Littauer, U. Z., Muench, K., Berg, P., Gilbert, W., and Spahr, P. F. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 157.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Maxwell, E. S., and Barnett, L. M. (1965), *Federation Proc. 24*, 483.
Miles, H. T., and Frazier, J. (1965), *Federation Proc. 24*, 483.
Neu, H. C., and Heppel, L. A. (1964a), *Biochem. Biophys. Res. Commun. 17*, 215.
Neu, H. C., and Heppel, L. A. (1964b), *Proc. Natl. Acad. Sci. U.S. 51*, 1267.
Nirenberg, M. W., and Matthaei, J. H. (1961), *Proc. Natl. Acad. Sci. U.S. 47*, 1588.
Rich, A. (1958), *Biochim. Biophys. Acta 29*, 502.
Sekiguchi, M., and Cohen, S. S. (1963), *J. Biol. Chem. 238*, 349.
Sigler, P. B., Davies, D. R., and Miles, H. T. (1962), *J. Mol. Biol. 5*, 709.
Singer, M. F. (1958), *J. Biol. Chem. 232*, 211.
Singer, M. F., and Guss, J. K. (1962), *J. Biol. Chem. 237*, 182.
Singer, M. F., Jones, O. W., and Nirenberg, M. W. (1963), *Proc. Natl. Acad. Sci. U.S. 49*, 392.
Singer, M. F., and Tolbert, G. (1964), *Science 145*, 593.
Spahr, P. F. (1964), *J. Biol. Chem. 239*, 3716.
Spahr, P. F., and Hollingworth, B. R. (1961), *J. Biol. Chem. 236*, 823.
Spahr, P. F., and Schlessinger, D. (1963), *J. Biol. Chem. 238*, PC 2251.
Szer, W., and Ochoa, S. (1964), *J. Mol. Biol. 8*, 823.
Tissières, A., and Watson, J. D. (1962), *Proc. Natl. Acad. Sci. U.S. 48*, 1061.
Tocchini-Valenti, G. P., Stodolsky, M., Aurisicchio, A., Sarnet, M., Graziosi, F., Weiss, S. B., and Geiduschek, E. P. (1963), *Proc. Natl. Acad. Sci. U.S. 50*, 935.
Vogel, H. J., and Bonner, D. M. (1956), *J. Biol. Chem. 218*, 97.
Von Hippel, P. H., and Felsenfeld, G. (1964), *Biochemistry 3*, 27.
Weissbach, A., and Korn, D. (1962), *J. Biol. Chem. 237*, PC 3312.

Catalytic Properties of Polymerized α-Chymotrypsin*

Tadashi Inagami and Julian M. Sturtevant

ABSTRACT: The catalysis of the hydrolysis of acetyl-L-tyrosine *p*-nitroanilide by α-chymotrypsin was investigated as a function of enzyme concentration in the range 7–2500 μM. The rate measurements were carried out under experimental conditions identical (except for the presence of substrate) with those employed by Rao and Kegeles in their ultracentrifugal study of the polymerization of α-chymotrypsin. Comparison of observed rates with rates predicted on the basis of many different sets of assumptions concerning the numbers and properties of the catalytic sites on the polymeric species present was made by means of an IBM 7094 computer.

Although the results do not permit a definitive statement concerning the effect of polymerization on the catalytic properties of the enzyme, it seems necessary to conclude that the polymeric species are not completely inactive. Satisfactory agreement between observed and predicted rates may be obtained with a number of different kinetic models.

The association of α-chymotrypsin has been studied by several investigators (Schwert, 1949; Schwert and Kaufman, 1951; Steiner, 1954; Massey *et al.*, 1955; Tinoco, 1957; Rao and Kegeles, 1958; Winzor and Scheraga, 1963, 1964), using light scattering, depolarization of fluorescence, ultracentrifugation, and gel filtration. Martin and Niemann (1958) determined the activity of the enzyme as a function of enzyme concentration under conditions where they considered that only monomeric and dimeric species were present; they concluded that although both species can combine with the substrates they employed, only the compounds formed with the monomeric enzyme can decompose to give reaction products at a significant rate.

