration could then be estimated from the rate of hydrolysis of the BAEE. The validity of this measure of the free trypsin was checked by adding a known

amount of trypsin to a mixture of one equivalent of inhibitor and 0.005 M-BAEE. The velocity remained constant for 3 or 4 min. and the measured slope was only 2% lower than that in the absence of inhibitor (Green, 1953). These experiments were made at the highest trypsin concentrations that were to be used; at lower concentrations the error was less since the concentrations of free inhibitor and trypsin were both reduced. No correction was made for this displacement of substrate by inhibitor since the error was small and difficult to estimate.

Most of the velocity measurements were made at reactant concentrations of 10^{-6} or 10^{-7} M. Typical reaction mixtures of ionic strength 0.0085 and 0.106 are shown in Table 1. The apparatus was set up containing the reaction mixture with the exception of the trypsin, which was added later from a calibrated syringe fitted with a glass jet bent at a right angle. The syringe was filled and immersed almost to the tip in the water bath to bring it and its contents to the correct temperature. It was then wiped, clamped in position and the trypsin squirted rapidly in. The pH came to 7.8 ± 0.05 and remained there until the reaction was stopped by the addition of 0.5 ml. of 0.1 M-BAEE. The rate of hydrolysis due to the free trypsin remaining at the time when the BAEE was added was then measured as described above, commencing within 1 min. of the addition of the BAEE.

RESULTS

Effect of temperature (T) on the hydrolysis of BAEE

A plot of $\log k_3$ against $1/T$ at two ionic strengths is shown in Fig. 2. Each point is the mean of five to ten independent measurements of k_3 at a substrate concentration of 0.005 M. The best straight line through the four points and the standard deviations of the derived constants were calculated by the method of least squares, a molecular weight of 24 000 for trypsin (Cunningham, Tietze, Green & Neurath, 1953) being used. No correction was made for the presence of inactive material in the trypsin preparation, although it is probable that some was present, since the value of k_3 (25° , $I = 0.03$) was only 19.3 sec.^{-1} , whereas a preparation used previously (Green, 1953) had a k_3 of 21.6 sec.^{-1} . However, since it cannot be assumed that this last figure corresponds to 100% trypsin, no correction was made. It

Table 1. Composition of reaction mixtures used for the determination of inhibitor-trypsin reaction velocity

Quantities are given in millilitres of the solution specified. The first two columns refer to experiments in which the initial concentration of the two reactants was 10^{-6} M. The corresponding figure for the second pair of columns is 10^{-7} M.

Ionic strength (I) ...	0.0085	0.106	0.0085	0.106
0.02 M-Tris, pH 7.8	2.0	2.0	0.2	0.2
0.4 M- CaCl_2	0.05	0.05	0.05	0.05
M-KCl	—	1.0	—	1.0
0.1 M-KCl	—	—	0.23	—
Inhibitor*	<i>v</i>	<i>v</i>	<i>v</i>	<i>v</i>
Water	7.45 - <i>v</i>	6.45 - <i>v</i>	9.0 - <i>v</i>	8.25 - <i>v</i>
Trypsin (2×10^{-5} M)	0.5	0.5	—	—
Trypsin (2×10^{-6} M)	—	—	0.5	0.5

* Volume of inhibitor solution (*v*) equivalent to the trypsin used was determined in a preliminary experiment.

can be said with some certainty the difference in rate constant is due to the variation in the amount of inactive trypsin present and not to true variation of the kinetic constants, as will be made clear in the next section. The heat and entropy of activation given in Table 2 agree well with previously published figures (Schwert & Eisenberg, 1949; Gutfreund, 1955).

Inhibitor equivalent to 1 mg. of trypsin

This quantity was frequently checked during the course of these experiments. The mean of twenty-four determinations at four temperatures (5°, 15°, 25° and 35°) was 0.242 ± 0.005 mg. This was appreciably lower than the value 0.270 determined previously (Green, 1953), and could best be accounted for by the higher specific activity of the trypsin sample used in the earlier investigation. It was, in general, found that trypsin solutions which had lost activity on standing required correspond-

ingly less inhibitor to inactivate them. The figure 0.270 was used to calculate a molecular weight for the inhibitor of 6500, from the trypsin molecular weight of trypsin (24 000). These molecular weights were used in subsequent calculations in this paper.

