The pH Dependence of the Pepsin-Catalyzed Hydrolysis of N-Acetyl-L-phenylalanyl-L-3,5-dibromotyrosine

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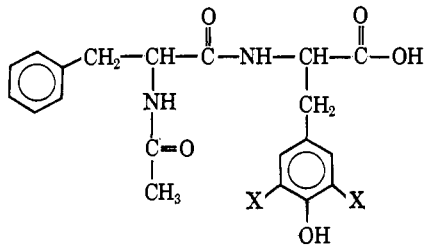
Abstract: In this paper we describe the pH dependence of the three kinetic parameters k_{cat}, k_{cat}/K_M, and K_M for the pepsin-catalyzed hydrolysis of the dipeptide, N-acetyl-L-phenylalanyl-L-3,5-dibromotyrosine, at 25°. The functions k_{cat} and k_{cat}/K_M each exhibit a bell-shaped pH dependence with the pH maximum being about 2.1 for k_{cat} and 1.9 for k_{cat}/K_M. The K_M *vs.* pH curve exhibits a broad minimum between pH 1 and 2. Inhibition by the product N-acetyl-L-phenylalanine has been reinvestigated at pH 2.0. The data have been interpreted in terms of mechanisms that involve the following: (a) binding of only the associated form of the carboxylic acid substrate, and (b) the participation of two catalytically important carboxyl groups, with pK values of 0.75 and 2.67 for the unbound enzyme (k_{cat}/K_M *vs.* pH) and 0.89 and 3.44 for the bound enzyme (k_{cat} *vs.* pH).

A previous report from this laboratory presented the kinetics of the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-3,5-dibromotyrosine (Ib) at pH 2.0 and 25°. Our data[3] showed this compound to



Ia, X = H
b, X = Br
c, X = I

be hydrolyzed at a rate intermediate between that of the unsubstituted dipeptide Ia[4] and the diiodo dipeptide Ic.[5] The cause of the rate acceleration for the halogenated dipeptides with respect to the unsubstituted one was primarily attributable to an increased strength of binding and only slightly attributable to more rapid catalysis; *i.e.*, in terms of the kinetic parameters, K_M was much more significantly decreased than k_{cat} was increased (see Table I, Results and Discussion). In order to gain a clearer understanding of what happens when pepsin hydrolyzes synthetic substrates we have studied in detail the pH dependence of the pepsin-catalyzed hydrolysis of compound Ib, and the results of that study are reported herein.

Experimental Section

Synthesis. The preparation of the substrate has been described by us earlier.[3]

Materials and Methods. The kinetic method and the materials used have also been described by us previously.[3] The following modifications were introduced: (1) pepsin was obtained from Sigma Chemical Co.; (2) daily solutions of the enzyme were prepared at 1×10^{-5} *M*, with concentrations being determined spectrophotometrically at 278 mμ on a Beckman Model DU spectrophotometer using a value of ϵ_{278} 51,500.[4] This called for samples which were approximately 0.7 ml to be added to 9.00 ml of the appropriate buffer to give an enzyme concentration of 7×10^{-7} *M*; (3) stock solutions of the substrate were prepared in methanol at concentrations of 0.02–0.025 *M* so that for most of the pH values studied, less than 100 μl of methanol was used for even the highest concentrations of substrate studied. The methanol content present in the reaction solutions ranged from 5.01 to 5.80% by volume.[6]

Phosphate buffers were employed over the whole pH range except at pH 1.08 and 4.04. The former pH was maintained by the use of an HCl–KCl solution of $\Gamma/2 = 0.09$ and the latter by the use of an acetic acid–sodium acetate solution of $\Gamma/2 = 0.02$. Control runs at pH 2 showed that the reaction rate was the same at $\Gamma/2 = 0.02$ and at $\Gamma/2 = 0.12$, using either KCl or $KClO_4$ to raise the value of $\Gamma/2$. This is in general agreement with the results of Jackson, *et al.*,[5] and implies that in this range, the effect of small variations of ionic strength is unimportant.

