The Mechanism of the Heat Inactivation of Pepsin[1]

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PROTEIN DENATURATIONS, including enzyme inactivations, have usually been considered to be first-order unimolecular processes (*1, 7*), although in a number of investigations marked deviations from first-order behavior have been observed (*2, 8*). The work has usually been carried out by calculating first-order rate coefficients and examining their constancy with respect to time. This procedure suffers from a disadvantage arising from the fact that impurities and products generated in a reaction may interfere with the progress of the reaction. In order to avoid this possibility we have recently made a study of the heat inactivation of pepsin in which we have measured the initial rate as a function of both initial concentration and temperature.

The results have been somewhat unusual, and appear to go some way towards explaining the anomalous and apparently inconsistent results obtained by previous workers. If the initial rate, v_0, of a reaction is related to the initial concentration, c_0, by the power law

$$v_0 = k\, c_0^n, \qquad (1)$$

where k and n are constants, a plot of log v_0 against log c_0 will give a straight line, the slope of which is the index n. When this procedure was applied to our data, the curve shown in Fig. 1 was obtained. It is evident that in fact the index n is not constant, but that it varies with the initial concentration. Over a certain range of high concentrations n is very close to unity (that is, the reaction is of the first order), but as the concentration is decreased, the value of n increases, and finally it reaches a value of about 5. It is clear from this analysis that in the low concentration region the usual first-order coefficients will not be constant but will decrease markedly with increasing time. On the other hand, in the high concentration region good first-order constants are to be expected, and we have confirmed with our own data that this is the case. It would therefore appear that those workers, such as Arrhenius (*1*) and Northrop (*3*), who obtained good first-order constants, were working in the region of high concentration; drifting constants, on the other hand, may be due to the fact that the work was done in the low concentration region (*2, 8*).

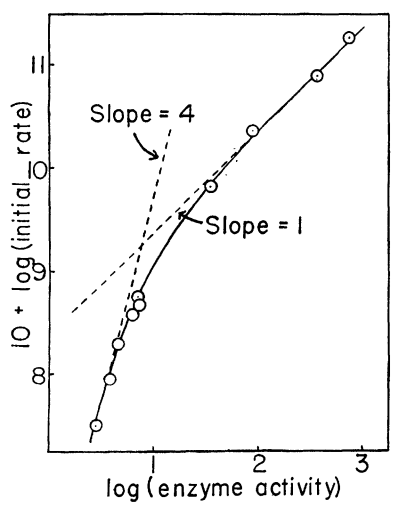

FIG. 1. Plot of log (initial rate) vs. log (initial enzyme activity) for the inactivation of pepsin at pH 4.83 and a temperature of 57.8° C.

TABLE 1

REACTION ORDERS AND ACTIVATION ENERGIES CORRESPONDING TO VARIOUS PEPSIN CONCENTRATIONS

% Pepsin by weight	Activity*	E_{exp}	n
0.004	2.90	147.0 ± 10	5.0
0.006	4.60	115.0 ± 3	3.0
0.008	6.20	109.0 ± 2	2.4
0.011	7.30	97.0 ± 2	2.1
0.050	25.9	80.0 ± 12	1.5
0.50	392	56.2 ± 0.5	1.0
4.00	2465	62.0 ± 10	1.0

* Expressed as meq. tyrosine per ml liberated from 4.0 ml of standard hemoglobin solution by 1.0 ml enzyme solution at 25.0° C and pH 1.9.

Some evidence as to the reason for this variation in apparent order with concentration is provided by the results of a study of the temperature dependence of the initial reaction rates. These were found to obey the Arrhenius law, but the resulting activation energy was influenced very markedly by the initial concentration; some values are included in Table 1, which gives also the order with respect to concentration corresponding to each enzyme concentration. The variation of activation energy with enzyme concentration is represented in Fig. 2.

The very considerable reduction in energy of activa-

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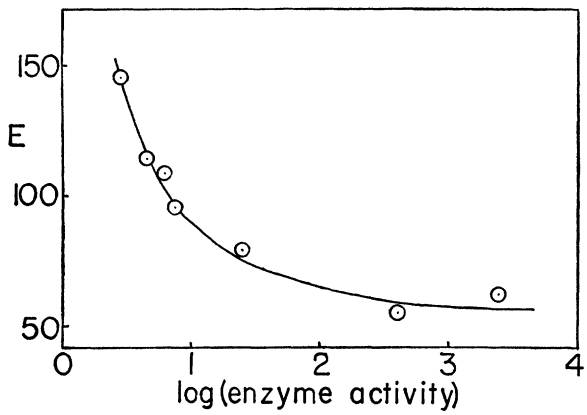

FIG. 2. Variation of activation energy E with enzyme activity for the inactivation of pepsin at pH 4.83.

tion with increasing concentration implies the existence of enormously strong repulsive forces acting between the pepsin molecules in solution. As a result of these, the protein molecules pass into states of higher and higher potential energy as the concentration increases, and consequently there is a diminution in the activation energy that it is necessary for them to acquire prior to becoming deactivated. We have confirmed that the magnitude of these repulsive forces is of the same order as that of those revealed by the osmotic pressure experiments of Scatchard *et al.* (*4, 5, 6*) on albumin. These forces are presumed to be electrostatic in nature, and to be due in part to the zwitterion character of the enzyme and in part to the presence of adsorbed ions.

The existence of these repulsive forces explains the variation in reaction rates with the initial concentration, since the process of activation is seen to be a cooperative phenomenon involving the presence of a group of protein molecules surrounding each protein molecule. At low concentrations the reduction in activation energy will be very sensitive to enzyme concentration, and the apparent order of the reaction will therefore be high; at sufficiently high concentrations, however, a saturation point will be reached, and the number of activated molecules will then be in proportion only to the first power of the enzyme concentration.

On the basis of the mechanism referred to above, it can be shown that the initial rate v_0 is related to the initial enzyme concentration c_0 by the approximate expression

$$v_0 = \frac{k_0 K c_0^{\,n}}{nK c_0^{\,n-1} + c_{H^+}{}^5} \qquad (2)$$

where c_{H^+} is the hydrogen ion concentration, K is the equilibrium constant for complex formation, and k_0 is the rate constant for the deactivation of the enzyme in the complex. The value of n is approximately 5. At low concentrations of enzyme, $c_{H^+}{}^5 \gg nKc_0^{\,n-1}$, so that the initial rate is

$$v_0 = \frac{k_0 K}{c_{H^+}{}^5}\, c_0^{\,n} \qquad (3)$$

and the reaction is of the nth (approximately fifth) order. At high concentrations, on the other hand, $nKc_0^{\,n-1} \gg c_{H^+}{}^5$, so that the rate is

$$v_0 = \frac{k_0}{n}\, c_0; \qquad (4)$$

the reaction is then of the first order. We have confirmed that the mechanism explains not only the variation of initial rates with initial enzyme concentration but also the change of concentration of active enzyme with time in an individual rate measurement.

A paper presenting full experimental details and a quantitative treatment of the data will shortly be submitted for publication in the *Journal of the American Chemical Society.*

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