[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE CATHOLIC UNIVERSITY OF AMERICA]

The Kinetics and Mechanism of the Heat Inactiviaton of Pepsin[1]

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The influence of enzyme concentration and temperature on the initial rate of pepsin inactivation has been investigated. The order of the reaction was found to be unity at high enzyme concentrations, but rises to approximately five at low concentrations. The experimental energy of activation decreases markedly with increase in enzyme concentration, the variation being ~90 kcal. per mole over a hundred-fold concentration range. The significance of the results is discussed briefly.

Introduction

Protein denaturations, including the inactivations of enzymes, have often been regarded as first-order processes,[2] in spite of the fact that marked drifts in the first-order coefficients are frequently obtained.[3] In the case of the heat inactivation of the enzyme pepsin, Arrhenius[4] quoted data which are consistent with the first-order law, but Tammann[5] had earlier investigated the reaction and found that the first-order coefficients decreased markedly with time, indicating an apparent order of greater than unity. Similar results were obtained by Michaelis and Rothstein,[6] who stated that the rate was proportional to the three-halves power of the enzyme concentration. Northrop[7] found that the pure enzyme follows the first-order law very closely, but that the coefficients decrease with increasing time in the presence of impurities. The reaction was reinvestigated in 1933 by Loughlin,[8] who measured activation energies over a range of *p*H values, but unfortunately he does not state whether the first-order law was obeyed or not. In a very careful kinetic study using crystalline pepsin Steinhardt[9] found that the first-order law was accurately obeyed.

A similar situation has existed with other proteins. Northrop[10] found that the deactivation of trypsin follows the first-order law when the enzyme is pure, but that certain substances decrease the rate of inactivation and cause the first-order coefficients to drift downward with increasing time.

With the object of obtaining information relative to the mechanism of enzyme deactivation, and in connection with a kinetic study of pepsin-catalyzed reactions,[11] we have carried out an investigation of the kinetics of pepsin inactivation by heat, and have in particular studied the influence of the initial concentration of enzyme on the initial rate of reaction. The advantage of using this method to study the rates of reaction in such systems will be discussed later. Temperature coefficients were determined over a wide range of concentration of enzyme. Since the effect of *p*H on the rate of reaction has been thoroughly investigated by Steinhardt,[9] the work was all done at the same *p*H, *viz.* 4.83.

Experimental

Procedure.—The studies were carried out with Armour crystalline pepsin, which was found by micro-Kjeldahl analysis to contain 14% total nitrogen. Solutions were made up in acetate buffer (0.10 *M*) at *p*H 4.83.

The enzyme activity was determined by the colorimetric technique developed by Anson[12] and described by Sumner and Somers.[13] The enzyme solution was suitably diluted and 1 ml. added to 4 ml. of a standard solution of hemoglobin, and allowed to hydrolyze the hemoglobin substrate for 10 minutes at 25°. The reaction was stopped by the addition of 10 ml. of 0.3 *N* trichloroacetic acid; the mixture was then filtered. To five ml. of the clear filtrate, 10 ml. of 0.5 *N* NaOH was added. Three ml. of the Folin phenol reagent was added to develop the color with the tyrosine present, the intensity of the color being a direct measure of the amount of tyrosine liberated, and hence a direct measure of the activity of the enzyme. The depth of the color was measured by means of a color comparator (Aminco Neutral Wedge Photometer, using a no. 68 filter). The blank for the color determination was taken by adding the trichloroacetic acid before adding the enzyme in the color development.

The rates of inactivation were measured in the following manner. Each of several test-tubes was charged with slightly more than 1 ml. of enzyme solution, and all were placed simultaneously in the constant temperature bath. At zero time, and at regular intervals of time later, a test-tube was withdrawn and cooled immediately in ice to stop the deactivation. A color development was made on each, as described above. From the graph relating activity *vs.* time, the initial slope was taken as the initial rate of reaction.

The critical range of temperature was found to be 50–62°; below 50° the rate of inactivation is too slow to be measured conveniently, while above 62° the enzyme becomes inactive in a matter of seconds. At about half of the enzyme concentration rates of inactivation were measured at four temperatures, usually 50.5, 54.0, 57.4 and 60.6°.