All reactions were followed for times of 100 to 120 min, and all behaved according to first-order kinetics over this time period. Since the per cent reaction at pH 3.5 and 4.0 in this time is less than 50%, one *cannot* say that at these pH values the reactions obeyed exclusively first-order kinetics. For the determination of initial rates, however, this time period was sufficient.

pK Determinations. pK_a' values for compound Ib were redetermined on a Radiometer Model TTTA3 automatic titrator at 25.0° in 5% methanol and at $\Gamma/2 = 0.02$ in NaCl, *i.e.*, the conditions used in the kinetic procedure. Under these conditions the pK values were determined to be $pK'_{A\text{-}COOH} = 4.06$ (±0.05) and $pK'_{A\text{-}OH} = 7.10$ (±0.05). This value for $pK'_{A\text{-}COOH}$ was used in the kinetic calculations. The pK_A for N-acetyl-L-phenylalanine under these conditions is 3.60 ± 0.05.

Kinetic Scheme. A scheme which is consistent with our results at a given pH is shown below.[3]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \qquad (1)$$

$$K_M = (k_{-1} + k_{cat})/k_1$$

$$E + P_1 \rightleftharpoons EP_1 \qquad (2)$$

K_I^1 = dissociation constant for EP_1 complex

$$E + P_2 \rightleftharpoons EP_2 \qquad (3)$$

K_I^2 = dissociation constant for EP_2 complex

$$V = k_{cat}[ES] = k_{cat}[E_0][S]/(K_M + [S_0]) \qquad (4)$$

Equation 4 results from the kinetic treatment when one assumes that $(K_M/K_I^1)[P_1] + (K_M/K_I^2)[P_2] + [S] = [S_0]$ or if one considers just the early stages of the reaction. Competitive inhibition by the product, P_1, N-acetyl-L-phenylalanine, has been observed by us[3]

(1) Predoctoral Fellow of the National Institutes of Health.
(2) To whom inquiries concerning this paper should be addressed.
(3) E. Zeffren and E. T. Kaiser, *J. Am. Chem. Soc.*, **88**, 3129 (1966).
(4) M. S. Silver, J. L. Denburg, and J. J. Steffens, *ibid.*, **87**, 886 (1965).
(5) W. T. Jackson, M. Schlamowitz, and A. Shaw, *Biochemistry*, **4**, 1537 (1965).
(6) Controls run in 100% aqueous buffer after completion of the pH profile showed the reaction to be from 40 to 60% faster than in 5.3% MeOH. There is a moderate linear retarding effect by MeOH.