Dissociation constant of the inhibitor-trypsin compound

An approximate figure for this dissociation constant (K_i) had previously been determined (Green, 1953) by measuring the concentration of free trypsin $[T_i]$ in the presence of one equivalent of inhibitor. In order to obtain an accurate value of this constant it was necessary to measure very low concentrations of free trypsin, and it was hoped that the method described above would be sufficiently sensitive for the purpose. However, it was soon discovered that the earlier value for the dissociation constant was much too high and that the true value was so low that an accurate estimate was impossible. The high figure had been obtained because insufficient time (3 min.) had been allowed for the inhibitor-trypsin reaction to come to equilibrium. In the present experiments it was found that the free trypsin continued to decrease for at least 20 min. at ionic strength 0.1. A further very slow decrease continued for some time longer, and even after an hour a steady value had not been reached. Since it could be calculated from the velocity constant that such a second-order reaction should be 99 % complete in 6 min., it seemed likely that the slow decrease of activity observed after 20 min. was due to spontaneous inactivation or autolysis of the trypsin, which is appreciable over long periods even in the presence of calcium salts. In addition to this source of error there was another due to the impossibility of determining the equivalence point with sufficient accuracy. For, at the lowest usable concentrations, only 0.5 % of the trypsin was in the free state, and therefore an error of 1 % in determining the correct proportion of inhibitor to trypsin gave errors of the order of 100 % in determining the free trypsin present at equilib-

Fig. 2. Effect of temperature on the hydrolysis of BAEE by trypsin. O, $I = 0.0085$; ●, $I = 0.106$.

Table 2. Kinetic constants calculated from Figs. 2 and 3 by the method of least squares

Free energies, heat contents and entropies given in this table refer to the formation of activated complexes from the reactants in their standard states (M aq. solution, pH 7.8, at the ionic strength quoted).

Reaction	Ionic strength	k_3 (25°) (sec. ⁻¹)	ΔG^* (25°) (kcal./mole)	ΔH^* (kcal./mole)	ΔS^* (e.u.)
Hydrolysis of BAEE	0.0085	20.2 ± 0.3	15.94 ± 0.01	10.48 ± 0.07	-18.3 ± 0.2
	0.106	18.9 ± 0.5	15.98 ± 0.02	10.5 ± 0.2	-18.2 ± 0.7
$10^{-4} k'$ (l./mole/sec.)					
Inhibitor-trypsin combination	0.0085	4.82 ± 0.15	11.34 ± 0.02	14.8 ± 0.15	11.6 ± 0.6
	0.0095	5.85 ± 0.25	11.22 ± 0.03	15.6 ± 0.1	14.8 ± 0.4
	0.035	15.7 ± 1.0	10.64 ± 0.05	12.6 ± 0.5	6.6 ± 1.5
	0.106	27.6 ± 1.5	10.30 ± 0.04	12.1 ± 0.3	6.0 ± 0.9

lence. Since trypsin solutions in 0.001M-HCl lost about 1% of their activity during the course of a day, an accurate determination of the equivalence point for a particular pair of solutions was made immediately before measuring the degree of dissociation. In this way eight determinations were made after allowing 10^{-6} M-inhibitor and trypsin to react for 20 min. ($I=0.1$, 25°). The calculated K_i values all lay between 10^{-11} and 10^{-12} M, corresponding to a free energy of association of 15–16 kcal./mol. More reproducible results could perhaps be obtained (1) by measuring the activity of purified inhibitor-trypsin compound, assuming no excess of inhibitor or trypsin to be present, (2) by using as substrate *p*-toluene-sulphonyl-L-arginine methyl ester (since it is hydrolysed five times as fast as BAEE) and lower concentrations of inhibitor-trypsin compound, to obtain correspondingly greater degrees of dissociation.

