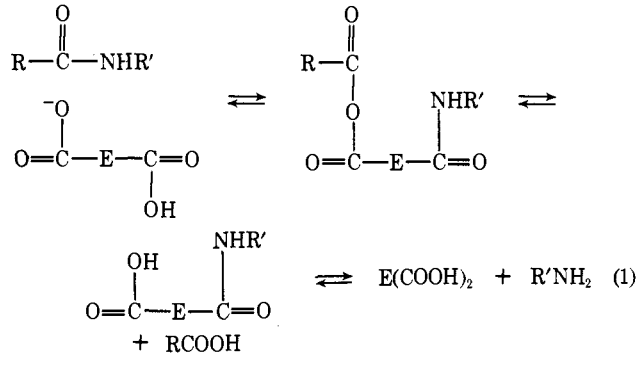
On the Mechanism of the Pepsin-Catalyzed Exchange of Carboxylic Acids with Water-^{18}O

Marc S. Silver,[1] Mai Stoddard,[1] and T. Peter Stein[2]

Contribution from the Departments of Chemistry, Amherst College, Amherst, Massachusetts 01002, and the University of California, Los Angeles, California 90024. *Received October 10, 1969*

Abstract: The pD dependence of the pepsin-catalyzed exchange of DL-acetylphenylalanine with $^{18}OD_2$ has been determined. The data at pD $\geq$ 4.0 are quantitatively accounted for by a kinetic scheme that postulates that the kinetic equivalent of the half-dissociated form of pepsin, ECOOHCOO$^-$, and the anionic form (RCOO$^-$) of the substrate undergoes reaction. Data at low pD require that an exchange path kinetically equivalent to reaction of ECOOHCOO$^-$ with the neutral form of the substrate (RCOOH) also be available. Possible mechanisms consistent with these data are discussed and evaluated for compatibility with other observations on pepsin's mechanism of action. The preferred mechanism for α-chymotrypsin-catalyzed exchange, a nucleophilic attack by enzyme upon undissociated substrate, is probably not operative with pepsin.

Studies on the kinetics[3-7] and inhibition[8] of pepsin action indicate that one or more carboxyl groups are crucial to the functioning of the enzyme, but their exact role remains unrevealed.[9,10] Equation 1 incorporates the essential ingredients of most proposed mechanisms for pepsin catalysis[3,5,11-13] and serves as a useful focus for subsequent discussion.



(1)

Formation of R''CONHR' when the hydrolysis of RCONHR' proceeds in the presence of an "acceptor," R''COOH, under conditions where added R'NH_2 is inert (the R's are suitable L-amino acid derivatives) suggests that pepsin has acylated the amino group of the peptide bond undergoing cleavage,[14-16] and such an intermediate may have been isolated recently.[13c,17] Evidence supporting the anhydride of eq 1 (an "acyl-enzyme") is more sparse. The prime justification[3,5,12] appears to be the pepsin-catalyzed exchange of $^{18}OH_2$ with the carboxyl group of acylated L-amino acids[18,19] but the exchange reaction *per se* does *not* establish the existence of a covalent enzyme–substrate intermediate.[13,20] Likewise, although some observations, such as the parallelism in the ability of R''COOH to function as a transpeptidation acceptor and in the exchange reaction,[19] are conveniently rationalized by the acyl-enzyme, other observations on pepsin's esterase behavior are not. The nature of the problem can be illustrated by contrasting α-chymotrypsin (CT) to pepsin.

Chymotryptic hydrolyses are primarily nucleophilic displacements by an enzymatic serine hydroxyl to yield an acyl-enzyme (eq 2).[21,22] The ease of hydrolysis of substrates is generally $RCO_2R' \gg$ RCONHR' and the exchange reaction (Y = OH) with $^{18}OH_2$ requires the neutral species RCOOH,[23,24] since the carboxylate anion is not readily attacked by nucleophiles.[25,26] A methyl ester, incapable of undergoing the unfavorable ionization, is therefore far more reactive