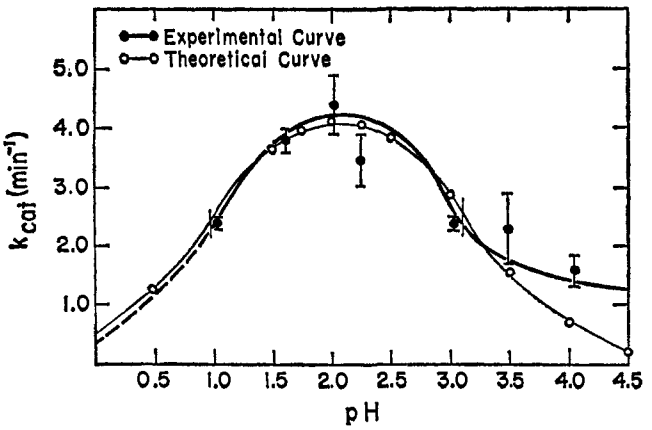


Figure 1. Plot of k_{cat} *vs.* pH at 25.0°

and by others,[4,7] and we have reported a value for K_I^1 at pH 2 and 25°. There are two possible complications, however, which make our former determination of K_I^1 somewhat ambiguous. In the first place, control experiments run in our laboratory after the completion of measurements on the pH–rate profile showed that the variation in the methanol content of the solutions used in obtaining the data reported in our earlier paper[3] would have caused approximately a 10–15% apparent inhibitory effect (over-all) on the reaction. For example, at one substrate concentration, the rate of reaction at a concentration of 7 vol % methanol was between 85 and 90% as fast as the rate in the presence of 5 vol % methanol.[6,8] The second complication is that one cannot at present rule out the possibility of inhibition by L-3,5-dibromotyrosine, product P_2 (*i.e.*, we cannot definitely assume that K_I^2 is very large). In this connection we have now determined that the apparent value for K_I^1 for N-acetyl-L-phenylalanine at pH 2 and 25° in a completely aqueous medium is $2 \pm 1 \times 10^{-3}$ *M* employing compound Ib as the substrate. While this is quite close to the value of K_M for compound Ia, it is far enough removed from that of Ib and Ic to suggest that inhibition by the other products, L-3,5-dibromotyrosine and L-3,5-diiodotyrosine, respectively, is required to give rise to the first-order kinetics observed. We should also mention here that using Ia as the substrate, Jackson, *et al.*, have found an apparent value of K_I^1 which is about ten times larger than ours.[9] The meaning of this divergence is unclear but it could indicate that we are actually observing mixed inhibition with L-3,5-dibromotyrosine while they are observing it with L-tyrosine.

In any event the decreased (and nearly constant) methanol content of the solutions used in obtaining the data for the pH–rate profiles described in this paper has brought the methanol effect inside the experimental error limits usually observed.

Results and Discussion

The results obtained at pH 2 for the related dipeptides Ia, b, and c are shown in Table I. Table II and Figures 1 and 2 illustrate the results gathered in this study of the pH dependence of the pepsin-catalyzed hydrolysis of compound Ib. The data for the individual runs were analyzed by a high-speed computer program described elsewhere.[3] The data shown in Table II for k_{cat} were treated by another high-speed computer program designed specifically for the analysis of enzymatic pH profiles through the use of eq 5, 6, and 7 (see below).[10] This program could not be used to calculate the theoretical pH dependence of the functions k_{cat}/K_M or K_M because the ionization of the substrate must be accounted for kinetically and, as written, the program does not consider this. However,

(7) L. E. Baker, *Nature*, 178, 145 (1956).
(8) J. Tang, *J. Biol. Chem.*, 240, 3810 (1967).
(9) W. T. Jackson, M. Schlamowitz, and A. Shaw, *Biochemistry*, 5, 4105 (1966).
(10) We wish to thank Mr. P. L. Hall who wrote this program and instructed us in its use.

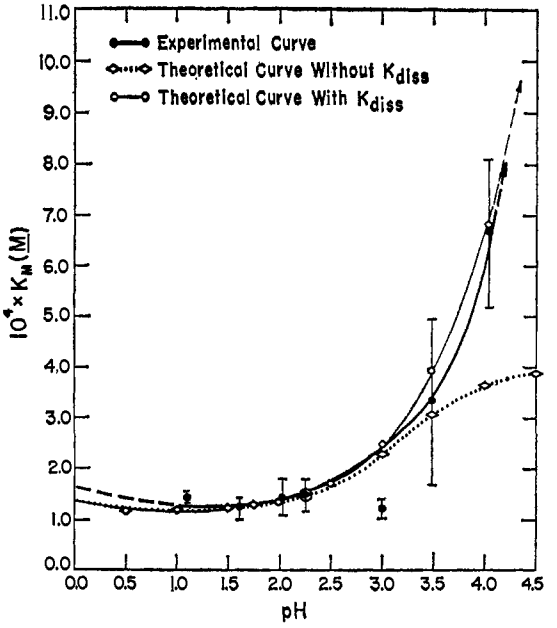


Figure 2. Plot of K_m *vs.* pH at 25.0°.

the values obtained from the computer treatment of k_{cat} were useful as an aid in estimating the necessary parameters used in calculating the other theoretical pH profiles.

