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The Molecular Kinetics of Trypsin Action

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Very little reliable information exists about the absolute rates and activation energies of enzyme reactions. In many cases in which measurements have been made, the reaction is sensitive to the hydrogen ion concentration and corrections for the effect of temperature on the *p*H are necessary before the true activation energy can be known. The object of this work was to determine these quantities for some typical proteolytic processes on both natural and synthetic substrates. Trypsin is particularly suited to a study of this kind because the same enzyme brings about a number of different reactions, *e. g.*, (a) the hydrolysis of ammonia from the synthetic peptide benzoyl-*l*-arginine amide[1]; (b) the conversion of chymotrypsinogen into chymotrypsin[2]; (c) the autocatalytic conversion of trypsinogen into trypsin[3]; (d) the digestion of native and denatured proteins. In the region of the optimum *p*H the effect of changes of *p*H is comparatively small and the effect of temperature change on the *p*H can probably be neglected in finding the activation energy of the reaction.

It was known that good unimolecular velocity constants, which are proportional to the trypsin concentration, are obtained for reactions (a) and (b). The action of trypsin on a protein is to be regarded as a group of simultaneous or consecutive reactions and it is not usually possible to find a velocity constant covering the whole course of the reaction. Northrop[4] showed that in the action of trypsin on casein the first stage could be distinguished by observing the change of viscosity and a fairly good velocity constant was calculated therefrom. For the comparison of reactions (a) and (b) with (d) we have used as the velocity constant of the latter the initial rate of digestion (measured by the number of acid groups liberated as determined by the formol titration), divided by the molecular concentration of the substrate. This would be identical with the true velocity constant of the reaction when each molecule of substrate gives rise to one equivalent of acid on complete hydrolysis. In other cases it seems a good basis of comparison (on the assumption that the action of trypsin is really primarily the breaking of peptide bonds) as it gives the number of bonds broken by a given concentration of enzyme with equal numbers of substrate molecules. If the protein contains different kinds of bonds which are acted on by the enzyme at different rates, the observed rate will be the sum of the rates of the various actions. In many cases, however, the observed rate would be predominantly that of one particular action and no great error will arise from treating it as such. Considerable caution is required however in the interpretation of the activation energy in such a case since the different processes may have different temperature coefficients. In such a case the Arrhenius equation will not hold.

The procedure can be applied only to the initial stage of the reaction where the protein is mainly intact. As the reaction proceeds new bonds may become accessible to the enzyme, the velocity constants may be influenced by the changes which have taken place in other parts of the molecule, as is well known[5] the products of the digestion may exert an inhibitory effect and when the protein breaks up the substrate concentration becomes indefinite.

The choice of a protein substrate presented some difficulty. It is known that trypsin frequently does not act appreciably on native proteins, *e. g.*, egg albumin, but it digests them rapidly when denatured. Casein is acted on rapidly, but is a mixture of proteins. We tried pepsin denatured by warming at *p*H 7.5 as a substrate; trypsin appears to have a real action on it but the amount is so small that the initial rate is very difficult to measure. Trypsin also digests protamines very easily[6] and sturin was found to be a very suitable substrate. Experiments were also made with chymotrypsin acting on the synthetic peptide benzoyl-*l*-tyrosyl-glycyl amide[7] and on denatured pepsin.

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(1) M. Bergmann, J. S. Fruton and H. Pollok, *J. Biol. Chem.*, **127**, 643 (1939); K. Hofmann and M. Bergmann, *ibid.*, **138**, 243 (1941).

(2) M. Kunitz and J. H. Northrop, *J. Gen. Physiol.*, **18**, 433 (1935).

(3) M. Kunitz and J. H. Northrop, *ibid.*, **19**, 991 (1936); M. Kunitz, *ibid.*, **22**, 293 (1939); *Enzymologia*, **7**, 1 (1939).

(4) J. H. Northrop, *J. Gen. Physiol.*, **16**, 339 (1932).

(5) J. H. Northrop, *ibid.*, **4**, 487 (1922).

(6) E. Waldschmidt-Leitz and T. Kollmann, *Z. physiol. Chem.*, **166**, 262 (1927).

(7) M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, **118**, 405 (1937); **124**, 321 (1938).