(1) Work at Amherst College supported by Grant No. AM 08005 of the U. S. Public Health Service.
(2) Work at U.C.L.A. supported by a postdoctoral fellowship of the U. S. Public Health Service.
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(4) J. L. Denburg, R. Nelson, and M. S. Silver, *ibid.*, 90, 479 (1968).
(5) G. E. Clement, S. L. Snyder, H. Price, and R. Cartmell, *ibid.*, **90**, 5603 (1968), and references therein.
(6) T. P. Stein and D. Fahrney, *Chem. Commun.*, 555 (1968).
(7) T. R. Hollands and J. S. Fruton, *Biochemistry*, 7, 2045 (1968).
(8) R. L. Lundblad and W. H. Stein, *J. Biol. Chem.*, **244**, 154 (1969), and references therein.
(9) L. S. Shkarenkova, L. M. Ginodman, L. V. Kozlov, and V. N. Orekhovich (*Biokhimiya*, 33, 154 (1968)) have established that one or more carboxyl groups of pepsin rapidly exchange with $^{18}OH_2$.
(10) L. M. Ginodman, T. A. Valueva, L. V. Kozlov, and L. Shkarenkova, *ibid.*, 34, 211 (1969).
(11) G. R. Delpierre and J. S. Fruton, *Proc. Natl. Acad. Sci. U. S.*, 54, 1161 (1965).
(12) M. L. Bender and F. J. Kezdy, *Ann. Rev. Biochem.*, **34**, 49 (1965).
(13) J. R. Knowles and coworkers, (a) *Biochem. J.*, **113**, 343 (1969); (b) *Phil. Trans. Roy. Soc. London, Ser. B*, in press; (c) "International Symposium on Structure-Function Relationships in the Proteolytic Enzymes," Copenhagen, 1969, in press. The last two references present comprehensive treatments of the mechanistic problem and reach conclusions, some of which anticipate parts of this paper.
(14) H. Neumann, Y. Levin, A. Berger, and E. Katchalski, *Biochem. J.*, 73, 33 (1959).
(15) J. S. Fruton, S. Fujii, and M. H. Knappenberger, *Proc. Natl. Acad. Sci. U. S.*, 47, 759 (1961).
(16) N. I. Mal'tsev, L. M. Ginodman, V. N. Orekhovich, T. A. Valueva, and L. M. Akimova, *Biokhimiya*, 31, 983 (1966), and preceding papers.
(17) Secure evidence is still lacking.
(18) N. Sharon, V. Grisaro, and H. Neumann, *Arch. Biochem. Biophys.*, 97, 219 (1962).
(19) L. V. Kozlov, L. M. Ginodman, and V. N. Orekhovich, *Dokl. Akad. Nauk SSSR*, 172, 1207 (1967), and earlier papers from this group.
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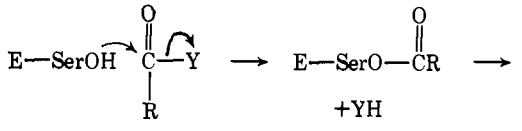
(21) M. L. Bender and F. J. Kezdy, *J. Amer. Chem. Soc.*, 86, 3704 (1964), and references therein.
(22) J. H. Wang, *Science*, **161**, 328 (1968).
(23) (a) M. L. Bender and K. C. Kemp, *J. Amer. Chem. Soc.*, **79**, 116 (1955); (b) M. L. Bender, *ibid.*, **84**, 2582 (1962).
(24) Reference 20, p 49.
(25) D. Samuel and B. L. Silver, *Advan. Phys. Org. Chem.*, 3, 168 (1965).
(26) A. F. Hegarty and T. C. Bruice (*J. Amer. Chem. Soc.*, **91**, 4924 (1969)) claim that the cyclization of N-(*o*-carboxyphenyl)urea proceeds at high pH *via* anionic nucleophilic attack upon a carboxylate anion.

than its corresponding carboxylic acid at pH 7–8, the region of maximum CT activity.[21] For example, CT catalyzes the exchange of AcTrp[27] and $^{18}OH_2$ with modest efficiency[23] and rapidly cleaves[21] AcTrpME.



$$E\text{—}SerOH + RCOOH \quad (2)$$

Y = NHR′, OR′, OH, etc.

Two differences between pepsin and CT reflect upon the likelihood of an acyl-enzyme intermediate in reactions of the former. First, pepsin must possess a more important electrophilic component in its mechanism, for the rates of hydrolysis of the elaborate ester Z-His-Phe(NO_2)≠PlaME and its amide analogy, Z-HisPhe-(NO_2)≠PheME, at the bonds shown, are nearly identical.[28,29] Second, pepsin suffers from the "esterase anomaly," for unlike CT, it does not catalyze the hydrolysis of simple ester (methyl, ethyl) or amide derivatives of acetyl L-amino acids despite the existence of the $^{18}OH_2$ exchange reaction.[13,30,31] We characterize this behavior as "anomalous" because we see no satisfactory explanation for it deriving from either (a) the acyl-enzyme formulation, in analogy to CT, or (b) an enzyme specificity argument, which requires that AcPhe meets the specificity demands of pepsin, but AcPheME or AcPheNH$_2$ do not.

The esterase anomaly is explained if the carboxylate anion RCO_2^-, a species uniquely formed by the acylated amino acids, is responsible for the exchange reaction. To explore this possibility, we have measured the pD dependence of the pepsin-catalyzed exchange of DL-AcPhe[32] with $^{18}OD_2$.

Results[33]

Analysis of carbon dioxide generated from DL-AcPhe which had been incubated with commercial pepsin and $^{18}OD_2$ gave the "per cent exchange" data of Table I. The exchange reactions should[23a] obey first-order kinetics, and as Figure 1 demonstrates, they do (see also ref 9). The slopes[34] of the first-order plots defined the experimental first-order rate constants, k_{exp}. Application of a buffer, solvent, or blank correction where appropriate provided the k_1's of Table I and the data points of Figures 2 and 3. Parentheses in Table I and open circles or triangles in Figures 2 and 3 distinguish those rate constants so corrected.