Table I. Kinetic Parameters at pH 2.0 for Compounds Ia,[a] b,[b,d] and c[c]

Compd	Temp, °C	$10^{-4}k_{cat}/K_M$, M^{-1} min^{-1}	k_{cat}, min^{-1}	$10^4 K_M$, *M*
Ia	35	0.144	2.80	19.2
Ib	25	3.04 ± 0.42	4.38 ± 0.46	1.44 ± 0.35
Ic	37	16	12	0.75

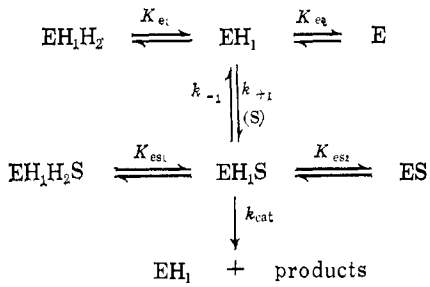
[a] Reference 4. The error limits are determined by a least-squares treatment of the data as incorporated in the computer program and are the values for one standard deviation at a 95% confidence level. [b] This work. [c] Reference 5. [d] Values determined in 100% aqueous medium at this temperature and pH are: k_{cat} = 7.7 min^{-1}; $K_M = 1.04 \times 10^{-4}$ *M*.

Table II. Kinetic Parameters for Compound Ib at the Various pH Values at 25.0 ± 0.2°

pH	$10^{-4}k_{cat}/K_M$, M^{-1} min^{-1}	k_{cat}, min^{-1}	$10^4 K_M$, *M*
1.08	1.67 ± 0.07	2.38 ± 0.07	1.43 ± 0.10
1.62	3.11 ± 0.037	3.78 ± 0.21	1.21 ± 0.21
1.85	3.43	4.46	1.3
2.01	3.05 ± 0.42	4.38 ± 0.47	1.44 ± 0.35
2.25	2.35 ± 0.20	3.46 ± 0.43	1.47 ± 0.30
3.03	1.93 ± 0.16	2.38 ± 0.13	1.23 ± 0.16
3.48	0.689 ± 0.16	2.29 ± 0.62	3.32 ± 1.64
4.04	0.24 ± 0.02	1.60 ± 0.24	6.66 ± 1.46

The somewhat large error limits at a few of the pH values unfortunately could cause a masking of subtleties in pepsin's mechanism of action. This is unavoidable, however, with the kinetic method used. One important point that was brought out by this study was the requirement that the substrate's carboxyl group be in the associated form. This became evident when an attempt was made to fit the data to the theoretical curves

for the kinetic parameters (K_M in particular). In the paper of Alberty and Massey[11] on the pH behavior of enzyme-catalyzed reactions, a derivation of the theoretical equations describing the dependence of k_{cat}/K_M, k_{cat}, and K_M on pH is given. The scheme to which their treatment was applied was



This scheme assumes that only EH_1 combines with the substrate and that only EH_1S breaks down to give products. The ionizations are also assumed to be much more rapid than catalysis. The equations for the theoretical pH dependence of the catalytic parameters that result from this treatment are

$$k_{cat} = \frac{(k_{cat})_{opt}}{1 + H^+/K_{es1} + K_{es2}/H^+} \quad (5)$$

$$k_{cat}/K_M = \frac{(k_{cat}/K_M)_{opt}}{1 + H^+/K_{e1} + K_{e2}/H^+} \quad (6)$$

$$K_M = (K_M)_{opt}\left[\frac{1 + (H^+/K_{e1}) + (K_{e2}/H^+)}{1 + (H^+/K_{es1}) + (K_{es2}/H^+)}\right] \quad (7)$$

This treatment, which has proven useful in the analysis of enzyme systems,[11,12] makes the final assumption that the substrate is either (a) undissociated, (b) is 100% in one ionic form, or (c) that all ionic forms have the same affinity for the enzyme. It has been successfully applied in the work of Clement and Snyder[13] in their study of the nonionizable substrate N-acetyl-L-phenylalanyl-L-tyrosine methyl ester. However, if the substrate can ionize, it is quite likely that assumption c will not hold, *i.e.*, the enzyme will show different affinities for the different ionic states of the substrate. The simplest assumption in this event is that the species EH_1 combines with only one form of the substrate. This requires (as pointed out in ref 12, p 136) that the theoretical expression for K_M be corrected by a factor to account for this substrate ionization. This factor is obtained through the evaluation of the appropriate Michaelis pH function for the substrate.[12] For a monobasic substrate such as Ib which can be assumed to react preferentially in the undissociated form, S, this function is given by

$$f_s = 1 + K_{1s}/H^+ \quad (8)$$

where K_{1s} is the ionization constant for the process $S \rightleftarrows S^- + H^+$. Incorporation of this factor changes eq 6 and 7 to 9 and 10, respectively. Equation 5 is unaffected.