The most extensive study of the polymerization of α-chymotrypsin is that of Rao and Kegeles (1958). These authors employed the Archibald (1947) ultracentrifuge method, and concluded that under the conditions of their experiments dimers and trimers are present in addition to the monomers. In an effort to obtain information concerning the catalytic properties of the polymeric species of the enzyme, we have carried out kinetic measurements over a wide range of enzyme

* From the Department of Molecular Biology and Biophysics, Yale University, New Haven, Conn. *Received February 18, 1965.* This work was supported in part by a grant (GM-04725) from the National Institutes of Health, U.S. Public Health Service.

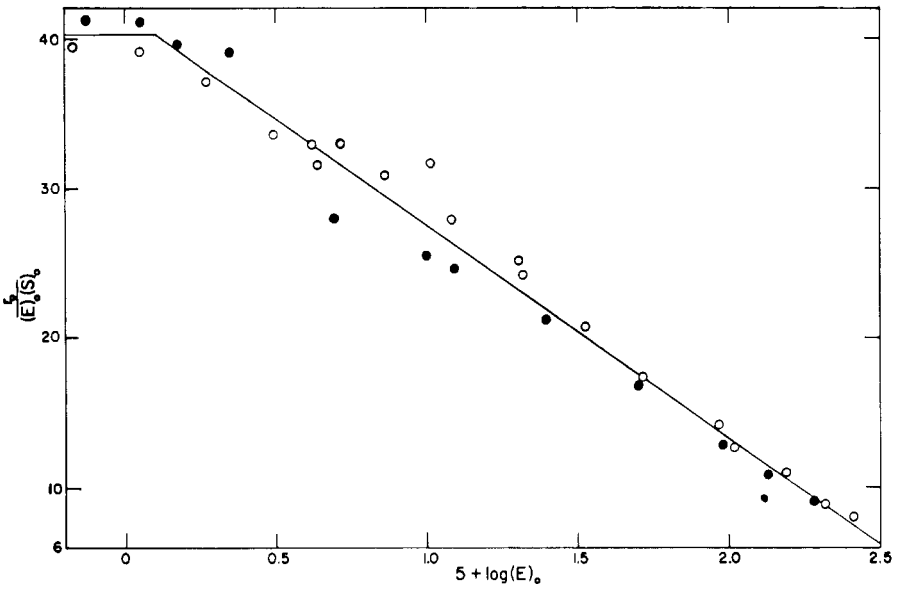

FIGURE 1: The initial rate of α-chymotrypsin-catalyzed hydrolysis of ATNA at $(S)_0 = 0.100$ (○), and at $(S)_0 = 0.189$ (●), in 0.029 M Na_2HPO_4, 0.114 M NaH_2PO_4, *p*H 6.1, 25.0°.

concentrations under experimental conditions differing from those used by Rao and Kegeles only in the presence of substrate.

Experimental

Selection of Substrate. It is obviously advantageous to use a relatively poor substrate when measurements at very high enzyme concentrations are to be made. At the same time it seemed advisable to use a substrate satisfying the usual specificity requirements of the enzyme. *N*-Acetyl-L-tyrosine *p*-nitroanilide (ATNA)[1] (Bundy, 1963) meets both of these requirements, and has the important added advantage of yielding a strongly absorbing product, *p*-nitroaniline ($\epsilon_{410} = 9.96 \times 10^3$ M^{-1} cm^{-1}). We have recently shown (Inagami and Sturtevant, 1964) that the hydrolysis of this substrate probably proceeds by the three-step mechanism usual for chymotrypsin-catalyzed hydrolyses, and is strongly rate limited at the second step, in which the Michaelis-Menten complex is decomposed to yield acylated enzyme and *p*-nitroaniline. Thus attempts to measure the initial rate of hydrolysis would not be complicated by a "burst" of *p*-nitroaniline liberation during the buildup of the steady-state concentration of acyl enzyme. The only drawback of this substrate is its limited solubility.

Materials. Crystalline salt-free α-chymotrypsin was purchased from Worthington Biochemical Corp. and was used without further purification. Enzyme stock solutions at concentrations up to 12.4% were prepared by dissolving the enzyme in a small volume of 10^{-3} M HCl, centrifuging to clarify the solution, carefully adding NaOH to *p*H 6.1 and then mixing with an equal volume of buffer of double the desired concentration. This solution was clarified again by centrifugation. Enzyme concentrations were determined by measuring the absorption at 280 mμ, using an absorption coefficient of $E^{1\%}_{1cm} = 20$ (Dixon and Neurath, 1957). All rate measurements were carried out in a buffer composed of 0.114 M NaH_2PO_4 + 0.029 M Na_2HPO_4, *p*H 6.1, at 25.0 ± 0.1°. Reagent-grade salts were used. The *p*H measurements were made with a Beckman Model 76 *p*H meter calibrated against a *p*H 6.86 phosphate buffer (Bates, 1964).