Kinetics of the inhibitor-trypsin reaction

The extent of combination of inhibitor and trypsin at pH 7.8 was measured as a function of time, ionic strength and temperature, the method described above being used. Second-order velocity

constants were calculated from the slope of $1/[T_i]$ against time, and five or six points were taken for each line. The points were usually spaced over the region from 30 to 70% reaction, since for a second-order reaction the errors are potentially the least in the neighbourhood of 50% reaction (Roseveare, 1931). The standard deviation of the slope varied between ± 1 and $\pm 4\%$ from one experiment to another. The slopes were converted into l./mol./sec. units with values for the specific activity of trypsin, determined at the appropriate temperature and ionic strength. Some representative second-order plots for the reaction at 25° are shown in Fig. 3. It can be seen that the reaction followed second-order kinetics up to at least 80% completion, that the second-order constants were independent of initial concentration and that they were strongly dependent on ionic strength.

The effect of ionic strength (I) on the second-order rate constants at 25° is shown in Fig. 4. The continuous curves were derived theoretically from the Brønsted-Bjerrum relation for the primary-salt effect in a reaction between ionic species A and B ,

$$\log k' = \log k_0 + \log \frac{f_A f_B}{f_{AB}},$$

Fig. 3. Reaction between equivalent amounts of inhibitor and trypsin at 25° , plotted according to second-order kinetics. $[T_i]$ is expressed in esterase activity units, moles of BAEE hydrolysed/l./min. One mole of trypsin at $I=0.1$ contains 1120 such units (the turnover number). The bottom and left-hand scales refer to the open points (10^{-7} M-reactants). The top and right-hand scales refer to the filled points (10^{-6} M-reactants). $\circ \bullet$, $I=0.106$; $\square$, $I=0.035$; $\triangle$, $I=0.0085$. The curves for the 10^{-6} M-reactants parallel those for the 10^{-7} M-reactants, showing that the velocity constants are independent of concentration.

and the Debye-Hückel expression for ionic activity coefficients,

$$\log f_i = -\frac{2.303z_i^2\epsilon^2}{2DkT} \left(\frac{\kappa}{1+\kappa a_i} \right)$$

(k_0 is the value of k' when $I=0$; f_i , z_i , a_i are the activity coefficient, net charge and radius of the ion i ; ϵ is the charge on the electron; k is the Boltzmann constant; D is the dielectric constant of the medium; T is the absolute temperature and κ the Debye-Hückel constant $= 3.28 \times 10^7 I^{\frac{1}{2}}$). For small ions κa_i is negligible compared with one, and a linear relation between $\log k'$ and $I^{\frac{1}{2}}$ results. However, this simplification cannot be made here, since for trypsin $\kappa a_i = 7.2I^{\frac{1}{2}}$ ($a_T = 22\text{\AA}$, see below) and the complete expression must be used.

$$\log k' = \log k_0 + \frac{\epsilon^2 \kappa}{2DkT(1+\kappa a_{IT})} \times \left[2z_I z_T - \frac{z_I^2(a_{IT}-a_I)\kappa}{1+\kappa a_I} - \frac{z_T^2(a_{IT}-a_T)\kappa}{1+\kappa a_T} \right],$$

Fig. 4. Effect of ionic strength on the rate of the inhibitor-trypsin reaction at 25° . The three curves were calculated from the equation for $\log k'$ given in the text, the following values for z_I , z_T and $\log k_0$ being used, the value of $\log k'$ at zero ionic strength.

	z_I	z_T	$\log k_0$
Curve (A)	3.5	7.5	3.39
Curve (B)	4.5	7.5	3.01
Curve (C)	5.5	7.5	2.63

The values of z_I and z_T for curve (B) were calculated from the amino acid composition of the two proteins. $\log k_0$ is arbitrary for all three curves.