Results.—Figure 1 is a plot of activity (milli-equivalents of tyrosine liberated from 4.0 ml. standard hemoglobin solution per ml. enzyme solution in 10 minutes at 25.0° and *p*H 1.9) *vs.* time for a typical run in which the initial pepsin concentration is low (in this case it is 0.006%). In this example the reaction has been allowed to continue for a much longer time than is necessary to determine initial rates; for the determination of initial rates more readings are taken during the early stages of the reaction.

In Table I are shown initial rates corresponding

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(2) *Cf.*, for example, J. B. Sumner and G. F. Somers, "Chemistry and Methods of Enzymes," Academic Press, Inc., New York, N. Y., 1947, p. 14.

(3) For a review of the kinetics of denaturation see H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944).

(4) S. Arrhenius, "Immunochemie," Leipzig, 1907, p. 57.

(5) G. Tammann, *Z. physik. Chem.*, **18**, 426 (1892).

(6) L. Michaelis and M. Rothstein, *Biochem. Z.*, **105**, 60 (1920).

(7) J. H. Northrop, *J. Gen. Physiol.*, **13**, 739 (1930).

(8) W. J. Loughlin, *Biochem. J.*, **27**, 1779 (1933).

(9) J. Steinhardt, *Nature*, **138**, 74 (1936); *Kgl. Danske Videnskab. Selskab Math-Fysik Medd.*, **14**, No. 11 (1937).

(10) J. H. Northrop, *J. Gen. Physiol.*, **4**, 261 (1922).

(11) E. J. Casey and K. J. Laidler, THIS JOURNAL, **72**, 2159 (1950).

(12) M. L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).

(13) J. B. Sumner and G. F. Somers, "Laboratory Experiments in Biological Chemistry," Academic Press, Inc., New York, N. Y., 1944, p. 127.

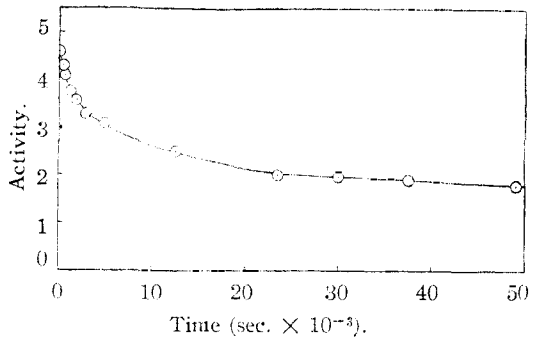

Fig. 1.—Plot of activity *vs.* time for a run corresponding to an initial pepsin concentration of 0.006%; *T* 57.4°, *p*H 4.83. This type of curve is typical for the runs corresponding to low initial concentrations of pepsin; the reaction appears to be approaching an equilibrium, but in fact after sufficient time almost complete deactivation has occurred. At high initial pepsin concentration the first-order law is followed.

to various initial enzyme concentrations. Figure 2 shows typical Arrhenius plots of log *v* *vs.* $1/T$ over a wide range of enzyme concentration. The

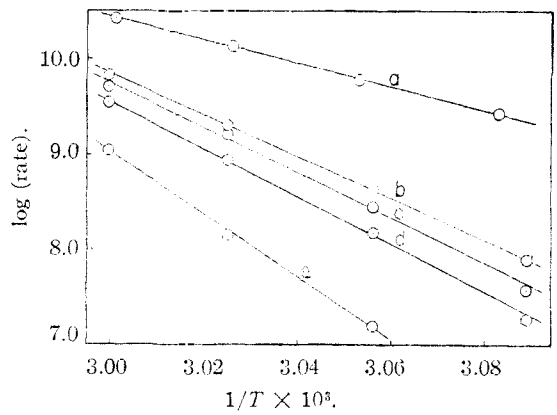

Fig. 2.—Arrhenius plots of log (rate) *vs.* $1/T$ for various initial pepsin concentrations, as follows, (a) 0.5%, (b) 0.01%, (c) 0.008%, (d) 0.006%, (e) 0.004%.

activation energies, calculated using the method of least squares, are included in Table I; they are seen to diminish markedly with increasing enzyme concentration. Figure 3 shows a plot of the activation energy as a function of enzyme concentration.

