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REFERENCES

Bach, M. L., Signer, E. R., Levinthal, C., and Sizer, I. W. (1961), *Federation Proc. 20*, 255.
Boyer, S. H. (1961), *Science 134*, 1002.
Bücher, T. (1947), *Biochim. Biophys. Acta 1*, 292.
Cox, R. P., and MacLeod, C. M. (1963), *Proc. Natl. Acad. Sci. U.S. 49*, 504.
Crowle, A. J. (1961), Immunodiffusion, New York, Academic.
Echols, H., Garen, A., Garen, S., and Torriani, A. (1961), *J. Mol. Biol. 3*, 425.
Garen, A., and Echols, H. (1962), *J. Bacteriol. 83*, 297.
Garen, A., and Levinthal, C. (1960), *Biochim. Biophys. Acta 38*, 470.
Harkness, D. R., and Hilmoe, R. J. (1962), *Biochem. Biophys. Res. Commun. 9*, 393.
Heppel, L. A., Harkness, D. R., and Hilmoe, R. J. (1962), *J. Biol. Chem. 237*, 841.
Levinthal, C., Signer, E. R., and Fetherolf, K. (1962), *Proc. Natl. Acad. Sci. U.S. 48*, 1230.
Malamy, M., and Horecker, B. L. (1961), *Biochem. Biophys. Res. Commun. 5*, 104.
Malamy, M., and Horecker, B. L. (1964), *Biochemistry 3*, 1889 (preceding paper; this issue).
Neu, H. C., and Heppel, L. A. (1964), *Biochem. Biophys. Res. Commun. 14*, 109.
Plocke, D. J., Levinthal, C., and Vallee, B. L. (1962), *Biochemistry 1*, 373.
Plocke, D. J., and Vallee, B. L. (1962), *Biochemistry 1*, 1039.
Schlesinger, M. J., Torriani, A., and Levinthal, C. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 539.
Schwartz, J. H., and Lipmann, F. (1961), *Proc. Natl. Acad. Sci. U.S. 47*, 1996.
Signer, E. R., Torriani, A., and Levinthal, C. (1961), *Cold Spring Harbor Symp. Quant. Biol. 26*, 31.

The Reaction of Carboxypeptidase A with Hippuryl-DL-β-Phenyllactate*

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The action of carboxypeptidase A on hippuryl-DL-β-phenyllactate has been examined over a 2860-fold range of substrate concentration. The system is characterized by a complex inhibition by excess substrate, which suggests either that the enzyme binds substrate to form several inactive complexes or that the enzyme exists as several differentially susceptible species. Initial velocities measured at substrate concentrations low enough to avoid substrate inhibition yield maximum velocities which are the highest yet reported for any substrate for carboxypeptidase A. In this range of substrate concentrations the rates are independent of *p*H between *p*H 7.5 and 9.7, indicating that the unusual *p*H rate profile previously reported for the action of carboxypeptidase on hippuryl-DL-β-phenyllactate (J. F. Riordan and B. L. Vallee [1963], *Biochemistry 2*, 1460) is not due to substrate inhibition. Measurements of the effect of temperature on the reaction velocity yield activation parameters which are of the same order of magnitude as those previously reported for peptide substrates.

In 1948, Snoke and Neurath reported that bovine carboxypeptidase A hydrolyzes hippuryl-DL-β-phenyllactate, the ester analog of the specific substrate, hippurylphenylalanine. The rate behavior was anomalous in that the velocity increased with decreasing substrate concentration over the concentration range examined—a finding consistent with inhibition by excess substrate. Excess substrate inhibition was also noted by Lumry *et al.* (1951) for the closely related substrate carbobenzoxyglycyl-DL-phenylalanine. Because of the widespread use of HPLA[1] as a substrate for native and modified carboxypeptidase A and for bovine carboxypeptidase B, it appeared of interest to re-examine in greater detail the kinetics of the reaction over a range of substrate concentrations which extended low enough to avoid excess substrate inhibition and high enough to define the kinetic parameters over the entire concentration range. The use of a spectrophotometric method in this work permitted measurements to be carried out over a 2860-fold variation in substrate concentration.