Kinetic Measurements. A stopped-flow apparatus (Sturtevant, 1964) was employed for the rate measurements. This apparatus is well suited to the determination of initial rates. In most of the present experiments observations were limited to the first 3% of the reaction. The actual rates observed ranged from 2.4×10^{-4} to 250×10^{-4} absorbance units per second. In all cases the absorbance varied linearly with time during the period of observation.

Results

Measurements of the initial rate, r_0, of hydrolysis of ATNA at various initial substrate concentrations, $(S)_0$, and enzyme concentrations, $(E)_0$, are listed in Table I. The rates are expressed in M sec^{-1}. As shown in Figure 1, the results of the experiments with $(S)_0 = 0.100$ and 0.189 mM are adequately represented by the equation

$$-\frac{r_0}{(E)_0(S)_0} = 29.3 + 14.2 \log (E)_0 \qquad (1)$$

[1] Abbreviation used in this work: ATNA, acetyl-L-tyrosine *p*-nitroanilide.

TABLE I: Initial Rate of the α-Chymotrypsin-catalyzed Hydrolysis of ATNA in Phosphate Buffer, pH 6.1, 25.0°.

Expt	Total Enzyme Concn (mM) $(E)_0$	Initial Substrate Concn (mM) $(S)_0$	Initial Rate (M sec^{-1} × 10^6) r_0	Expt	Total Enzyme Concn (mM) $(E)_0$	Initial Substrate Concn (mM) $(S)_0$	Initial Rate (M sec^{-1} × 10^6) r_0
1	2.58	0.100	2.06	24	0.246	0.189	0.988
2	2.07	0.100	1.84	25	0.123	0.189	0.569
3	1.55	0.100	1.71	26	0.0994	0.189	0.479
4	1.037	0.100	1.32	27	0.0492	0.189	0.261
5	0.929	0.100	1.32	28	0.0223	0.189	0.165
6	0.516	0.100	0.898	29	0.0149	0.189	0.112
7	0.335	0.100	0.698	30	0.0112	0.189	0.0871
8	0.207	0.100	0.501	31	0.00743	0.189	0.0581
9	0.201	0.100	0.508	32	0.397	0.446	2.82
10	0.1205	0.100	0.337	33	0.397	0.357	2.48
11	0.1037	0.100	0.328	34	0.397	0.268	1.94
12	0.0723	0.100	0.222	35	0.397	0.223	1.68
13	0.0516	0.100	0.171	36	0.397	0.214	1.61
14	0.0434	0.100	0.137	37	0.397	0.171	1.27
15	0.0415	0.100	0.137	38	0.397	0.134	1.07
16	0.0310	0.100	0.104	39	0.397	0.128	1.03
17	0.0186	0.100	0.0692	40	0.397	0.0892	0.770
18	0.0112	0.100	0.0439	41	0.397	0.0856	0.675
19	0.00671	0.100	0.0265	42	0.397	0.0642	0.518
20	1.91	0.189	3.29	43	0.397	0.0446	0.380
21	1.34	0.189	2.76	44	0.397	0.0428	0.350
22	0.955	0.189	2.33	45	0.397	0.0321	0.263
23	0.477	0.189	1.53	46	0.397	0.0214	0.176