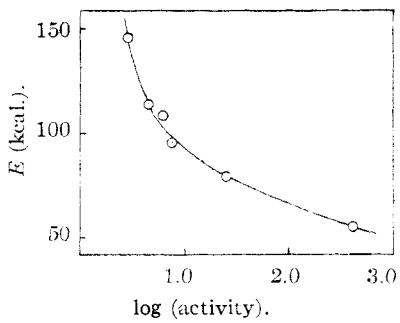

Fig. 3.—Variation of activation energy with enzyme concentration for the inactivation of pepsin; *p*H 4.83.

TABLE I

INITIAL RATES, ACTIVATION ENERGIES AND APPARENT ORDERS AT VARIOUS PEPSIN CONCENTRATIONS AT 57.4° AND *p*H 4.83

Enzyme by weight (approx.), %	Activity, activity units	Initial rate, activity units per min.	*E*, kcal.	n_c
0.004	2.90	0.014	147	5.0
.005	3.82	.055	...	3.8
.006	4.60	.090	115	2.0
.008	6.20	.170	109	1.6
.009	6.63	.250	...	1.2
.01	7.30	.210	97	1.2
.04	25.9	.760	80	1.0
.05	34.7	.940	...	1.0
.10	73.7	2.43	...	1.0
.5	377	13.7	...	1.0
.5	392	8.65	56	1.0
1.0	759	19.6	...	1.0

Discussion

The Order of the Reaction.—In complicated kinetic systems such as those involving enzymes it is often advantageous[14] to analyze the experimental data using a graphical version of the van't Hoff differential method.[15] The general type of relationship between the rate, *v*, and the concentration, *c*, is

$$v = kc^n \qquad (1)$$

although in practice it may not be possible to assign constant values to both *k* and *n*. If a plot of log *v* *vs.* log *c* does not give a straight line, the reaction does not have a simple order. This procedure can be applied not only to a single rate determination but also to initial rates obtained at a series of initial concentrations. Accordingly, log (initial rate) was plotted against log (initial concentration) for our data (Fig. 4), and it was seen that the re-

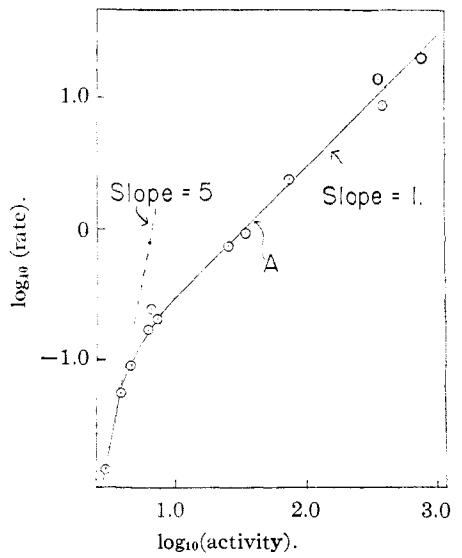

Fig. 4.—Plot of log (initial rate) *vs.* log (initial concentration) for the inactivation of pepsin; *p*H 4.83, *T* 57.4°.

(14) *Cf.* K. J. Laidler, "Chemical Kinetics," McGraw-Hill Book Co., Inc., New York, N. Y., 1950, p. 15; K. J. Laidler and J. P. Hoare, THIS JOURNAL, **71**, 2699 (1949).

(15) J. H. van't Hoff, "Études de dynamique chimique," F. Muller and Company, Amsterdam, 1884, p. 87.

action does not admit of a simple order; the apparent order with respect to concentration, n_c, decreases from a value of about 5 to a limiting value of unity as the initial concentration of enzyme is increased. Some values of n_c (determined from the slope of the curve in Fig. 4) are included in Table I.

Mechanism of Inactivation.—The facts that the apparent order of the reaction, n_c, varies with enzyme concentration, and that the energy of activation also varies markedly, imply the existence of strong intermolecular forces acting between enzyme molecules. That there is a decrease of E with increasing pepsin concentration requires that these are of a repulsive nature, the system going to a higher potential-energy state with increasing enzyme concentration. The existence of very strong forces between protein molecules has also been indicated by the osmotic pressure experiments of Scatchard, *et al.*,[16] and we have verified that the forces postulated in the present work are of the same order of magnitude as those of Scatchard. These forces are most probably electrostatic in nature and due in part to the zwitterion character of the enzyme and partly to the presence of adsorbed ions.