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† The data presented are taken from a dissertation submitted to the Graduate Faculty of the University of Washington by W. O. McClure in partial fulfillment of the requirements for the Ph.D. degree. Present address: Rockefeller Institute, New York, N. Y.

[1] The following abbreviations are used: HPLA, hippuryl-DL-β-phenyllactate; CGP, carbobenzoxyglycylphenylalanine; carboxypeptidase, bovine pancreatic zinc carboxypeptidase A_γ; DFP, diisopropylphosphorofluoridate; ammediol, 2-amino-2-methyl-1,3-propanediol.

EXPERIMENTAL

Bovine pancreatic zinc carboxypeptidase A_γ purified by the method of Anson (1937) as modified by Putnam and Neurath (1946) was purchased from Worthington (Freehold, N. J.) and stored as an aqueous suspension under a toluene atmosphere. Stock solutions of about 30 mg ml were made by dissolving crystals in 3 M sodium chloride, after washing three times with distilled water. Such solutions were stable at 4° for periods of at least 3 weeks, but were never used for longer than 7 days. Dilutions of these stock solutions were made as needed with 0.005 M Tris-HCl–0.1 M sodium chloride, *p*H 7.50. Diluted solutions of the enzyme were dis-

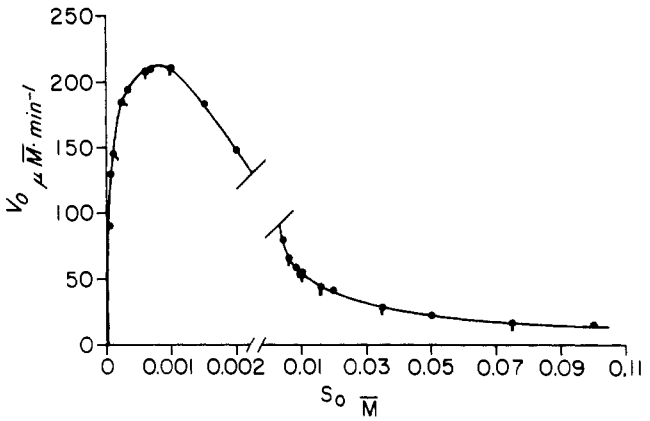

FIG. 1.—Dependence of initial velocity v_0 upon the concentration of substrate S_0 from 3.5×10^{-5} to 0.1 M. Enzyme concentrations were varied between 1.3 and 8.0×10^{-9} M in order to encompass the observed range of initial velocities. The velocities given are corrected to an enzyme concentration of 8.0×10^{-9} M. The flagged points represent multiple determinations (usually triplicates) which agreed within ±2%. The square point corresponds to the usual assay conditions for carboxypeptidase. The curve is plotted from equation (5) using the parameters of Table III.

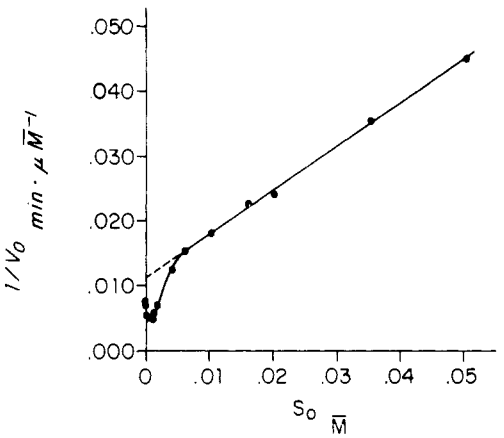

FIG. 2.—Relation between the reciprocal of the observed velocity and the substrate concentration, using the data given in Figure 1.

carded after 1 day. Treatment of the enzyme with 5×10^{-3} M DFP produced no change in the reported results.

Hippuryl-DL-β-phenyllactate was synthesized according to the method of McClure (1964). Solutions were prepared by weighing material which had been previously dried over silica gel. Except as noted, all concentrations refer to the total concentration of racemic substrate.