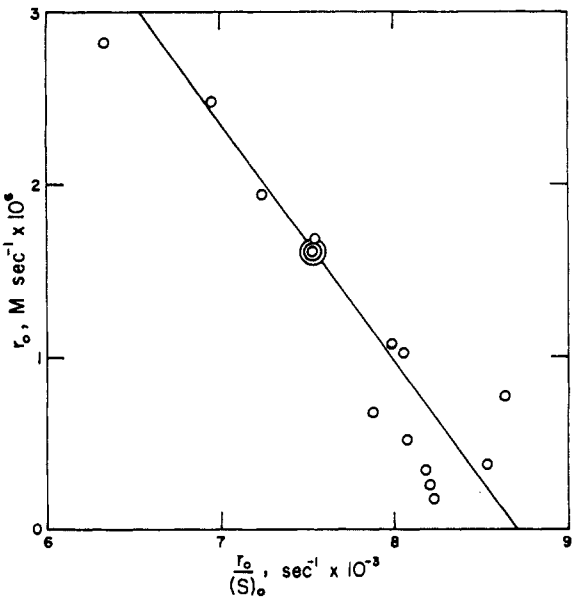

FIGURE 2: Eadie (1942) plot showing the variation with $(S)_0$ of the initial rate of the α-chymotrypsin-catalyzed hydrolysis of ATNA. $(E)_0$ = 0.397 mM; 0.029 M Na_2HPO_4, 0.114 M NaH_2PO_4, pH 6.1, 25.0°.

in the range $1.3 \times 10^{-5} < (E)_0 < 3.2 \times 10^{-3}$, the variance over this range amounting to 4.8%. A set of runs in which $(E)_0$ was held constant and $(S)_0$ was varied from 0.02 to 0.45 mM showed that this equation is valid only over a restricted range of values of $(S)_0$. The data of this latter set are shown in an Eadie (1942) plot in Figure 2.

Discussion

It is evident that the rate of hydrolysis of ATNA does not increase in proportion to the enzyme concentration over the range covered by our experiments. At a substrate concentration of about 0.15 mM, the ratio $r_0/(E)_0$ varies from 6.4 sec^{-1} at $(E)_0 = 10^{-5}$ M to 1.1 sec^{-1} at $(E)_0 = 2.6 \times 10^{-3}$ M.

It is of interest to investigate whether the observed behavior can be accounted for on the basis of a reasonably simple treatment. We have noted that at low enzyme concentrations it is permissible with the substrate ATNA to employ the simple Michaelis-Menten equation because the acylation step is rate limiting. We shall assume that acylation is rate limiting at all enzyme concentrations, and that substrate-binding and enzyme-polymerization equilibria are established very rapidly

compared to the rate of acylation of the enzyme. Thus the enzyme polymerization is assumed to be determined by the equations

$$2\,E \rightleftarrows E_2 \qquad K_D = (E)^2/(E_2) = 0.925\ \text{M}^{-1} \quad (2)$$

$$E_2 + E \rightleftarrows E_3 \qquad K_T = (E)(E_2)/(E_3) = 0.281\ \text{M}^{-1} \quad (3)$$

where the equilibrium constants are derived from those reported (in terms of weight concentrations) by Rao and Kegeles (1958), taking the molecular weight of α-chymotrypsin to be 24,000 (Wilcox *et al.*, 1957). Figure 3 shows the distribution of enzyme, in the absence of substrate, between monomers, dimers, and trimers calculated on the basis of these equilibrium constants. If K_1, K_2, and K_3 are the intrinsic dissociation constants of the enzyme-substrate complexes involving monomers, dimers, and trimers, and k_1, k_2, and k_3 are the acylation rate constants for these complexes, and if we assume (case I) that there are two independent active sites per dimer molecule and three independent active sites per trimer molecule, then the initial rate is given by the expression

$$r_0 = \frac{k_1(E)(S)}{K_1} + \frac{2k_2(E)^2(S)}{K_DK_2}\left[1 + \frac{(S)}{K_2}\right] + \frac{3k_3(E)^3(S)}{K_DK_TK_3}\left[1 + \frac{(S)}{K_3}\right]^2 \quad (4)$$

The concentrations of free enzyme, (E), and free substrate, (S), satisfy the conservation equations

$$(E)_0 = (E)\left[1 + \frac{(S)}{K_1}\right] + \frac{2(E)^2}{K_D}\left[1 + \frac{(S)}{K_2}\right]^2 + \frac{3(E)^3}{K_DK_T}\left[1 + \frac{(S)}{K_3}\right]^3 \quad (5)$$

$$(S)_0 = (S)\left[1 + \frac{(E)}{K_1}\right] + \frac{2(E)^2(S)}{K_DK_2}\left[1 + \frac{(S)}{K_2}\right] + \frac{3(E)^3(S)}{K_DK_TK_3}\left[1 + \frac{(S)}{K_3}\right]^2 \quad (6)$$

In deriving these equations the statistical factors appropriate to the assumption of noninteracting sites have been included. For example, the equilibrium constant for the reaction

$$E_3S_3 \rightleftarrows E_3S_2 + S \quad (7)$$

is set equal to $3K_3$, and the rate constant for the decomposition of E_3S_3

$$E_3S_3 \rightarrow E_3S_2 + P \quad (8)$$

is set equal to $3k_3$.