The control experiments of Table I established that exchange or cleavage reactions of pepsin itself never significantly contributed to the observed ^{18}O enrichment of DL-AcPhe (runs 11, 17, and 32) and that correction for the rate of nonenzymatic exchange by DL-AcPhe is only appreciable for pD 2.13 (runs 5, 18, 27, 30, 31). Run 21, which utilized preincubated pepsin, and run 24,

Table I. Rate of Pepsin-Catalyzed Exchange of DL-Acetylphenylalanine[a] with $^{18}OD_2$ at 35°

Run[b]	pD[c]	Time, hr	% exchanged[d]	$k_1 \times 10^5$, sec^{-1}	Comments
1	5.92A	1	8 ± 0		
2		2	12 ± 2	2.06 ± 0.16	
3		2	17 ± 1		
4		3.5	25 ± 2		*e*
5C		4	1 ± 0		*f*
6	5.60A	3	18 ± 0	3.0	*g*
7	5.35A	1	15 ± 2		
8		2	36 ± 5	6.1 ± 0.4	*e*
9		2	35 ± 2		*e*
10		4	59 ± 4		*e*
11C		4	2 ± 0		*h*
12	5.12A	3	32 ± 4	6.8	*e,g*
13	4.82A	1	37 ± 2	9.5 ± 0.9	
14		2	51 ± 4		*i*
15	4.32A	1	33 ± 1	8.4 ± 0.6	
16		2	47 ± 1		
17C		4	4 ± 1		*h*
18C		4	3 ± 1		*f*
19	5.42C	2	44 ± 2	6.9 ± 0.4	*j*
20		3	53 ± 1	(5.5 ± 0.3)	
21C	4.51C	2	64 ± 0	14	*e,k*
22	4.42C	1	51 ± 3	17.0 ± 0.9	*j*
23		2	71 ± 0	(11.3 ± 0.6)	
24C		3	66 ± 0	20.4	*l*
25	3.20P	5	37 ± 2	2.04 ± 0.13	*e,j*
26		10	53 ± 0	(12.9)	
27C		10.5	6 ± 1	0.05	*e,f,m*
28	2.13P	5	29 ± 2	0.93	*e,n*
29		10	44 ± 0	(6.0)	
30C		10	21 ± 1	0.24 ± 0.01	*f,m*
31C		20	34 ± 1		*f*
32C		19	2 ± 1		*e,h*

[a] All runs contained 10 mg/ml of DL-AcPhe and of commercial pepsin unless otherwise stated. Experimental details, nature of corrections applied, etc. are described fully in the text. [b] C implies a control run. [c] Buffer A = 0.5 *M* (total) acetate; C = 0.5 *M* (total) citrate; P = 0.4 *M* (total) phosphate plus 0.3 *M* KCl diluted to 89% with ethanol. [d] Average of two combustion analyses unless an exception is noted, with average deviations. The percentage was calculated from the formula: 100 (% $^{18}O_{obsd}$ − 0.202)/(% $^{18}O_\infty$ − 0.202). [e] Three analyses. [f] Substrate alone was incubated in buffer for the time indicated, enzyme added, and work-up begun immediately. [g] [Pepsin] = 5 mg/ml, so the k_1 entered is twice that observed. [h] Pepsin alone was incubated in buffer for the time indicated, substrate added, and work-up begun immediately. Four analyses. [i] The unparenthesized k_1 is that observed, while the parenthesized k_1 is corrected for buffer or solvent effect. [k] Pepsin (20 mg/ml) was preincubated for 3 hr in 4.51C, an equal volume of substrate solution (20 mg/ml) added, and incubation continued for 2 hr. [l] Pepsin (4.8 mg/ml) from pepsinogen was employed. The tabulated k_1 is 2.1 times that observed. [m] The k_1 was calculated with the assumption that both stereoisomers undergo exchange but the per cent exchange was not. [n] The unparenthesized k_1 has been corrected for spontaneous exchange, while the parenthesized k_1 has been corrected for solvent effect also.

(27) Abbreviations used: Ac, acetyl; Z, benzyloxycarbonyl; Trp, tryptophan; Phe, phenylalanine; Phe(NO_2), *p*-nitrophenylalanine; His, histidine; Tyr, tyrosine; Pla, β-phenyllactic acid; ME, methyl ester; EE, ethyl ester; NH_2, amide. All configurations are L unless otherwise stated. Occasionally, abbreviations such as AcPheCOOH or AcPheCO_2^- are used to indicate a particular ionic or covalent species.

(28) K. Inouye and J. S. Fruton, *Biochemistry*, **6**, 1765 (1967).