With the exception of the *p*H studies, all data were obtained in *p*H 7.50, 0.005 M Tris-HCl–0.1 M sodium chloride. Owing to the contribution of high substrate concentrations to the ionic strength, it was occasionally necessary to reduce the concentration of sodium chloride in order to maintain an ionic strength of 0.105. In the *p*H studies, buffers of 0.1 total ionic strength were employed. The following buffer salts were used: *p*H 7.65, Tris-HCl; *p*H 8.70 and 9.70, ammediol-HCl.

Enzyme concentrations were determined by measuring the absorbance at 278 mμ of suitably diluted solutions. A value of the molar extinction coefficient of 6.49×10^4 liters/mole cm was taken from Bargetzi *et al.* (1963).

Reactions were followed either in a Perkin-Elmer Model 350 recording ultraviolet spectrophotometer or on a *p*H-stat as described by Bargetzi *et al.* (1963). Initial velocities were evaluated by either visual examination or by utilizing the fact that

$$v_o = \frac{S_o}{2} \lim_{t \to 0} \left[\frac{d \ln (S_o/S)}{dt} \right] \quad (1)$$

when S_o is the initial substrate concentration, v_o is the initial velocity, and S is the substrate concentration observed at some time t. When used, plots of ln (S_o/S) against t were linear to at least 50% completion of the reaction, and gave values for v_o which agreed to within 3–4% of those measured directly. Kinetic parameters evaluated by extrapolation to zero time were used in order to avoid interference by one of the products, L-phenyllactic acid, which is known to inhibit this reaction (Snoke *et al.*, 1948).

Spectrophotometric data were obtained by following the increase in absorbance at 254 mμ which occurs when benzoylamino acid derivatives are subjected to hydrolysis (Schwert and Takenaka, 1955). Rectangular quartz cells of 10-mm light path were filled with 3.00 ml of substrate and used as samples upon which to zero the instrument at 254 mμ. Thereafter the chart drive was engaged, and a calibrated micropipet was used to introduce 100 μl of buffer into the reference solution. At zero time an equal amount of enzyme solution was added to the reaction vessel, and the pen was placed in contact with the chart paper. Recording was commenced after the 5–10 seconds required to mix the solution and insert the cell in the spectrophotometer. In general, the enzyme concentration was adjusted to a level allowing about 20–40 minutes for complete hydrolysis.

The Beer-Lambert law was obeyed by both reactants and products at all substrate levels employed. At very low substrate concentrations (less than 10^{-4} M) it was necessary to use the expanded transmission scales of the instrument. In such instances proper attention to the light intensity, such as cleaning and aligning the entrance mirrors, enabled operation with signal-to-noise ratios of 10–11 at the lowest concentration (3.5×10^{-5} M) and 20 or greater at all higher concentrations.

Data obtained by spectrophotometric means were converted to initial velocities by using an observed $\Delta\epsilon$ at 254 mμ of 592 liters/mole cm. The value was obtained by repeated measurements of the change in absorbance upon complete hydrolysis of several known concentrations of HPLA. This extinction coefficient represents the change in absorbance of the L isomer only (since the D isomer is neither hydrolyzed nor inhibitory) (Snoke and Neurath, 1949), allowing direct comparison with *p*H-stat measurements.

During all measurements, the temperature was maintained at 25.0 ± 0.1° by means of circulating water controlled with a Haake thermostat. All linear relationships were determined by the method of least squares, and the reported errors were taken at ±1 standard deviation.

RESULTS

Dependence on Enzyme Concentration.—It has been well established that the initial rate of hydrolysis of HPLA is directly proportional to the concentration of enzyme if measurements are conducted at substrate concentrations of 10^{-2} M (Snoke *et al.*, 1948). In view of the marked substrate inhibition characterizing this system, the proportionality was examined at substrate levels giving the maximal rate of hydrolysis. Measurements carried out from $1–8 \times 10^{-9}$ M carboxypeptidase, at a substrate concentration of 10^{-3} M HPLA, yielded