It seems equally likely that the enzyme polymerization proceeds in such a way that there is only *one* active site left on each molecule, whether it is a monomer, dimer, or trimer molecule. For this case (case II) the equations corresponding to equations (4), (5), and (6) are

$$r_0 = \frac{k_1(E)(S)}{K_1} + \frac{k_2(E)^2(S)}{K_DK_2} + \frac{k_3(E)^3(S)}{K_DK_TK_3} \quad (9)$$

$$(E)_0 = (E)\left[1 + \frac{(S)}{K_1}\right] + \frac{2(E)^2}{K_D}\left[1 + \frac{(S)}{K_2}\right] + \frac{3(E)^3}{K_DK_T}\left[1 + \frac{(S)}{K_3}\right] \quad (10)$$

$$(S)_0 = (S)\left[1 + \frac{(E)}{K_1} + \frac{(E)^2}{K_DK_2} + \frac{(E)^3}{K_DK_TK_3}\right] \quad (11)$$

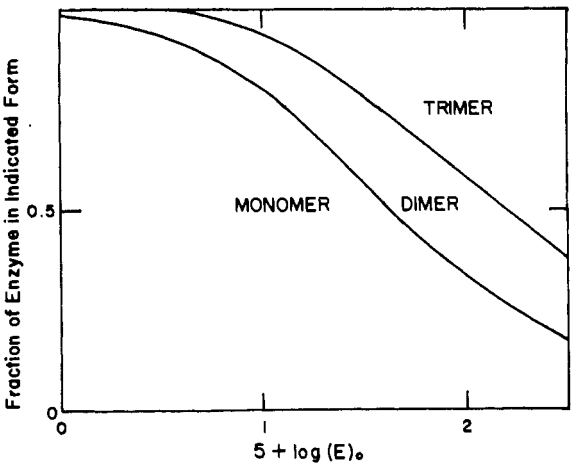

FIGURE 3: Distribution of α-chymotrypin between monomeric and polymeric forms, in the absence of substrate, according to the data of Rao and Kegeles (1958). In 0.029 M Na_2HPO_4, 0.114 M NaH_2PO_4, *p*H 6.1, 25.0°.

Initial rates, r_0, have been calculated with an IBM 7094 computer for several hundred sets of the six parameters k_1, k_2, k_3, K_1, K_2, and K_3. Table II summarizes several of the more successful sets of parameters for both cases I and II, the adequacy of the model being judged by the variance of the experimental points. These sets of parameters, and doubtless many others, give values of the variance which are quite acceptable in view of the expected uncertainty of the experimental data. Figures 4 and 5 give deviation plots for the best representatives of cases I and II which we have found. Although slightly better fits could undoubtedly be found by further variations of parameters, it is quite unlikely that it would become possible to assert on the basis of the present data that one of these models is to be preferred over the other. Furthermore, other conceivable models would probably prove to be equally acceptable.

Three extreme cases are readily derived from the equations already given. For case III it is assumed

TABLE II: Comparison of Observed Rates of Hydrolysis of ATNA with Rates Predicted on the Basis of Various Kinetic Models.

Case[a]	Dissociation Constants[b] (mM) K_1	K_2	K_3	Decomposition Rate Constants[c] (k, sec^{-1}) k_1	k_2	k_3	Variance[d] of r_0 (%)
I	1.0	3.0	5.0	40	40	40	7.34
I	1.1	5.0	5.0	45	40	40	7.03
I	1.1	5.2	5.0	45	40	40	7.03
I	1.1	4.8	5.0	45	40	40	7.05
I	1.1	5.0	5.0	43	40	40	8.00
I	1.1	5.0	5.0	47	40	40	8.07
I	1.1	5.2	4.8	45	38	42	6.98 (5.20)
II	0.65	0.65	0.65	28	28	28	7.55 (4.95)
II	0.7	0.7	0.7	29.3	29.3	29.3	7.52 (4.98)
II	1.1	3.0	3.0	47	40	40	7.82 (7.00)
III	0.7	0.7	0.7	25	25	25	12.6 (8.61)
IV	1.2		$\gg(S)_0, (E)_0$	53	0	0	11.1
V	1.0	5.0	5.0	50	0	0	16.4