We have formulated a tentative mechanism to explain the main kinetic features of the denaturation, but since it involves rather unusual and arbitrary assumptions, and since similar data for other systems are not available, the details will not be published at the present stage. The essential feature of the proposed mechanism is that inactivation is a coöperative phenomenon, involving the participation of five or more enzyme molecules. The final expression obtained for the rate v is[17]

$$v = \frac{k_0 K c^n}{c_{H^+}^5 (1 + n^2 K c^{n-1})} \qquad (2)$$

where k_0, K and n are constants. This law corresponds to an n-th order reaction at low concentrations and to a first-order reaction at high ones.

Equation (2) applies to rates at any time, and not only to initial rates. It is therefore to be expected that good first-order constants will be obtained in the high concentration region, and we have found that this is so; in Fig. 5, a plot of log (active enzyme remaining) *vs.* time, the points are linear for the first 60% of reaction, indicating first-order behavior. In the low concentration region eq. (2) indicates more complex behavior, and this is in fact found, there being now no simple order. It may be seen from Fig. 1 that as reaction proceeds the rate becomes progressively less and less. It might be suggested[18] that this may be due to an approach to equilibrium; however we have found that after a sufficiently long period of time practically complete deactivation does occur. On the other hand the integrated form of eq. (2), *viz.*

$$\frac{k_0 K t}{c_{H^+}^5} = \frac{1}{n-1}\left\{\frac{1}{c^{n-1}} - \frac{1}{c_0^{n-1}}\right\} + n^2 K \ln c_0/c \qquad (3)$$

has been found to fit satisfactorily several of the concentration–time curves. The curve shown in Fig. 1 is in fact a theoretical curve based on eq. (3), with $n = 5$, and is seen to pass as close to the experimental points as can be expected.

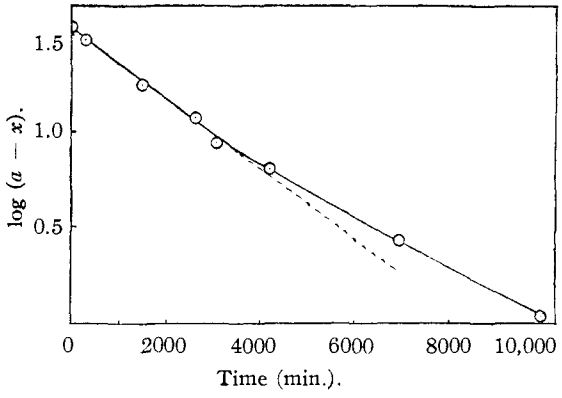

Fig. 5.—Plot of log (active pepsin remaining) *vs.* time for a 0.25% pepsin solution; *p*H 4.83, *T* 54.8°. The process under these conditions obeys the first-order law during the early stages of reaction.

In the light of the above results and discussion it would appear that those previous workers who obtained good first-order constants in individual runs were working in the high concentration region where the true order is unity. This is certainly true of Northrop's work,[7] the concentration at which he worked being represented by point A in Fig. 4, and it is true of Steinhardt's investigation.[9] The same presumably applies to the work quoted by Arrhenius[4]; the enzyme concentration is not known, but the activation energy quoted, 75.6 kcal., suggests that the concentration was fairly high (*cf.* Fig. 3). On the other hand, Tammann,[5] and Michaelis and Rothstein,[6] were presumably working in the region of lower concentration where first-order behavior is no longer obtained.

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(16) G. Scatchard, THIS JOURNAL, **68**, 2315 (1946); G. Scatchard, A. C. Batchelder and A. Brown, *ibid.*, **68**, 2320 (1946); G. Scatchard, A. C. Batchelder, A. Brown and M. Zosa, *ibid.*, **68**, 2610 (1946); G. Scatchard, I. H. Scheinberg and S. H. Armstrong, *ibid.*, **72**, 535, 540 (1950).

(17) In a preliminary note on these results (Casey and Laidler, *Science*, **111**, 110 (1950)) we suggested a law that, as pointed out to us by Dr. Steinhardt, gave the wrong *p*H dependence.

(18) *Cf.* J. H. Northrop, *J. Gen. Physiol.*, **16**, 323 (1932) (for trypsin).