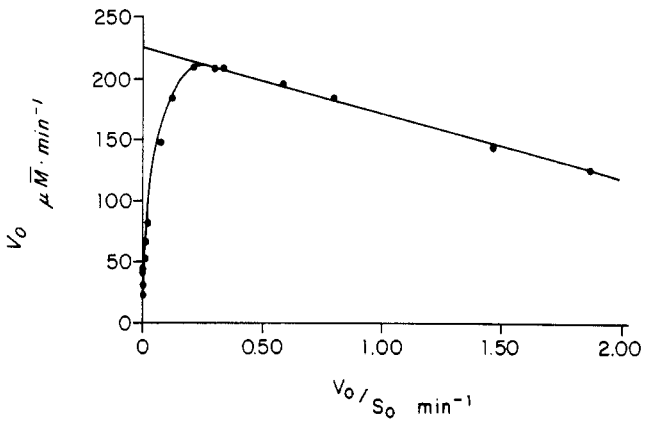

FIG. 3.—Relation between the observed velocity and v_0/S_0 for HPLA and carboxypeptidase using the data given in Figure 1.

TABLE I

EFFECT OF TEMPERATURE ON THE CARBOXYPEPTIDASE-CATALYZED HYDROLYSIS OF HPLA

	k_o (min^{-1})	K_m (M)	Parameter[a]	Value
20.0	2.06×10^4	40.6×10^{-5}	$\Delta S^{\ddagger}$	-11.8[b]
25.0	2.80×10^4	52.8×10^{-5}	$\Delta H^{\ddagger}$	9.9[c]
30.0	3.75×10^4	56.9×10^{-5}	$\Delta F^{\ddagger}$	13.4[c]
35.0	4.82×10^4	54.7×10^{-5}	ΔE_{act}	10.5[c]

[a] Calculated at 25°. [b] eu. [c] kcal/mole.

velocities directly proportional to the enzyme concentration in accord with expectation. The proportionality constant was found to be 2.61×10^4 min^{-1}.

Effect of Substrate Concentration.—The dependence of the observed reaction velocity upon a 2860-fold range of substrate concentrations (3.5×10^{-5} to 0.10 M) is given in Figure 1. The marked inhibition by excess substrate originally observed by Snoke and Neurath (1949) is confirmed. In addition, a plot of $1/v_o$ against S is found to be linear (Fig. 2) at high substrate concentrations (above 0.005 M). A conventional dependence of the velocity upon substrate concentration (with K_m of $5.10 \pm 1.3 \times 10^{-5}$ M and k_o of $2.80 \pm 0.02 \times 10^4$ min^{-1}) is observed only at very low substrate concentrations (below 0.001 M) using the spectrophotometric method and an Eadie (1942) plot of v_o against v_o/S_o (Fig. 3). The constants K_m and k_o were obtained from the slope and intercept respectively of the Eadie plot at low concentrations, and represent observed values. However, an anomalous dependence of the velocity on substrate concentration is observed at intermediate concentrations of substrate (between 0.001 and 0.005 M) as is particularly apparent in Figure 2. Since both the intermediate range and the higher range of substrate concentrations lead to anomalous behavior reflecting a complex mechanism involving the interaction of the enzyme with more than one substrate molecule, the following features of the catalysis were examined at low substrate concentrations to avoid these complexities.

Effect of Temperature.—The constants K_m and k_o were determined using four substrate concentrations at each of four temperatures. The substrate concentrations chosen were in all cases limited to those values showing no inhibition. The data were analyzed as above and are presented in Table I. The large variations in k_o are seen to be both quantitatively and qualitatively different from the small corresponding changes in K_m.

An Arrhenius plot of these data, given in Figure 4, yields an observed activation energy of 10.5 kcal/mole

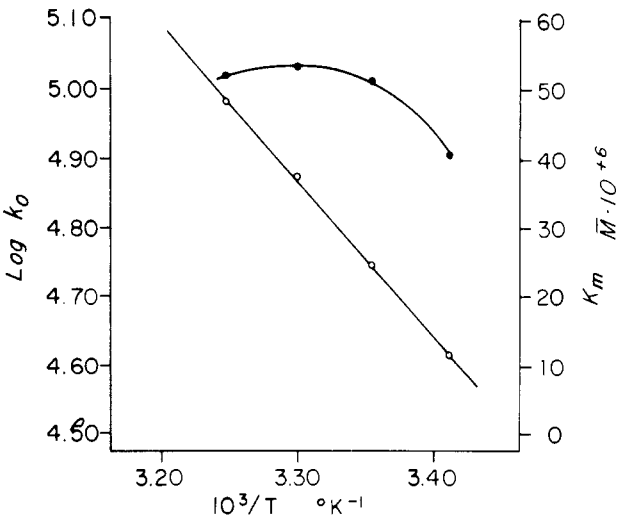

FIG. 4.—An Arrhenius plot giving the effect of temperature on k_0 (O). For purposes of comparison, the values of K_m(●) at the four temperatures are also given.