[a] See text for specification of cases and further definition of symbols. [b] Equilibrium constants for dissociation of enzyme-substrate complexes. [c] Rate constants for formation of acyl enzyme from enzyme-substrate complexes. [d] Variance $= [(1/N)\Sigma(r_0 - r_{0(obs)})^2/r_0^2]^{1/2}$, where $N = 46$, the number of experiments. Values in parentheses computed omitting experiments 28-31 (Table I).

that polymerization has no effect on the activity of the enzyme, that is, that $k_1 = k_2 = k_3$, $K_1 = K_2 = K_3$ in case I. It can be shown that equations (4), (5), and (6) lead in this case to

$$r_0 = \frac{k_1(E)_0(S)_0}{K_1 + (E)_0 + (S)_0} \qquad (12)$$

provided that $K_1 + (E)_0 + (S)_0 \gg 2\sqrt{(E)_0(S)_0}$. Actually this approximation is not valid in all situations of interest here, and calculations by the complete equations are listed in Table II. For case IV it is assumed that the dimers and trimers are totally inactive, having K_2, $K_3 \gg (E)_0$, $(S)_0$ and $k_2 = k_3 = 0$; for this degenerate case one can equally well apply equations (4)–(6) or equations (9)–(11). Finally, for case V, all the sites, numbering one, two, and three, respectively, on monomers, dimers, and trimers, are assumed to bind substrate significantly, but only the sites on the monomers undergo acylation. The best sets of parameters found for each of these three cases are listed in Table II, and it is seen that much less satisfactory agreement is obtained than with cases I and II. In these cases unreasonably large deviations were observed at one or the other extremes of enzyme concentration. Again it cannot be claimed that slightly better agreement for these cases could not be found; however, enough computations were made so that we can be confident that no instance of substantially better agreement could be obtained.

For experiments 1 through 31 (Table I) the rate is proportional to the concentration of monomeric enzyme with a variance of only 6.9%. However, that this is an unsatisfactory description of the true situation is evidenced by the fact that the ratio $r_0/(S)_0$ varies from 6.3 to 8.2 $\times 10^{-3}$ sec^{-1} for experiments 32 through 46; this ratio should obviously be constant at constant $(E)_0$ if the rate is proportional to the concentration of monomeric enzyme.

Previous work with this substrate has been done under conditions very different from those employed here. Bundy (1963) found for the parameters of the simple Michaelis-Menten equation $K_m = 1.1$ mM and $k_2 = 0.30$ sec^{-1} at 35°, *p*H 8, in dilute Tris buffer, and T. Inagami (to be published) found $K_m = 0.9$ mM, $k_2 = 0.058$ sec^{-1} at 25°, *p*H 8, in 5% dimethylformamide in the absence of a buffer.

Martin and Niemann (1958) studied the activity of α-chymotrypsin at 25° and *p*H 7.0 in 0.3 M and 1.0 M NaCl, at enzyme concentrations up to 8×10^{-4} M. They considered that under these conditions the enzyme is present only in monomeric and dimeric forms. One of the two models which best fitted their data is analogous to our case V, in Table II, and involves the assumption that the substrate is bound at the site on the monomer and at both sites on the dimer, but that only the monomer complex decomposes to give products. The other model which they found to be successful omits the statistical factors appropriate to the above-mentioned model. It is interesting that this type of model is in poor agreement with our data. It appears that the polymerization of the enzyme under the conditions of our experiments has very different kinetic results from those observed by Martin and Niemann (1958).