TABLE I

Enzyme	Substrate	$\log_{10} k$ (0°)	ΔH	ΔS
Trypsin	Benzoyl-*l*-arginine amide	0.40	14,900	− 6.2
Trypsin	Chymotrypsinogen	2.6	16,300	+ 8.5
Trypsin	Sturin	3.33	11,800	− 4.7
Chymotrypsin	Benzoyltyrosylglycyl amide	1.57	10,500	−17.4
Chymotrypsin	Pepsin	2.34	11,200	−11.5
(Hydrogen ion)	Acetylglycine	−6.47 (60°)	21,200	−24.8

Discussion

The evidence previously available appeared to indicate that enzyme reactions are frequently abnormal kinetically. In a review of the available information[8] Stearn found that in most cases the entropy of activation of enzyme reactions was considerably more negative than that of the same reactions catalyzed by acids, *etc.* This means that the increase in the reaction rate brought about by the enzyme is not so great as would be expected from the decrease of activation energy, so that there is a factor which might be a stringent condition of mutual orientation of the substrate and enzyme molecules which "interferes" with the reaction.

This conclusion is not borne out by the data obtained here. Figure 1 shows that over the range of temperatures studied the Arrhenius expression holds within the experimental error. Table I summarizes the characteristics of all the reactions and gives the entropy of activation, ΔS, calculated by

$$k = \frac{RT}{N_0 h} e^{-\Delta H/RT} e^{\Delta S/R}$$

or, for 0°

$$k^0 = 5.7 \times 10^{13} e^{-\Delta H/RT} e^{\Delta S/R}$$

The activation energies of these reactions are rather high for enzymatic processes (10–16 kcal.), although considerably lower than that of the hydrolysis of the peptide bond by acids which is about 21 kcal.[9] The entropies of activation are, with one exception, reasonably close to the range (−5 to −10) usually taken as representing normal reactions.

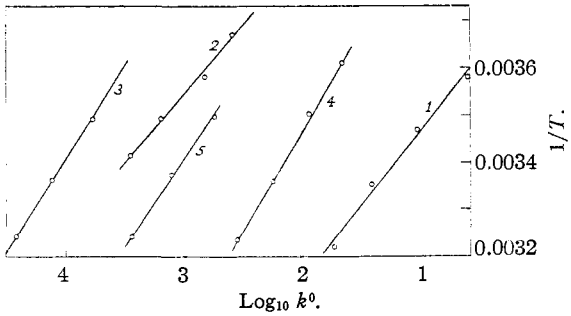

Fig. 1.—Plots of $\log_{10} k^0$ against $1/T$ for: (1) trypsin on benzoyl-*l*-arginine amide; (2) trypsin on chymotrypsinogen; (3) trypsin on sturin; (4) chymotrypsin on benzoyltyrosyl-glycylamide; (5) chymotrypsin on denatured pepsin.

The rates are, in fact, not very different in most cases from that calculated on the simple collision theory, *viz.*

$$k = Ze^{-\Delta H/RT}$$

where Z, the number of collisions between the reacting molecules per cc., is given by

$$Z = n_1 n_2 \left(\frac{\sigma_1 + \sigma_2}{2}\right)^2 \left\{8\pi RT \left(\frac{1}{M_1} + \frac{1}{M_2}\right)\right\}^{1/2}$$

where n_1, n_2 are the numbers of the reacting molecules per cc.; $\sigma_1\sigma_2$ their diameters and M_1, M_2 their molecular weights. Taking for trypsin $\sigma_1 = 6 \times 10^{-7}$ cm., $M_1 = 36{,}000$, we find that the calculated rate, which is not much influenced by the size of the substrate molecule, corresponds to $\Delta S = -6$ to -8. The conversion of chymotrypsinogen, which has a positive entropy, is considerably faster than the other reactions when allowance is made for its high activation energy.

In reactions involving one or two large molecules it can hardly be expected that every collision having the necessary energy will lead to reaction, since there must inevitably be steric or orientational factors tending to reduce the rate. It follows that even for reactions coming within the normal range for small molecules, there must be a factor producing an enhanced rate. Such a factor may be the complex formation between enzyme and substrate which is a very general feature of enzyme reactions. The formation of a stable complex will evidently increase the rate above that calculated by the collision theory, because, if the molecules spend an appreciable time in union, the chance of reaction is obviously greater than if they separate immediately on collision.

The effect on the reaction velocity of the formation of stable complexes between the reactants does not appear to have been considered closely.

(8) A. E. Stearn, *Ergebn. der Enzymforschung*, **7**, 1 (1938).

(9) A. I. Escolme and W. C. M. Lewis, *Trans. Faraday Soc.*, **23**, 651 (1927).