TABLE II

EFFECT OF pH ON v_o AT VARIOUS VALUES OF S_o[a]

	S_o			
pH	7.0×10^{-5}	2.3×10^{-4}	7.0×10^{-4}	1.0×10^{-2}[b]
7.65	15.4	21.8	25.2	7
8.75	15.4	21.9	24.5	7
9.70	15.4	22.1	24.5	9

[a] Initial velocities (μM min^{-1}) were obtained at 25° with 9.4×10^{-10} enzyme under the conditions given under Methods. [b] Those values listed at S_o = 0.01 M were taken from the data of Riordan and Vallee (1963) and have been corrected to an enzyme concentration of 9.4×10^{-10} M.

from which the activation parameters given in Table I have been calculated according to considerations of the transition-state theory of chemical reactions as set forth by Eyring and Stearn (1939).

Effect of pH.—The studies of Riordan and Vallee (1963) have shown that the pH dependence of the hydrolysis of HPLA by carboxypeptidase differs from the bell-shaped pH-rate profile observed with peptide substrates. The two pK_a values corresponding to the rising and falling limbs of the bell-shaped curve for carbobenzoxyglycylphenylalanine are replaced in the case of HPLA by a plateau from pH 7.5 to 9.5. Since pH-rate profiles are known to vary with substrate concentration (Laidler, 1958a), the effect of pH on the initial velocities of this reaction was determined at three different substrate concentrations. In particular, substrate concentrations which did not cause substrate inhibition were included to test the hypothesis that a molecule of substrate bound to the enzyme in an inhibitory manner was competing with hydroxyl ions for that site on the enzyme responsible for the observed inflection point at pH 9.1 (Bruice and Schmir, 1959). As shown in Table II, the rate of hydrolysis at all substrate concentrations is independent of the pH within the range 7.5–9.7, in agreement with the results of Riordan and Vallee (1963) obtained at high substrate levels.

DISCUSSION

The wide range of substrate concentrations examined in this study permits a re-evaluation of both the conventional conditions of assay of carboxypeptidase and of current ideas of its mechanism of action. The present data indicate that the usual assay is carried out

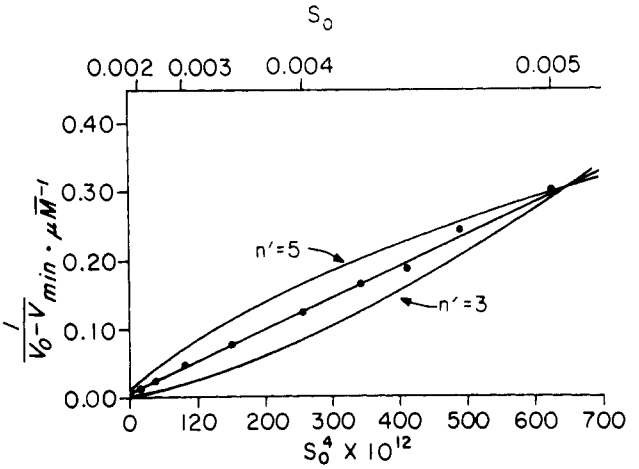

FIG. 5.—A plot of $1/(v_0 - v)$ as a function of S_0^4. For purposes of comparison smoothed curves evaluated as a function of S_0^3 and S_0^5 are also given. The abscissa for the S_0^3 and S_0^5 plots is set in such a way that 0.005 M is represented by the same abscissa value in all three cases. See text for details.

under conditions (S_o = 0.010 M) in which initial velocities are reduced to only 25% of those measurable at the optimal substrate concentration. More sensitive determinations could be carried out at substrate levels of 7–10 × 10^{-4} M, using a spectrophotometer at 254 mμ. Using these conditions, it is possible to assay as little as 0.2 μg of carboxypeptidase A. The maximum velocity calculated from the measurements in this low range of substrate concentrations is the highest yet reported for any substrate for carboxypeptidase (Neurath and Schwert, 1950).