(29) k_2 (ethyl acetate)/k_2(acetamide) is ~2500 for OH^-, only ~20 for H^+. Data for 25°, H_2O are in "Tables of Chemical Kinetics," U. S. National Bureau of Standards, Circular 510, pp 230, 231, 235, and its Supplement 1, pp 98, 99.

(30) L. A. Lokshina, V. N. Orekhovich, and V. A. Sklyankina, *Nature*, **204**, 580 (1964).

(31) (a) Unpublished observations of M. S. Silver; (b) private communication from H. Neumann and V. Grisaro. Dr. Knowles and ourselves have also been unable to detect the pepsin-catalyzed hydrolysis of the *p*-nitrophenyl ester of AcPhe.

(32) Use of the DL isomer was dictated by the need to compare the exchange data with those for other experiments underway which require the racemate. Exchange is known[18,19] to be stereospecific.

(33) The Experimental Section supplies details relative to several points raised here.

(34) Determined by the method of least squares and an *ad hoc* weighting scheme where more than one point other than $t = 0$ was available.

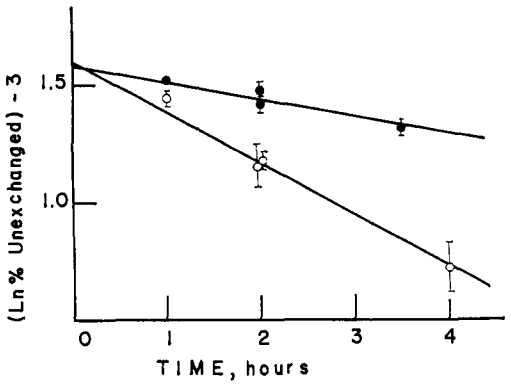


Figure 1. Plot of experimental data according to the first-order rate law: ●, pD 5.92A run; ○, pD 5.35A run.

which employed pepsin obtained from the activation of pepsinogen,[35] gave k_1's in agreement with those from routine experiments. These observations plus the reasonably good adherence of the data for each run to first-order kinetics strongly suggest that the exchange is a genuine pepsin-promoted reaction and not an artifact introduced by the products of pepsin's autodigestion, but the latter possibility has yet to be absolutely ruled out.[36]

Kinetic Analysis. Equation 3, where S = AcPhe-^{16}O and P = AcPhe-^{18}O, formulates a symmetrical exchange reaction in which free substrate and enzyme rapidly equilibrate with enzyme–substrate complexes and isotope effects are negligible.[37] Equation 4, under the stipulated conditions, defines the rate of appearance of labeled AcPhe if E_0 and S_0 are, respectively, the stoichiometric concentrations of pepsin and AcPhe (regardless of oxygen isotope present).[37] Equation 5 therefore specifies the relationship between the corrected experimental first-order rate constant, k_1, and the quantities of eq 3 and 4.

$$\mathrm{E} + \mathrm{S} \overset{K_0}{\rightleftharpoons} \mathrm{ES} \underset{k_x[^{18}\mathrm{OD}_2]}{\overset{k_x[^{18}\mathrm{OD}_2]}{\rightleftharpoons}} \mathrm{EP} \overset{K_0}{\rightleftharpoons} \mathrm{E} + \mathrm{P} \qquad (3)$$

Since $[^{16}\mathrm{OD}_2] \gg [^{18}\mathrm{OD}_2]$

$$k_0 = k_x[^{16}\mathrm{OD}_2] \gg k_x[^{18}\mathrm{OD}_2]$$

and

$$\mathrm{d[P]}/\mathrm{d}t = k_x[^{18}\mathrm{OD}_2]E_0S_0/(K_0 + S_0) - k_0E_0[\mathrm{P}]/(K_0 + S_0) \qquad (4)$$

$$k_1 = k_0E_0/(K_0 + S_0) \qquad (5)$$

If $K_0 \gg S_0$

$$k_1 = k_0E_0/K_0 \qquad (6)$$

Equation 5 reduces to eq 6 with the approximation $K_0 \gg S_0$, and our successful analysis of the pH dependence of k_1 will assume the validity of eq 6 for all pH's. In this approximation the analysis requires only three known pK_a's. Some futile efforts to utilize eq 5 were made, but its application demands knowledge of four additional, ill-defined[4] pK_a's of the enzyme–substrate complexes.

(35) T. G. Rajagopalan, S. Moore, and W. H. Stein, *J. Biol. Chem.*, 241, 4940 (1966).

(36) L. V. Kozlov, L. M. Ginodman, and V. N. Orekhovich (*Dokl. Akad. Nauk SSSR*, 161, 1455 (1965)) first raised this question.

(37) Reference 23a offers a more complete discussion.