where subscripts I, T and IT refer to inhibitor, trypsin and the compound of the two. If it is assumed that $a_I = a_T = a_{IT}$, the last two terms in the square brackets vanish. These terms were included in the present calculations, although they were only about 10 % of the term $2z_I z_T$ and so did not have any detectable effect on the agreement between experiment and theory. The protein radii were calculated from the molecular weights, spherical molecules being assumed, with the data of Bragg & Perutz (1952) for haemoglobin to provide the density of the hydrated protein. The calculated radii were, $a_I = 15\text{\AA}$, $a_T = 22\text{\AA}$ and $a_{IT} = 24\text{\AA}$. Three curves were calculated for different values of z_I . For curve (B) values of z_I (4.5) and z_T (7.5) were estimated from the analytical data for the inhibitor (Green & Work, 1953a) and trypsin (Green & Neurath, 1954). Curve (A) was calculated with $z_I = 3.5$ and curve (C) with $z_I = 5.5$. z_T was not varied independently since the slope of the curve depends mainly on the product $z_I z_T$. Since k_0 could not be determined experimentally the vertical positions of the curves were adjusted arbitrarily to make $\log k' = 4.76$ when $I^{\frac{1}{2}} = 0.1$. Although it appears that curve (A) gives the closest fit with the experimental points, it should be remembered that the Debye-Hückel theory does not hold at high ionic strengths and greatest weight should be given to the four points at low ionic strength, which lie more nearly on curve (B). The agreement between the observed slope and that calculated from the analytical data is good, but unfortunately it cannot be taken as a critical test of the theory since either of the analytical figures might be in error by ± 2 units of charge.

A few rate measurements were made at higher ionic strengths, not shown in Fig. 4. The highest rate was observed when $I = 0.21$ ($k' = 29.2 \times 10^4$ l./mole/sec.). Further increase in ionic strength decreased the rate (when $I = 0.51$, $k' = 18.9 \times 10^4$ l./mole/sec.).

The second-order velocity constants were also determined at 5° , 15° , 25° and 35° at ionic strengths of 0.0085, 0.0095, 0.035 and 0.106. The ionic strengths of 0.0085 and 0.106 were chosen for most detailed measurements, since the first was the lowest that could conveniently be used in the presence of 2 mM-calcium chloride, and at ionic strength 0.1 the rate constant approached its maximum value and hence the electrostatic contribution to the free energy of activation should have been small.

Fig. 5 shows the graphs of $\log k'$ against $1/T$, and Table 2 gives the free energies, heats and entropies of activation calculated by the method of least squares. The electrostatic contribution to these constants is theoretically obtainable by differentiation with respect to temperature of the Brønsted-Christiansen-Scatchard equation for the velocity

constant (Amis, 1949). The resulting equation for ΔH^* is

$$\Delta H^* = \Delta H_0^* + \frac{Nz_A z_B e^2}{Dj} \left(1 + \frac{\partial \log D}{\partial \log T} \right) \left[\frac{1}{r} - \frac{\kappa(\frac{3}{2} + \kappa a_i)}{(1 + \kappa a_i)^2} \right]$$

(ΔH_0^* is the hypothetical heat of activation when $I=0$ and $D=\infty$, r is the radius of the activated complex and j the mechanical equivalent of heat). Since $\partial \log D / \partial \log T = -1.37$ for water, the sign of the electrostatic contribution to the heat of activation is the reverse of what it would be in a medium of constant dielectric constant, and so for a reaction between ions of like charge the heat of activation should increase as the ionic strength increases. This has been verified by Amis & LaMer (1939) for some reactions between small ions. They found that the predicted relation between ΔH^* and $I^{\frac{1}{2}}$ held at low ionic strength, but that when $I > 0.01$ the slope changed sign and ΔH^* decreased quite rapidly. Qualitatively the same effects were observed here, though the magnitude of the change was much greater than the few hundred calories found by Amis & LaMer. Further comparison with this theory cannot profitably be made without more data at low ionic strength, where the Debye-Hückel limiting law is valid.

Dissociation of the inhibitor-trypsin compound at acid pH

Earlier observations (Kunitz & Northrop, 1936; Green & Work, 1953*b*), showing that the inhibitor-trypsin compound dissociated in the region of pH 3, suggested that combination did not occur unless

Fig. 5. Effect of temperature on the rate of the inhibitor-trypsin reaction at different ionic strengths. Δ , $I=0.0085$; $\blacksquare$, $I=0.0095$; $\square$, $I=0.035$; $\circ$, $I=0.106$.

certain carboxyl groups were negatively charged. In order to obtain some idea of the number of such groups involved, a preliminary study was made of the effect of pH on the dissociation constant of the compound.