Carbobenzoxyglycyl-L-phenylalanine is one of the most widely used peptide substrates for carboxypeptidase (Neurath and Schwert, 1950). HPLA was originally introduced as an ester analog of the peptide hippurylphenylalanine and of CGP, and offers the convenience of rate measurements by use of the *p*H-stat. It differs structurally from CGP in the replacement of the —NH— function of the peptide by an oxygen atom in the ester, and in the deletion of the —CH_2O— group in the carbobenzoxy moiety of CGP. The latter change is probably of much less significance than the former, as indicated by the similar values of K_m and k_o for hippuryl-L-phenylalanine (K_m = 0.011 M, k_o = 1.09 × 10^4 min^{-1}) and CGP (K_m = 0.033 M, k_o = 1.15 × 10^4 min^{-1}) (Snoke and Neurath, 1949). The replacement of the susceptible amide bond in CGP by an ester bond in HPLA causes a lower K_m and a higher k_o for HPLA as compared to CGP. Although by no means an invariable rule, it is usually true that in hydrolyses catalyzed by proteolytic enzymes esters are more labile than the analogous peptides, in keeping with the relative stabilities of the two compounds.

The marked substrate inhibition found with these two substrates is very similar in character. An exhaustive analysis of the data presented in Figures 1 and 2 has enabled us to rule out completely all simple schemes of excess substrate inhibition for which the rate law of

$$\frac{E_o}{v_o} = (1 + K_m/S_o + K_I S_o) \qquad (2)$$

is predicted (Webb, 1963), as is readily seen in the anomalous behavior of a plot of the reciprocal of the initial velocity against the substrate concentration (Fig. 2). The present data give many points lying below the extended line, while equation (2) specifically predicts that no points will lie in this region of the graph.

A mechanism yielding kinetics which can adequately explain more complex substrate inhibitions has been discussed by Botts and Morales (1953) and amplified by Morales (1955) and Botts (1958). Extensive analog and digital computation has been employed to show that even this scheme is insufficient to satisfy these data.

Further evaluation of the data revealed several schemes which could be formulated as rate laws entirely consistent with the experimental measurements. These expressions were generated by assuming that either several different inactive complexes are formed by interaction of excess substrate with the enzyme or that several species of enzyme coexist. The former assumption invokes the stepwise noncompetitive binding of five substrate molecules to form five complexes each capable of binding (but not hydrolyzing) substrate in the usual manner. The latter assumption invokes a maximum of four species of enzyme. Of course several mechanisms, intermediate in character between these two extreme cases, would also generate a rate law of identical form. Without more data on this system it is impossible to choose any one of these mechanisms, but an interesting parallel between the carboxypeptidase-catalyzed hydrolysis of HPLA and CGP can be obtained by generalizing the mechanism proposed by Lumry *et al.* (1951) for the reaction with the latter substrate.

Assume that the population of enzyme molecules is composed of two species of enzyme, E and E', both of which are capable of being inhibited by binding an undefined number of substrate molecules. If we impose no further restraints upon the mechanism, we may write

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} C_1 \xrightarrow{k_r} E + P$$

$$C_1 + n\mathrm{S} \overset{K_I}{\rightleftarrows} C_2$$

$$E' + S \underset{k_{-1}'}{\overset{k_1'}{\rightleftarrows}} C_3 \xrightarrow{k_r'} E' + P$$

$$C_3 + n'\mathrm{S} \overset{K_I'}{\rightleftarrows} C_4 \qquad (3)$$

Define

$$E_t = E + C_1 + C_2 = E_o\alpha$$
$$E_t' = E' + C_3 + C_4 = (1 - \alpha)E_o \qquad (4)$$

The usual derivation assuming C_1 and C_3 to be in the steady state and $S_o >> E_o$ yields a rate law of

$$v_0 = v + v' = \frac{\alpha k_r S_o E_o}{K + S_o + K_I S_o^{(n+1)}} + \frac{(1 - \alpha)k_r' S_o E_o}{K' + S_o + K_I' S_o^{(n'+1)}} \qquad (5)$$

when v and v' refer to the contributions of enzymes E and E' to the observed velocity v_o, E_o = the total enzyme concentration, $K = (k_r + k_{-1})/k_1$, $K' = (k_{-1}' + k_r'\lambda)/k_1'$, and K_I and K_I' refer to the respective association constants.