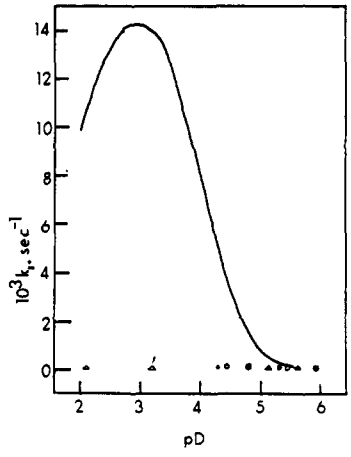


Figure 2. Failure of a mechanism postulating exchange implicating EH + AcPheCOOH exclusively to explain the experimental data. The theoretical curve was calculated from eq 8 with $k_{-6}E_0/K_{ESH}$ = 1.67×10^{-2} sec^{-1} and the pK_a's of the text. The experimental points are: ●, acetate buffer, E_0 = 10 mg/ml; ○, citrate buffer, E_0 = 10 mg/ml (corrected for buffer effect); ▲, acetate buffer, E_0 = 5 mg/ml (corrected to E_0 = 10 mg/ml); △, phosphate buffer, E_0 = 10 mg/ml, 11% ethanol (corrected to 0% ethanol).

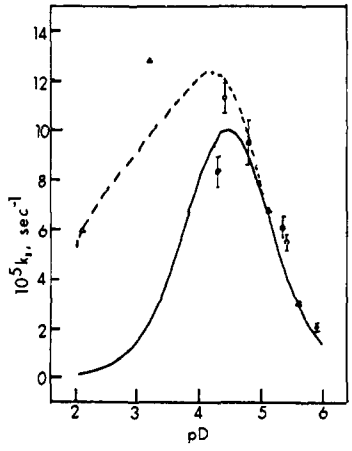


Figure 3. Comparison of experimental points (indicated as in Figure 2) and a theoretical curve (solid line) for an exchange reaction involving EH + AcPheCO$_2^-$ exclusively (eq 9 with $k_{-3}E_0/K_{ES}$ = 2.0×10^{-4} sec^{-1}). The dotted curve represents the summation of the solid line and a contribution from exchange between EH + AcPheCOOH, calculated from eq 8 with $k_{-6}E_0/K_{ESH}$ = 8.9×10^{-5} sec^{-1}. The dotted curve therefore plots eq 7 with the indicated values for the parameters.

How likely is the assumption $K_0 \gg S_0$? K_0 may represent the dissociation constant of AcPhe from pepsin,[38] and be identical with K_i, the inhibitor constant of AcPhe for pepsin, or it may be a complex mixture of rate and equilibrium constants and bear no resemblance to K_i. If K_0 is essentially K_i, the assumption $K_0 \gg S_0$ is reasonable for the high pD data of Figures 2 and 3 and for our purposes is not significantly in error even at low pD. In the present experiments, L-[S_0] = 2.4×10^{-2} M, while K_i for L-AcPhe is [5,13,39,40] $>5 \times 10^{-2}$ M for pH $\geq$ 4, but $\sim 2 \times 10^{-2}$ M at pH 2. Hence even at pH 2 ignoring S_0 relative to K_0 results in only a 50% error in k_1, while we shall be

(38) The experimental K_0 arising in the hydrolysis of peptides by pepsin appears to be a simple equilibrium constant.[4,5,13]

(39) K. Inouye and J. S. Fruton, *Biochemistry*, 7, 1611 (1968).

(40) M. Schlamowitz, A. Shaw, and W. T. Jackson, *J. Biol. Chem.*, 243, 2821 (1968).

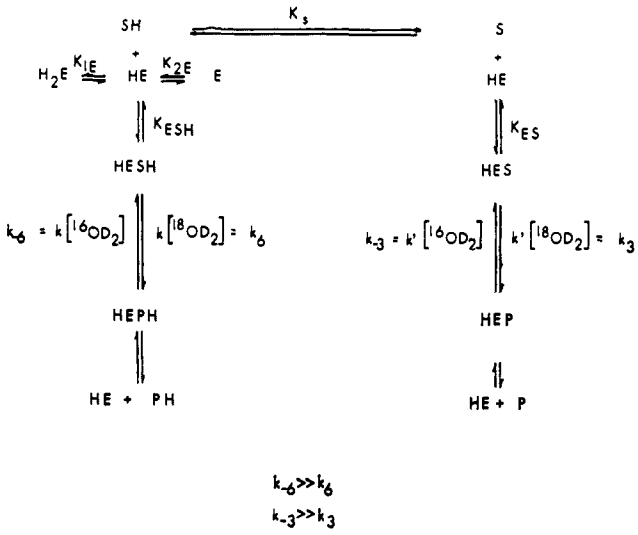


Figure 4. Kinetic scheme for analysis of the kinetics of the exchange reaction. The ionization equilibrium of the enzyme–substrate complexes are omitted for simplicity since they are not required in the data analysis.

concerned with much larger effects in distinguishing between mechanisms for the exchange reaction.