If the course of the reaction is formulated as

$$E + S \underset{k'C}{\overset{k_1ES}{\rightleftharpoons}} C \underset{k'C^*}{\overset{k_2C}{\rightleftharpoons}} C^* \xrightarrow{k_4C^*} X + S$$

where C is the stable complex between enzyme and substrate and C* the "activated" complex, which gives rise to the reaction products, we can assume that C is present at an equilibrium concentration (this implies that $k_2 < k_2'$). There are now two cases: (1) The complex C* is also present in an equilibrium concentration which will be the case if $k_4 < k_2'$. It can, of course, be assumed that the concentration of the energy-rich complex C* is very small. The rate of reaction will be unaffected by the concentration of C, since the equilibrium concentration of C* will be the same whether it is derived directly from E or S or from C. (2) If $k_4 > k_2'$ the activated state will not reach an equilibrium concentration and the rate of the reaction will be k_2C, which is the rate of formation of C*. In this case the concentration of the stable complex may be expected to be a significant factor.

It is not, however, possible at present to make any correlation between reaction rates and the complex stability which is indicated by the Michaelis constant. The formation of a complex has been demonstrated in the action of trypsin on sturin (Fig. 3), where the Michaelis constant is of the order of 7.5×10^{-3} mole per liter. In the case of trypsin on chymotrypsinogen the constant is $> 1.3 \times 10^{-3}$, and it follows that the stability of the latter complex is not so much greater than the former as to account by itself for the greater rate of the latter reaction.[10] A great deal more data will have to be collected before the connection can profitably be discussed in detail.

Experimental

Trypsin on Benzoyl-*l*-arginine Amide.—The course of the reaction was followed by a modified formol titration. Five-tenths of a milliliter of the sample was added to 0.5 ml. of 40% formaldehyde with 5 drops of 0.1% phenolphthalein and titrated with 0.01 *N* aqueous sodium hydroxide. If θ_0, θ_t, θ_∞ are titers at times 0, t and ∞, the fraction hydrolyzed at time t is $x = (\theta_t - \theta_0)(\theta_\infty - \theta_0)$. Figure 2 shows log x plotted against t. From the slopes of these lines we obtain the constants in Table II.

TABLE II

Reaction mixture: 5 ml. 0.05 *M* benzoyl-*l*-arginine amide, 1 ml. 0.1 *M* phosphate buffer, *p*H 7.8; 1 ml. of trypsin solution containing 0.281 mg. trypsin nitrogen per ml. (as determined by hemoglobin activity). Concentration of trypsin in reaction mixture 6.9×10^{-6} mole per liter.

T, °C.	6.0	15.2	25.5	37.5
k, min.$^{-1} \times 10^3$	1.68	4.62	11.2	23.0
k sec.$^{-1}$ for 1 mole trypsin per liter	4.06	11.2	27.0	55.6
$k^0 = 2.54$	$\Delta H = 14{,}900$			

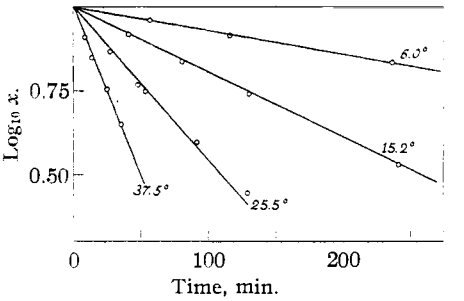

Fig. 2.—Hydrolysis of benzoyl-*l*-arginine amide by trypsin.

Trypsin on Sturin.—The rate of hydrolysis was followed by the modified formol titration described above. Since the initial quantity of alkali required is comparatively large, a stronger solution was made up of a strength such that 1 cc. was just insufficient for the initial titer. This quantity was added to every sample. The initial rate of hydrolysis is proportional to the sturin concentration but becomes independent of it at concentrations above about 0.0075 *M* (Fig. 3). The temperature coefficient was determined at a sturin concentration of about 2×10^{-3} *M*, taking molecular weight as 3550.[11] The increase of formol titration with time in the first stages of the reaction is shown in Fig. 4.

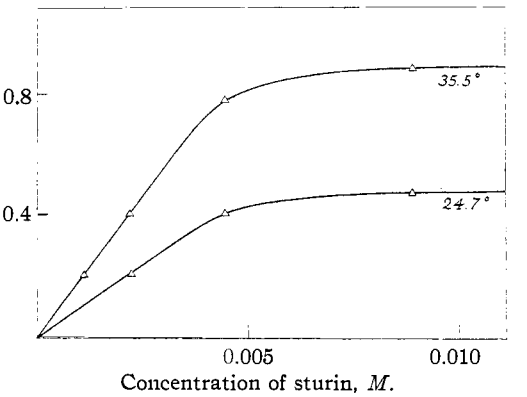

Fig. 3.—Effect of substrate concentration on the rate of hydrolysis of sturin by trypsin: ordinate represents increase of formol titration, equiv. $\times 10^{-7}$, per ml. per min.