The following conclusions may be drawn with

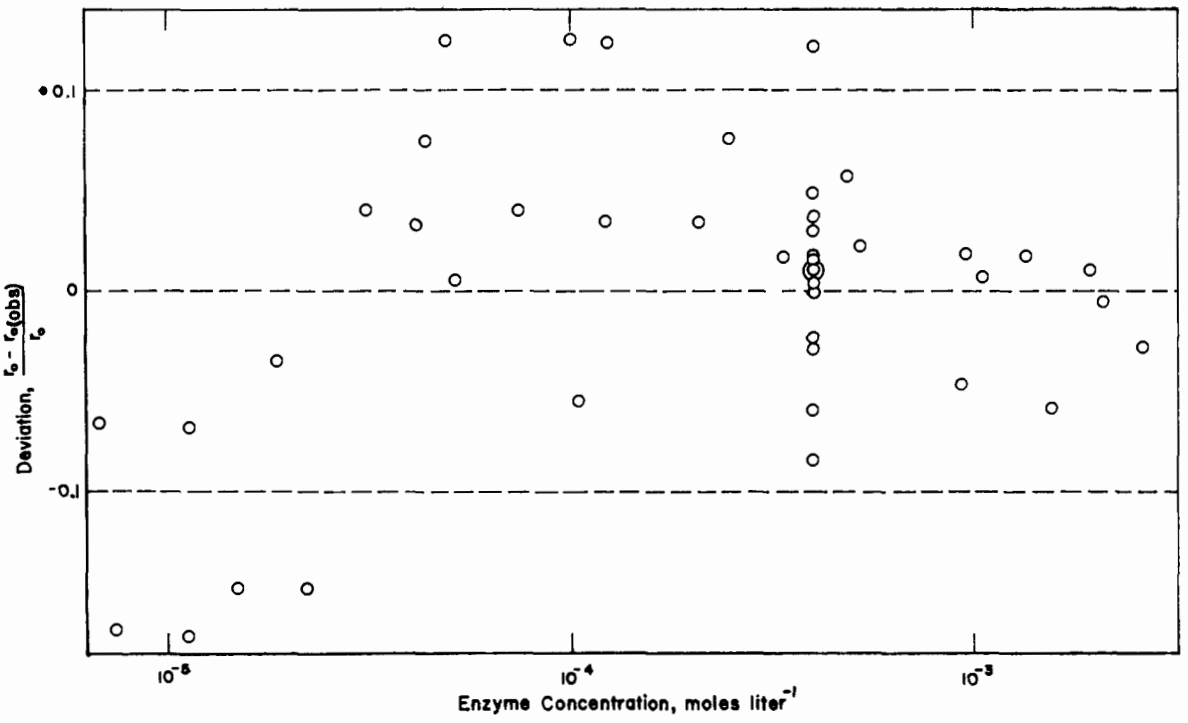

FIGURE 4: Deviation plot for initial rates calculated according to equations (4), (5), and (6). With K_1 = 1.1 mM, K_2 = 5.2 mM, K_3 = 4.8 mM, k_1 = 0.045 sec^{-1}, k_2 = 0.038 sec^{-1}, k_3 = 0.042 sec^{-1}.