The constants in equation (5) may be evaluated as follows: since a plot of $(1/v_o)$ versus S_o is linear, n must equal 1 for one species of enzyme. From the extrapolated straight-line portion of this graph (Fig. 2) we may evaluate K_I and αk_r. Comparing αk_r = 1.12 × 10^4 min^{-1} with the observed value of k_o from Figure 3 (2.80 × 10^4 min^{-1}), it is apparent that both E and E' are being saturated with substrate in the same concentration range. Since Figure 3 shows no evidence of two sites,[2] K must be nearly equal to K'. Therefore

[2] For a critical discussion of this argument see Reiner (1959), who has shown that it is not possible, in general, to observe deviations from normal Michaelis-Menten kinetics in a system in which two enzymes are acting on a single substrate unless the two K_m values vary by a factor of 2 or more.

TABLE III

VALUES CALCULATED FOR THE PARAMETERS OF EQUATION (5)[a]

$K = 5.10 \times 10^{-5}$ moles liter^{-1}	$K' = 5.10 \times 10^{-5}$ moles liter^{-1}
$K_I = 61.7$ liters mole^{-1}	$K_I' = 6.22 \times 10^{10}$ liters^4 moles^{-4}
$\alpha k_r = 1.12 \times 10^4$ min^{-1}	$(1 - \alpha)\, k_r' = 1.77 \times 10^4$ min^{-1}

[a] Calculated at 25°.

the value of k_o obtained from Figure 3 represents saturation of both enzyme species and allows the calculation of $k_r'(1 - \alpha)$ from the relation:

$$k_o = k_r'(1 - \alpha) + \alpha k_r \qquad (6)$$

The evaluation of K_I' and n' may be accomplished by using a rearranged form of equation (5):

$$\frac{1}{v_0 - v} = \frac{1}{v'} = \frac{K'/S_o + 1 + K_I' S_o^{n'}}{k_r'(1 - \alpha)E_o} \qquad (7)$$

which predicts a linear relationship between $(1/v')$ and $S_o^{n'}$ at values of $S_o >> K'$. Having obtained v by extrapolation in Figure 2, it is possible to apply equation (7) to the data, producing the plot given in Figure 5. As shown by the curvature at $n' = 3$ or 5, this quantity must be taken equal to 4,[3] yielding a value of $(n' + 1) = 5$ and, from the slope of the line, a value of K_I'. Since the low substrate ranges produce a linear plot of v_o versus v_o/S_o, we have no justification for assuming that K and K' are different from each other or from K_m,[2] although the two need not be exactly the same in order to make the derivation above remain valid. Values obtained in this manner are presented in Table III and have been used to calculate the theoretical line of Figure 1. It can be seen that the fit is excellent over the entire substrate concentration range of 3.5×10^{-5} to 0.10 M, there being no measurable difference between the theoretical curve and the points obtained by experiment. If in the present formulation k_r were equal to k_r', the respective amounts of the two enzymes could be calculated by evaluating α using equation (6). These species would be found to be present in a ratio of 61:39, in marked similarity to the distribution assumed by Lumry *et al.* (1951). The parallel between this mechanism and that proposed by Lumry is further evidence for the similarity of the carboxypeptidase-catalyzed hydrolyses of HPLA and of CGP.

In the present kinetic scheme, the nature of the substrate inhibition is quite complex. In view of this fact, any change in the activity of the enzyme toward HPLA through chemical modification could be subject to several interpretations. It should be noted that the 4-fold increase in activity expected upon release of substrate inhibition at 0.01 M concentrations is clearly less than the 7-fold stimulation occurring upon acetylation of the enzyme (Simpson *et al.*, 1963; Riordan and Vallee, 1963). However, since in cases of substrate inhibition the observed maximum velocity may be less than that theoretically attainable if substrate inhibition were absent (Laidler, 1958b; Botts and Morales, 1953) it is not possible to evaluate precisely the net increase in activity which is solely due to chemical modification, in contrast to that which results merely from release of substrate inhibition.