$$\frac{k_{cat}}{K_M} = \frac{(k_{cat}/K_M)_{opt}}{[1 + H^+/K_{e1} + K_{e2}/H^+][1 + K_{1s}/H^+]} \quad (9)$$

$$K_M = (K_M)_{opt}\left[\frac{1 + H^+/K_{e1} + K_{e2}/H^+}{1 + H^+/K_{es1} + K_{es2}H^+}\right]\left[1 + \frac{K_{1s}}{H^+}\right] \quad (10)$$

Application of eq 9 and 10 to our data affected the theoretical behavior of the function k_{cat}/K_M only slightly but it caused a great improvement in the agreement between the theoretical behavior of K_M and the experimental observations (see Figure 2). This improvement shows that the ionization of the substrate is a significant factor affecting the kinetics and implies that the protonation of the substrate's carboxyl terminus is important in binding. This might be due to the electrostatic repulsion of the anion (COO^-) of the substrate by a carboxylate anion on the enzyme.

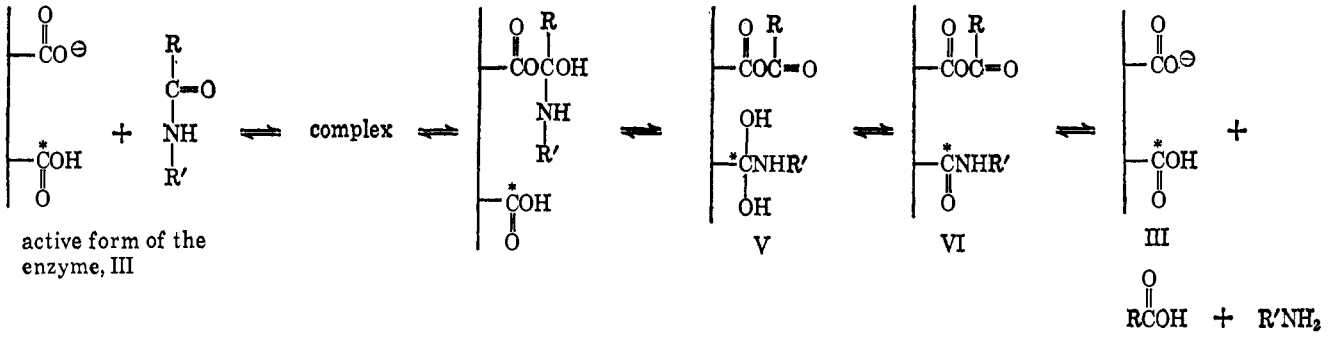
It might be mentioned here that the value for $K'_{A\text{-}COOH}$ for compound Ib which was used in these calculations was slightly different from that determined by us earlier.[3] The old value was determined in 50% MeOH; the new was determined in 5% MeOH at $\Gamma/2 \approx 0.025$, the experimental conditions used in the kinetic procedure.

The data show that the decrease in the strength of binding on changing from pH_{max} to 4.0 is 5.5-fold ($1.2\text{–}6.6 \times 10^{-4}$ *M*) while the change in k_{cat} is about 2.8-fold (4.38–1.60 min^{-1}), or approximately one-half the change in K_M. Because of the divergence of the theoretical and experimental k_{cat} *vs.* pH curves on the basic side of the maximum, any extrapolation of the theoretical curve to pH values outside the range studied is rather tenuous. However, since the K_M *vs.* pH curves agree more closely, a calculation of the theoretical point on this curve at pH 4.5 allows a comparison of the ratio (K_M–pH 2)(K_M–pH 4.5) for compounds Ib and Ic.[5] The value for Ic is about 11.25 and that for Ib is about 12. While this similarity of ratios may be somewhat fortuitous, it is what one would expect from the similarity of the substrates. Extrapolation of the experimental curve of k_{cat} *vs.* pH to pH 4.5 and calculation of the similar k_{cat} ratio show a factor of about 3 for Ic and 3.5 for Ib. Thus our results agree quite closely with those of Jackson, *et al.*,[5] in that the effect of pH on K_M is more pronounced than the effect of pH on k_{cat}.