A Scheme to Explain the pH Dependence of k_1. If the exchange and peptidase reactions catalyzed by pepsin possess a common mechanistic basis, a simple modification of the kinetic scheme which quantitatively accounts[4] for pH effects in the hydrolysis of AcPheTyr, AcPheTrp, and AcPheTyrNH$_2$ should explain the pH dependence of k_1. Figure 4, an expanded version of eq 3 in which the important acid–base equilibria are explicitly recognized,[41] is identical with the scheme used in ref 4 to analyze the peptidase kinetics except that it recognizes the reversibility of the exchange reaction. The mechanism of Figure 4 does the following: (a) assigns to pepsin, both alone and complexed to substrate, two kinetically significant ionizable groups;[41] (b) assumes only the monoprotic form of pepsin, HE, is reactive; (c) takes into account the ionization of the C-terminal carboxyl group of AcPhe, as was done previously for AcPheTyr and AcPheTrp.[4]

Solution[42] of the kinetic scheme of Figure 4 produces eq 7 to express the relationship between the corrected experimental first-order rate constant, k_1, and the mechanistic parameters of Figure 4. Equation 7 es-

$$k_1 = k_0E_0/K_0 = \frac{(k_{-6}/K_{ESH} + k_{-3}K_S/[H^+]K_{ES})E_0}{(1 + K_S/[H^+])f_E} \quad (7)$$

$$f_E = (1 + [H^+]/K_{1E} + K_{2E}/[H^+])$$

tablishes that k_1 should depend only upon pH and the known[43] quantities K_{1E}, K_{2E}, and K_S: pK_{1E}, pK_{2E}, pK_s in H_2O 1.4,[4] 4.35,[4] 3.60;[3,13,31a] in D_2O 1.84,[44] 4.85,[44] 4.08.[44]

(41) The ionization constants of the enzyme–substrate complexes are omitted from Figure 4 since the ratio k_0/K_0 of concern here does not depend upon them.[4]

(42) See ref 4 for a more detailed development of an equation for k_0/K_0 which is characteristic of the peptidase reaction, which uses the same notation as Figure 4, and whose form is identical with that of eq 7.

(43) The data of ref 4 have been used. Reference 13a supports ref 4 in almost all respects and reports pK_{1E} = 1.1 and pK_{2E} = 4.7.

(44) The required pK's for D_2O were calculated from the equation pK_{D_2O} = 1.02pK_{H_2O} + 0.41 of R. P. Bell, "The Proton in Chemistry," Cornell University Press, Ithaca, N. Y., 1959, pp 188–189. The calculated ΔpK's are in accord with those experimentally measured (+0.3–0.5 pK units) for the hydrolysis of AcPheTyrME[5] and methyl phenyl sulfite (T. W. Reid and D. Fahrney, *J. Amer. Chem. Soc.*, 89, 3941 (1967)) by pepsin.

Comparison of Experimental Data and Theoretical Kinetic Scheme. Let us concentrate on interpretation of the more reliable data for pD >4.0 with the aid of eq 7. In the hydrolysis of AcPheTyr and AcPheTrp, the anionic species AcPheTyrCO$_2^-$ and AcPheTrpCO$_2^-$ were unreactive.[4] If by analogy AcPheCO$_2^-$ does not undergo exchange and only AcPheCOOH (SH) is reactive, $k_{-3} = 0$ and eq 7 reduces to eq 8. Does eq 8 fit the experimental data? Figure 2 compares the theoretical curve for eq 8 to the experimental data when theory and experiment are made to coincide at pD 5.92 by setting $k_{-6}E_0/K_{ESH} = 1.67 \times 10^{-2}$ sec^{-1}. The calculated k_1 at pD 4.32 is approximately 50 times greater than that observed, and the discrepancy between theory and experiment is still larger at lower pD.

$$k_1 = \frac{k_{-6}E_0}{K_{ESH}(1 + K_S/[H^+])f_E} \quad (8)$$

Equation 9, a second simplified expression for the pH dependence of k_1, results from allowing AcPheCO$_2^-$ (S) alone to undergo reaction with EH so that in eq 7, $k_{-6} = 0$. The solid line of Figure 3 reveals excellent agreement between experimental data and theoretical curve for eq 9 at pD > 4.0 if $k_{-3}E_0/K_{ES} = 2.0 \times 10^{-4}$ sec^{-1}. At pD <4.0, eq 9 predicts a diminution in k_1

$$k_1 = \frac{k_{-3}K_SE_0}{K_{ES}[H^+](1 + K_S/[H^+])f_E} \quad (9)$$

that is more rapid than observed (Figure 3). We attribute this discrepancy to a contribution to exchange from reaction between EH and SH, which is relatively unimportant at pD >4.0. This contribution is represented by the k_{-6} term of eq 7 or by eq 8 so that eq 7 or its equivalent, eq 8 *plus* eq 9, is necessary for treatment of the entire pD range. Quantitatively, the calculated contribution of the k_{-3} term to k_1 at pD 2.13 is negligible, so this k_1 defines $k_{-6}E_0/K_{ESH} = 8.9 \times 10^{-5}$ sec^{-1}. The experimental k_1 at pD 3.20 establishes the internal consistency of the kinetic analysis since at this pD both the k_{-3} and k_{-6} terms should contribute appreciably. Equation 7 predicts $k_1 = 9.5 \times 10^{-5}$ sec^{-1} (alternatively, 7.3×10^{-5} from eq 8 plus 2.2×10^{-5} from eq 9) which compares favorably to the observed value of 13×10^{-5} sec^{-1} (runs 25 and 26).