In most of the experiments the inhibitor-trypsin compound was prepared from equivalent amounts of inhibitor and trypsin (10^{-5} M) in 0.002 M-borate buffer, pH 7.9, in the absence of Ca^{2+} ions. The solution of the compound was mixed with an equal volume of 0.1 M-glycine buffer of the desired pH and samples were taken at intervals and squirted into 0.005 M-BAEE in borate buffer, pH 7.8, containing 0.01 M- CaCl_2 . The rate of hydrolysis of the BAEE measured the amount of free trypsin present. Equilibrium constants were calculated from the steady values for free trypsin reached after 4 or 5 hr. These checked well with the constants obtained by starting from free inhibitor and trypsin and following their combination. The resulting values of $\log K_i$ are plotted against pH in Fig. 6. The points have not been joined by a line since they were not sufficiently accurate to determine the shape of the curve. However, it can be seen that in the neighbourhood of pH 3 the slope is 2.5.

This slope is most simply explained by assuming that three specific carboxyl groups must be negatively charged for combination to take place (cf. Steinhardt, 1937). If the reaction is written,

and if $K_1 > K_2 > K_3$ are the apparent dissociation constants of the three trypsin carboxyl groups,

$$\log K_i = \log K - \log \left(1 + \frac{h}{K_3} + \frac{h^2}{K_3 K_2} + \frac{h^3}{K_3 K_2 K_1} \right),$$

Fig. 6. Effect of pH on the apparent dissociation constant of the inhibitor-trypsin compound at 25°.

where h is the hydrogen-ion concentration. The equation retains the same form whether all the carboxyl groups are assumed to be on the same molecule or whether there are two on one and one on the other. In the latter case, however, the constants become composite ones. By differentiating this expression with respect to pH the slope of the pH- pK_i graph is obtained.

$$\frac{d pK_i}{d \text{pH}} = \frac{\left(\frac{h}{K_3} + \frac{2h^2}{K_3 K_2} + \frac{3h^3}{K_3 K_2 K_1} \right)}{\left(1 + \frac{h}{K_3} + \frac{h^2}{K_3 K_2} + \frac{h^3}{K_3 K_2 K_1} \right)}.$$

When $h > K_1$ the slope will reach a maximum value of 3, and when $h = K_1 > K_2 > K_3$ the slope will be approximately 2.5. Although the theoretical curve would not be linear, model calculations show that quite a close approach to linearity can be obtained over a range of one or two pH units if the relative value of K_1 , K_2 and K_3 are appropriate. Accurate measurements of K_i at lower pH values, where the slope should approach 3, are difficult since the amount of inhibitor-trypsin compound present can only be found as a small difference between two large quantities. However, the data presented here are consistent with a reaction scheme in which combination between inhibitor and trypsin is dependent on the ionization of three specific carboxyl groups.

Reaction between the soya-bean inhibitor and trypsin

Previous experiments had shown that at trypsin concentrations of the order of 10^{-6} M this reaction was complete in a matter of seconds or less. However, by using the methods described above it was possible to study the reaction at concentrations of 10^{-8} M, where the velocity was just measurable. The half-time of the reaction was about 4 sec. and the second-order velocity constant (25° , pH 7.8, $I = 0.11$) was 2×10^7 l./mol./sec., about 100 times as fast as the pancreatic inhibitor-trypsin reaction. The method of stopping the reaction by addition of BAEE was checked as described above under Methods. It was found that addition of the inhibitor (10^{-8} M) after the substrate had no measurable effect on the rate for 2 or 3 min.

DISCUSSION

Several analyses of the electrostatic contribution to the free energy of interaction of charged protein molecules have been made (Edelhoc, Katchalski, Maybury, Hughes & Edsall, 1953; Steiner, 1953; Ehrenpreis & Warner, 1956), and it has often been possible to account for most of this quantity in terms of coulombic attraction. However, the free energies involved have been relatively small (5–6 kcal./mole),