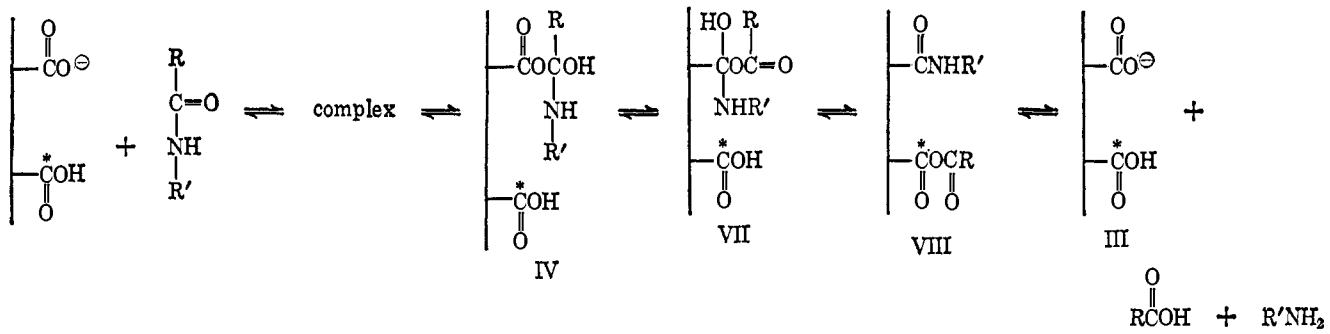
Considering the pH optimum for this and other pepsin substrates (between 2 and 4), the hypothesis that the functional groups responsible for catalytic action of this enzyme are carboxylic acid groups seems reasonable. Although it was experimentally impossible to extend the study to pH values more acid than pH 1, a short extrapolation allowed the pK values for these groups to be estimated from the data. These values are: $K_{e1} = 1.16 \times 10^{-1}$ (p$K_{e1} = 0.75$), $K_{e2} = 2.16 \times 10^{-3}$ (p$K_{e2} = 2.67$), $K_{es1} = 1.23 \times 10^{-1}$ (p$K_{es1} = 0.89$), and $K_{es2} = 3.60 \times 10^{-4}$ (p$K_{es2} = 3.44$). The similarity of K_{e1} and K_{es1} implies that these are dissociation constants for one carboxyl group with the slight shift being caused by binding of the substrate. The same deduction applies to K_{e2} and K_{es2}, although the shift is somewhat larger. These values, especially those for K_{e1} and K_{es1}, are rather large for normal organic acids. Although K_{e2} is in the general vicinity of a carboxylic acid dissociation constant for a free amino acid (or the carboxyl group of a small unblocked dipeptide; see

(11) R. A. Alberty and V. Massey, *Biochim. Biophys. Acta*, **13**, 347 (1954).
(12) M. Dixon and E. C. Webb, "Enzymes," 2nd ed, Academic Press Inc., New York, N. Y., 1964, pp 121ff.
(13) G. E. Clement and S. L. Snyder, *J. Am. Chem. Soc.*, **88**, 5338 (1966).

Scheme I



Scheme II



ref 14), K_{e1} is abnormally large. This strongly suggests a great influence of environmental factors on the ionization of this group. It is of interest to note here that in their study of the hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine methyl ester, Clement and Snyder obtained values for pK_{es1} and pK_{es2} of 1.62 and 3.48, respectively.[13] While their value for pK_{es2} is quite close to ours (probably the same within experimental error), their value of pK_{es1} is approximately 0.7 pK unit higher than the value obtained here. If these pK_{es1} values are for the same anionic carboxyl group on the enzyme then there would appear to be a large substrate dependence of the ionization of this group. This could be caused by hydrogen bonding of the protonated COOH of the substrate (required for binding) to the anionic form of this peculiar carboxyl group (stabilizing the anionic form and thus lowering the pK).