The activation parameters given in Table I are of the order of magnitude previously reported for the breakdown of the enzyme-substrate complex in carboxypeptidase-catalyzed reactions (Snoke and Neurath, 1949; Lumry *et al.*, 1951). It is important to bear in mind the complexity of k_o, since this constant is almost certainly a collection of terms involving many of the constants of equation (3), and may be even more complex for the other possible mechanisms. The experimentally observed energy of activation of 10.5 kcal/mole reported herein for the hydrolysis of HPLA indicates that an error of ±1.0° in the control of temperature will introduce an error of ±6.0% in the measured rate.

Turning now to the possible effect of the substrate concentration upon the *p*H dependence of the initial velocity, it is clear that no decrease in rate is observed between *p*H 7.5 and 9.7 at any of the substrate concentrations tested. This result would rule out the possibility of suppressed ionization upon the enzyme surface owing to the binding of an inhibiting molecule of substrate, but does not allow any statements to be made concerning the possibility of an altered ionization which is present for other reasons.

We would like to extend our thanks to Dr. M. F. Morales for his generous assistance in dealing with the analog computation involved in this problem.

[3] In order to account for the rapid fall in velocity upon the initiation of substrate inhibition, Lumry *et al.* (1951) invoked a rate law in which four molecules of substrate were bound to the enzyme to produce an inactive complex. Owing to an oversimplification in the derivation of the rate law used in their studies, however, the value of four should be raised to five, in perfect accord with the present results. Lumry *et al.* (1951) have assumed that the concentrations of the active complexes in their system are insignificant with respect to the free enzyme and the inhibited complex. In addition, it is stated that the observed rate of the reaction is equal to $k_o S_o E_{\text{"effective"}}$ (symbols of Lumry *et al.*, 1951), which amounts to the mathematical limitation that $(K_m + S_o) \equiv 1$ at all values of S_o.

REFERENCES

Anson, M. L. (1937), *J. Gen. Physiol. 20*, 777.

Bargetzi, J.-P., Sampath Kumar, K. S. V., Cox, D. J., Walsh, K. A., and Neurath, H. (1963), *Biochemistry 2*, 1468.

Botts, J. (1958), *Trans. Faraday Soc. 54*, 593.

Botts, J., and Morales, M. F. (1953), *Trans. Faraday Soc. 49*, 696.

Bruice, T. C., and Schmir, G. L. (1959), *J. Am. Chem. Soc. 81*, 4552.

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

Eyring, H., and Stearn, A. E. (1939), *Chem. Rev. 24*, 253.

Laidler, K. J. (1958a), The Chemical Kinetics of Enzyme Action, London, Oxford, p. 117.

Laidler, K. J. (1958b) The Chemical Kinetics of Enzyme Action, London, Oxford, pp. 73–75.

Lumry, R., Smith, E. L., and Glantz, R. R. (1951), *J. Am. Chem. Soc. 73*, 4330.

McClure, W. O. (1964), *Biochem. Prepn. 11, in press.*

Morales, M. F. (1955), *J. Am. Chem. Soc. 77*, 4169.

Neurath, H., and Schwert, G. W. (1950), *Chem. Rev. 46*, 69.

Putnam, F. W., and Neurath, H. (1946), *J. Biol. Chem. 166*, 603.

Reiner, J. M. (1959), Behavior of Enzyme Systems, New York, Burgess, p. 98.

Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 1460.

Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 16*, 570.

Simpson, R. T., Riordan, J. F., and Valleee, B. L. (1963), *Biochemistry 2*, 616.

Snoke, J. F., and Neurath, H. (1949), *J. Biol. Chem. 181*, 789.

Snoke, J. F., Schwert, G. W., and Neurath, H. (1948), *J. Biol. Chem. 175*, 7.

Webb, J. L. (1963), Enzyme and Metabolic Inhibitors, New York, Academic, p. 114.