whereas the free energies of formation of the various inhibitor-trypsin compounds are in the neighbourhood of 11–16 kcal./mole. The calculations made so far have assumed that the net charge is concentrated at the centre of a spherical protein molecule. While this may be adequate for long-distance interactions or even for non-specific short-distance interactions, it is clearly not valid where there is close association between specific ionic groups. When local-charge distribution permits such association there are several new factors which must be taken into account. In particular, the effective dielectric constant will be much lower than that of water in bulk owing to orientation of the water molecules in the intense electrostatic field (Waugh, 1954). For example, if two charged groups approach within 5 Å of each other the effective dielectric constant may be as little as one-tenth of that in the bulk of the solution. In such a case the interaction energy would be about 8 kcal./mole. Since it is likely that three such pairs are involved in the combination of the pancreatic inhibitor with trypsin, binding of this type would be adequate to explain the observed equilibrium constant and would account for the apparently electrostatic nature of the forces holding inhibitor and trypsin together in spite of their similar net charges.

Although this combination of long-range electrostatic repulsion with specific short-range electrostatic attraction could give a potential-energy curve which goes through a maximum as the molecules approach each other, nevertheless it would be impossible to explain the observed kinetic constants in terms of such a simple mechanism. In the first place, owing to the low inverse power of the distance governing coulombic interactions, this potential-energy maximum would be about 10–20 Å from the molecular surface, and would therefore reduce the collision number rather than increase the activation energy. The number of effective collisions would be still further reduced by the stringent steric requirements for combination between three specific pairs of groups. Both these factors would be expected to produce a large negative entropy of activation, contrary to the experimental findings.

Unexpectedly positive entropies of reaction (Edelhoc *et al.* 1953; Doty & Myers, 1953; Steiner, 1954) and activation (Edsall, Maybury, Simpson & Straessle, 1954) for associations between protein molecules have been observed on several occasions; in fact they appear to be the rule rather than the exception. They have usually been explained by postulating an increase in solvent entropy when water molecules are displaced from the combining protein surfaces. A similar assumption was made by Eisenberg & Schwert (1951) to explain the high positive heat and entropy of denaturation of chymotrypsinogen. In the same way this postulate

may be invoked here to account for both the positive entropy and the heat of activation, which is high for a reaction in which formation of electrostatic links appears to be the primary result. If assumptions similar to those of Eisenberg & Schwert (1951) are made concerning the magnitude of the heat of hydration, then displacement of ten water molecules from the two proteins would be sufficient to account for the heat of activation.

The increase of rate constant with ionic strength (Fig. 4) shows that electrostatic repulsion considerably reduces the rate of combination of inhibitor and trypsin. Although, as we have just seen, this repulsion cannot account for the kinetic constants of the reaction it may well explain the low rate relative to that of the other inhibitor-trypsin reactions. For example, the preliminary results given above show that the soya-bean inhibitor reacts about 100 times faster than the pancreatic inhibitor ($I = 0.1$). Calculations from the equation used in deriving the curve of Fig. 4 show that a negative charge of three units on the soya-bean inhibitor at pH 7.8 would be sufficient to account for the difference in rate constant between the two reactions, assuming that other factors affecting the reaction are unchanged. Since the isoelectric point of the soya-bean inhibitor is at pH 4.5 (Kunitz, 1947), a charge of this magnitude would be quite reasonable. If this interpretation is correct the reaction between trypsin and the soya-bean inhibitor should have a heat of activation of about the same magnitude as that with the pancreatic inhibitor and a considerably more positive entropy of activation.

Although no direct mention has been made of the possibility of hydrogen bonding between trypsin and inhibitor, electrostatic interaction between $-\text{NH}_3^+$ and $-\text{CO}_2^-$ probably implies simultaneous hydrogen-bond formation. Other types of hydrogen bond may also be involved, but there is no evidence for or against such an assumption.

SUMMARY

1. A potentiometric method of high sensitivity is described for following reactions in which H^+ ions are liberated or absorbed. It has been used to measure the extent of dissociation of the pancreatic inhibitor-trypsin compound.

2. The kinetics of the reaction between trypsin and the pancreatic inhibitor has been followed as a

function of ionic strength and temperature at pH 7.8.

3. The effect of ionic strength on the rate at 25° was in accord with the Brønsted-Bjerrum theory for reaction between ions of like charge, values of the net charge derived from analytical data being used.

4. The interpretation of the heats and entropies of activation is discussed in terms of current theories of protein interaction.

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