Figure 4 presents a kinetic scheme whose solution is eq 7 and which allows the reactive partners to be EH + S and EH + SH. Similar schemes and solutions are possible for reaction between E and SH (equivalent to EH + S) and between EH$_2$ and S (equivalent to EH + SH). The maximum rate of reaction for each pair of reactants is characterized by a ratio $k\alpha E_0/K\alpha$, defined in Table II. This table shows that the maximum rate constants for the reactions which predominate at low and high pD are essentially equal regardless of which pairs of reactants actually produce exchange.

Discussion

Postulation that the pepsin-catalyzed exchange of AcPhe with $^{18}OD_2$ involves both the reaction of S with EH (or SH + E) and SH with EH (or S + EH$_2$) results

Table II. Rate Constant Ratios for the Exchange Reaction with Alternative Reaction Partners

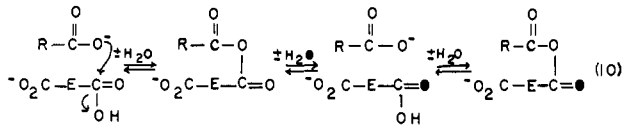
pD region	Reactive species	$(k_\alpha E_0/K_\alpha)^a$ $sec^{-1} \times 10^5$
Low	EH + SH	8.9[b]
	EH_2 + S	18
High	EH + S	20[c]
	E + SH	120

[a] Value required to fit the experimental data, where K_α is the dissociation constant for the complex between the two reactive species and k_α is $[^{18}OD_2]$ times the rate constant for formation of the enzyme–reactive species complex from the corresponding enzyme–product complex. See Scheme I and footnotes *b* and *c*. [b] Equation 8 and $k_{-6}E_0/K_{ESH}$. [c] Equation 9 and $k_{-3}E_0/K_{ES}$.

in a theoretical curve which excellently reproduces the pD dependence of the experimental first-order rate constant, k_1. At pD >4.0, reaction between S and EH dominates while at pD 2.13 it is SH + EH. A mechanism in which only SH and EH undergo exchange is clearly incompatible with the data (Figure 2). The preferred explanation is probably the simplest one available since it employs a kinetic scheme and pK_a's that were previously determined.[4]

All observations to date suggest that pepsin possesses a single active site which is responsible for the enzyme's various reactions. Assumption that S + EH and SH + EH are the true reactive entities therefore both preserves the possibility of a unique ionic species of reactive enzyme and offers the many other advantages enumerated below. Note that discarding SH + E as a reactive pair effectively removes from consideration (but see ref 26) the usual acyl-enzyme type of exchange mechanism, which in the case of pepsin would probably be represented as nucleophilic attack by an enzymatic carboxyl group upon AcPheCOOH to yield a mixed anhydride.

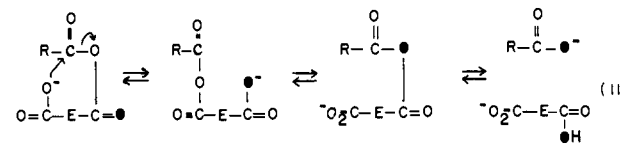
Consider first the reaction of S with EH. Equation 10 illustrates a possible interaction, where the resemblance to eq 1 is emphasized and only that bond made between enzyme and substrate is allowed to break.[45] Anhydride formation as in eq 10 should be akin to an



(10)

intramolecular reaction, and the energetics of the formation of intramolecular anhydrides are considerably more favorable than those for the intermolecular process.[46] Higuchi[47] has postulated the intermediacy of succinic anhydride in the production of derivatives of succinic acid, and Lipscomb and Vallee[48] have suggested that reactions of carboxypeptidase A may proceed *via* an anhydride between substrate and enzyme.

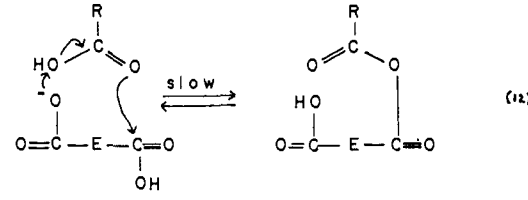
Equation 10 results in the exchange of a carboxyl group of pepsin with water. To achieve exchange of AcPhe, another reaction of the mixed anhydride must be introduced. Equation 11 describes our choice, a



(11)

nucleophilic mechanism which requires exchange of pepsin with solvent prior to the introduction of label into AcPhe.