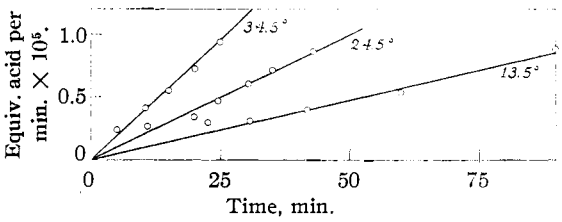

Fig. 4.—Initial stages of digestion of sturin by trypsin.

(10) The stability of the complex is inversely proportional to the Michaelis constant.

(11) K. Felix and A. Lang, *Z. physiol. Chem.*, **188**, 96 (1930).

TABLE III

Reaction mixture: 1 ml. sturin solution, 0.01 *M*, 3 ml. 0.1 *M* phosphate buffer, *p*H 7.5; 0.5 ml. trypsin solution containing 3.2×10^{-2} mg. per ml. trypsin as determined by rate of hydrolysis of benzoyl-arginine amide.

T, °C.	13.5	24.5	35.5
Δ, equiv./ml./min. $\times 10^{7}$	3.88	1.96	0.90
k' sec.$^{-1}$ for 1 mole trypsin per liter $\times 10^{-3}$	6.0	13.1	25.9
k' (0°) $= 2.14 \times 10^{3}$	$\Delta H = 11{,}800$		

Chymotrypsin on Benzoyltyrosylglycylamide.—The substrate solution contained approximately 2 mg. of the substance per ml. The reaction was also followed by the formol titration. Only a small quantity of the substrate was available, sufficient for determinations of the initial and final titers and that of one or two intermediate points, which were taken in the region of half change. From these the period of half change was estimated. The accuracy of this procedure is inferior to that of the other data in this paper and the activation energy calculated from the temperature coefficient is to be regarded as an approximation.

TABLE IV

Reaction mixture: 2 ml. of the substrate solution, 0.4 ml. 0.1 *M* phosphate buffer, *p*H 7.5; 0.4 ml. of beef chymotrypsin solution containing 0.105 mg. protein nitrogen per ml. in 7.5 phosphate buffer, diluted either 1/2 or 1/5.

T, °C.	35.7	24.7	13.3	4.0
1/2 period C.T. × 1/5	8.7	17.5	35.0	..
1/2 period C.T. × 1/2	..	6.5	[13.5] [12.5]	25.0
k (× 1/5 min.$^{-1}$)	0.080	0.041	0.021	0.011
k, sec.$^{-1}$ for 1 mole C.T. per liter $\times 10^{-2}$	3.5	1.8	0.93	0.49
k (0°) $= 3.7 \times 10^{2}$	$\Delta H = 10{,}800$			

Chymotrypsin on Denatured Pepsin.—The pepsin was a crystalline specimen for which I am indebted to Dr. R. M. Herriott. A solution was dialyzed until practically free from salt and then 1 *M* K_2HPO_4 was added until the *p*H was near 7.5. This solution, which contained 7.2 mg. of nitrogen per ml., was warmed at 60° for ten minutes. The initial stage of the reaction was followed by the formol titration.

TABLE V

Reaction mixture: 4 ml. pepsin solution, 1 ml. chymotrypsin solution (0.021 mg. protein nitrogen per ml.). Taking molecular weight as 36,000, the molar concentration of chymotrypsin in reaction mixture is 7.5×10^{-5} mole per liter.

T, °C.	13.0	24.0	35
Δ, equiv./ml./min. $\times 10^{8}$	3.75	7.85	16.2
k' sec.$^{-1}$ for 1 mole C.T. per l. $\times 10^{-2}$	5.8	13.4	27.6
k' (0°) $= 2.2 \times 10^{2}$	$\Delta H = 11{,}200$		

I have again to thank Dr. John H. Northrop for continued hospitality of his laboratory and for much valuable guidance. I am indebted to Dr. M. Kunitz for several of the substances used in this work.

Summary

1. The velocity constants of the following reactions, (1) trypsin on benzoyl-*l*-arginine amide, (2) trypsin on sturin, (3) chymotrypsin on benzoyltyrosylglycylamide, (4) chymotrypsin on denatured pepsin, have been determined at various temperatures and the heats and entropy of activation have been calculated.

2. All these reactions come reasonably close to the range regarded as that of normal reactions among small molecules and, in fact, fairly close to the rates calculated by the simple collision hypothesis. The conversion of chymotrypsinogen by trypsin is considerably more rapid.

3. Since in highly specific reactions involving large molecules, it can hardly be expected that all collisions having the proper energy will lead to reaction, it is suggested that there is a compensating factor which produces an enhanced rate. This factor may arise from the formation of stable complex between enzyme and substrate.

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