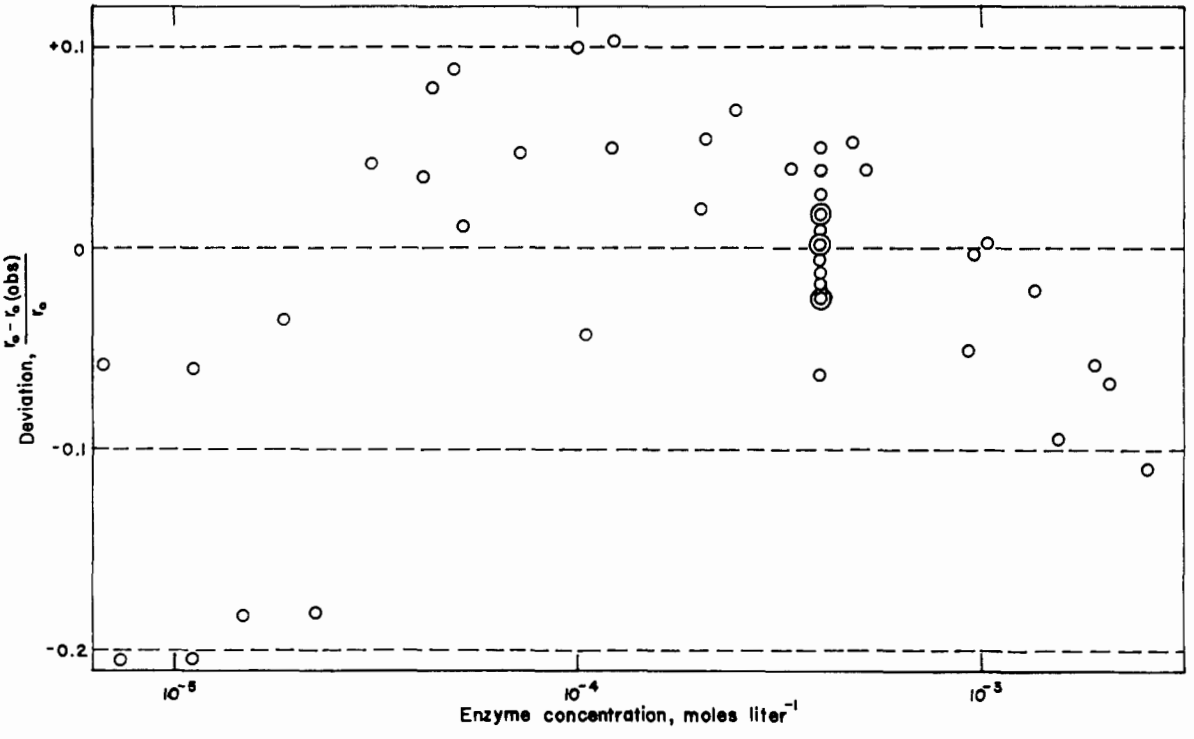

FIGURE 5: Deviation plot for initial rates calculated according to equations (9), (10) and (11). With $K_1 = K_2 = K_3$ = 0.7 mM, $k_1 = k_2 = k_3$ = 0.0293 sec^{-1}.

moderate confidence: (a) The active sites in polymerized α-chymotrypsin retain some activity; and (b) if the number of active sites is not reduced by polymerization, the sites on the polymeric species have impaired catalytic properties with respect either to substrate binding or to decomposition rate constant, or to both. In addition it is clear that it is practically impossible, on the basis of kinetic experiments, to provide a uniquely satisfactory

analysis of the actual situation. This is not surprising in view of the fact that at least six adjustable parameters are available.

A number of examples are known of modifications of enzymes which produce different changes in catalytic activity with different substrates (see, for example, Wellner *et al.*, 1963). It is quite possible that the association of chymotrypsin would be found to have different kinetic results from those described in this paper if a different substrate, in particular a protein substrate, were employed.

Acknowledgments

The authors are indebted to Dr. H. F. Bundy for the supply of acetyltyrosine *p*-nitroanilide used in the experiments reported here. Dr. Martin Saunders wrote the program used in the computations of the data summarized in Table II, and in addition contributed much in discussions of the problems arising in this work.

References

Archibald, W. J. (1947), *J. Phys. & Colloid Chem.* (now *J. Phys. Chem.*) *51*, 1204.

Bates, R. G. (1964), Determination of *p*H, New York, Wiley, p. 123.

Bundy, H. F. (1963), *Arch. Biochem. Biophys. 102*, 416.

Dixon, H. G., and Neurath, H. (1957), *J. Biol. Chem. 225*, 1049.

Eadie, G. S. (1942), *J. Biol. Chem. 146*, 85.

Inagami, T., and Sturtevant, J. M. (1964), *Biochem. Biophys. Res. Commun. 14*, 69.

Martin, R. B., and Niemann, C. (1958), *J. Am. Chem. Soc. 80*, 1473.

Massey, V., Harrington, W. F., and Hartley, B. S. (1955), *Discussions Faraday Soc. 20*, 24.

Rao, N. S., and Kegeles, G. (1958), *J. Am. Chem. Soc. 80*, 5724.

Schwert, G. W. (1949), *J. Biol. Chem. 179*, 655.

Schwert, G. W., and Kaufman, S. (1951), *J. Biol. Chem. 190*, 807.

Steiner, R. F. (1954), *Arch. Biochem. Biophys. 53*, 457.

Sturtevant, J. M. (1964), *in* Rapid Mixing and Sampling Techniques in Biochemistry, Chance, B., Gibson, Q. H., Eisenhardt, R. H., and Lonberg-Holm, K. K., eds., New York, Academic, p. 89.

Tinoco, I. (1957), *Arch. Biochem. Biophys. 68*, 367.

Wellner, D., Silman, H. I., and Sela, M. (1963), *J. Biol. Chem. 238*, 1324.

Wilcox, P. E., Kraut, J., Wade, R. D., and Neurath, H. (1957), *Biochim. Biophys. Acta 24*, 72.

Winzor, D. J., and Scheraga, H. A. (1963), *Biochemistry 2*, 1263.

Winzor, D. J., and Scheraga, H. A. (1964), *J. Phys. Chem. 68*, 338.