Whatever the detailed reason for this phenomenon, on the basis of the results of Erlanger, *et al.*,[15] and Gross and Morell,[16] it is tempting to speculate that one of the carboxyl groups stems from an aspartate residue and the other from a nearby glutamate residue. Erlanger, *et al.*, isolated an inactivator-labeled pentapeptide (Gly-Gly-Asp-Ser-Glu)[1*p*-$BrC_6H_4COCH_2$] from the enzymic digestion of *p*-$BrC_6H_4COCH_2Br$-inactivated pepsin, with the sequence of the pentapeptide and the position of the modifier indefinite. Gross and Morell later showed that the modifier had esterified the carboxyl of the aspartate residue.

If, at this point, one is allowed to make mechanistic speculations as a basis for further investigation, Scheme I is suggested from the available data on pepsin.

This mechanism incorporates all of the known data concerning peptic hydrolysis of synthetic substrates, including oxygen-18 exchange results[17] and imino and carbonyl type transpeptidations.[18,19] The requirement for two catalytically important groups is implied by the bell-shape of Figure 1. This is in contrast to the suggestion of Fruton that only one carboxyl group is necessary for catalysis.[20]

An alternative to Scheme I is represented by Scheme II. One argument which could be raised against this latter mechanism is that it requires the carboxyl group labeled with an asterisk in VII to act as a nucleophilic species in its protonated form rather than in its dissociated form. In other words, one might have anticipated that in a mechanism of the type shown in Scheme II, both of the enzyme's reactive carboxyl groups would be catalytically active in their anionic forms.

In neither Scheme I nor II have we specified the nature of intermediates formed between the decomposition of the species VI or VIII and the regeneration of the active enzyme III. It is reasonable to suppose that species such as IX and X could intervene as reactive intermediates.[21] In Schemes I and II we postulate that the rate-determining step occurs prior to the decomposition of species VI and VIII, respectively.[22]

(14) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961, Chapter 4.

(15) B. F. Erlanger, S. M. Vratsanos, N. Wasserman, and A. G. Cooper, *Biochem. Biophys. Res. Commun.*, **23**, 243 (1966).

(16) E. Gross and J. L. Morell, *J. Biol. Chem.*, **241**, 3638 (1966).

(17) N. Sharon, N. Grisaro, and H. Neumann, *Arch. Biochem. Biophys.*, **97**, 219 (1962).

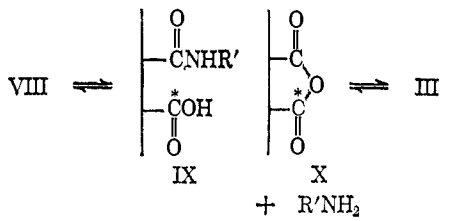
(18) J. S. Fruton, S. Fujii, and M. H. Knappenberger, *Proc. Natl. Acad. Sci. U. S.*, **47**, 759 (1961).

(19) H. Neumann, Y. Levin, A. Berger, and E. Katchalski, *Biochem. J.*, **73**, 33 (1959).

(20) G. R. Delpierre and J. S. Fruton, *Proc. Natl. Acad. Sci. U. S.*, **54**, 1161 (1965).

(21) An example of the interaction of neighboring carboxyl and amide functions like those shown in structure IX was seen in the hydrolysis of phthalamic acid and some of its derivatives: (a) J. Brown, S. C. K. Su, and J. A. Shafer, *J. Am. Chem. Soc*, **88**, 4468 (1966); (b) M. L. Bender, *ibid.*, **79**, 1258 (1957); (c) M. L. Bender, Y. L. Chow, and F. Chloupek, *ibid.*, **80**, 5380 (1958); (d) B. Zerner and M. L. Bender, *ibid.*, **83**, 2267 (1961).

(22) This postulate appears to be consistent with the report of Clement and Snyder[13] that a deuterium solvent isotope effect was absent in the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine methyl ester.



One last mechanistic point should be made. In Schemes I and II we have assumed that one of the reactive groups on the enzyme with a pK of 0.75 is active in its dissociated form while the other with a pK of 2.67 is active in its associated form. However, there is another rather different possibility that is also in agreement with our kinetic results. This is the hypothesis that a catalytically important group on the enzyme with a pK of 0.75 is active in its associated form while the other group with pK = 2.67 is active in its dissociated form. This hypothesis can be shown to give rise to the same kinetic results as would be expected from the previously considered models. There are some difficulties, however. In particular, it is not clear what the group of pK = 0.75 could be. The protonation of amide groups is frequently significant[23] in the pH range near 1, and one could suggest that a protonated amide species is catalytically important in pepsin's mechanism of action. It is hard to envisage though why a protonated amide group would be likely to be catalytically important, unless perhaps it is required to hold pepsin in a reactive conformation.

(23) J. T. Edward and I. C. Wang, *Can. J. Chem.*, **40**, 966 (1962).

Adrenocorticotropins. XXXVII. The Synthesis of Lysine^{8}-α^{1-17NH_2}-ACTH and Its Biological Properties[1]

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Abstract: The synthesis of the heptadecapeptide amide corresponding to the first 17 amino acid residues in the adrenocorticotropin (ACTH) molecule with the replacement of arginine in position 8 by lysine (Lys^{8}-α^{1-17NH_2}-ACTH) is described. The synthetic product possesses much lower adrenal-stimulating and melanocyte-stimulating activities as compared with that of the parent molecule. This indicates that arginine position 8 is essential for the biological function of adrenocorticotropins.

Earlier work has established some biologically nonessential features of the amino-terminal section of the adrenocorticotropins (ACTH) by synthetic means. Li, Schwyzer, and their co-workers[2,3] have synthesized the amino-terminal nonadecapeptide α^{1-19}-ACTH with glutamine substituted for glutamic acid in amino acid residue number 5 (see Figure 1) with no marked change in biological activities. Similarly, Hofmann, *et al.*,[4] have replaced methionine in position 4 by α-amino butyric acid in α^{1-20NH_2}-ACTH. Geiger, *et al.*,[5] have made glycine,[1] phenylalanine,[2] alanine,[3] and deserine[1] analogs in α^{1-23NH_2}-ACTH; and Otsuka, *et al.*,[6] have made a glycine[1] analog of α^{1-17NH_2}-ACTH. When these analogs were assayed and compared with the parent

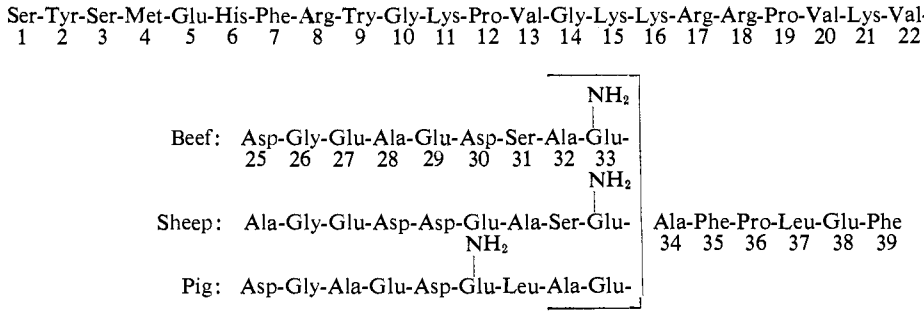

Figure 1. Structure of adrenocorticotropins.

(1) For paper XXXVI, see W. Oelofsen and C. H. Li, *J. Am. Chem. Soc.*, **88**, 4254 (1966). All amino acids occurring in the peptides mentioned in this paper are of the L configuration with the exception of glycine.

(2) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T. B. Lo, and J. Ramachandran, *ibid.*, **82**, 5760 (1960); C. H. Li, *Recent Progr. Hormone Res.*, **18**, 1 (1962).

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