Accommodation of the low pD data necessitates introduction of one further complexity. If we assume reaction between SH and EH and require that proton loss from SH be essential to reaction,[49] a mechanism like eq 12 results. The equivalent rate constant ratios for the (EH + S) and (EH + SH) reactions (Table II) could then fortuitously arise because the unfavorable electrostatic interactions of eq 10 are offset by the stereochemical demands for proton removal in eq 12.



(12)

A mechanism for the exchange reaction requiring a rate-controlling step 10 or 12 succeeded by a rapid step 11 possesses the following advantages: (a) a single reactive form of pepsin, EH, participates in both the exchange and peptidase reactions. (b) The requirement that exchange be necessarily linked to loss of the acidic proton of AcPheCOOH explains the esterase anomaly. (c) The spontaneous exchange[9,51] of pepsin with $^{18}OH_2$ could arise when the carboxylate anion of the enzyme functions in the role of $AcPheCO_2^-$ in eq 10. (d) Equation 10 accounts for the AcPhe-promoted exchange[9,51] of pepsin with $^{18}OH_2$. (e) If eq 11 is very rapid, the second-order rate constant for introduction of ^{18}O into (or loss of ^{18}O from) pepsin and for exchange of AcPhe with $^{18}OH_2$ should be essentially the same, as is observed.[9] The rate constant for eq 11 could easily exceed 30 sec^{-1} if analogy to formation of maleic anhydride from *p*-methoxyphenyl hydrogen maleate is appropriate. The maleate system has $k_1 = 3.3 \times 10^{-2}$ sec^{-1} (H_2O, 25°) and replacement of *p*-methoxyphenolate with the carboxylate leaving group characteristic of the enzyme system should result in a rate enhancement of 10^3–10^4. The estimate for a leaving group effect relies on the fact that nucleophilic displacements by acetate ion with carboxylate leaving groups are at least 100 times more rapid than with *p*-nitrophenolate,[52]

(45) In equations 10, 11, 13, and 15, ● symbolizes ^{18}O and the attainment of equivalence of the oxygen atoms of carboxyl groups is taken as rapid.

(46) W. P. Jencks, F. Barley, R. Barnett, and M. Gilchrist, *J. Amer. Chem. Soc.*, 4464 (1966).

(47) T. Higuchi, J. D. McRae, and A. C. Shah, *ibid.*, 88, 4015 (1966), and earlier papers.

(48) W. N. Lipscomb, *et al.*, *Brookhaven Symp. Biol.*, **21**, 24 (1969); (b) B. L. Vallee and J. F. Riordan, *ibid.*, **21**, 91 (1969).

(49) The reported rates of exchange of DL-AcPhe with $^{18}OH_2$ appear considerably faster than ours at both low (pH 2.4, 10% ethanol, 1 *M* acetic acid)[36] and high pH (4.7).[36,50] A detailed comparison of the pH and pD profiles for the entire pH range by a single laboratory is clearly required.

(50) N. I. Mal'tsev, L. M. Ginodman, and V. N. Orekhovich, *Dokl. Akad. Nauk SSSR*, **165**, 1192 (1965).

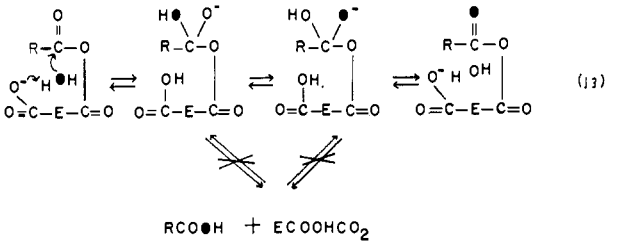
(51) We believe the balance of the present experimental evidence favors the view that one carboxyl group of pepsin undergoes exchange. The proposed mechanism can be reluctantly modified to explain the exchange of two carboxyls, if necessary.[9,10]

(52) Acetate ion is 100 times more effective in the nucleophilic catalysis of the hydrolysis of acetic propionic anhydride than in the general-base catalyzed hydrolysis of *p*-nitrophenyl acetate, which sets a lower limit to the difference between the two substrates in the nucleo-

and in the maleate model system *p*-bromophenolate is 20 times more reactive than *p*-methoxy and *p*-nitro should be even more reactive. (f) Although the hydrolysis of sulfite esters requires a nucleophilic carboxyl group on the enzyme, an electrophilic carboxyl also appears to be present.[8]

The following two serious objections to the mechanism can be partially countered. (g) Efforts to trap an anhydride between pepsin and AcPhe with radioactive methanol[13] or hydroxylamine[31] have failed. The methanol experiment is the reverse of the esterase studies and the negative result is to be expected. Equations 10 and 12 require that methanol esterify only the carboxyl group of pepsin. This ester should rapidly equilibrate with free enzyme and solvent,[54] and the existence of this equilibrium could explain the competitive inhibition of pepsin by aliphatic alcohols.[55] (h) Equation 10 makes no obvious use of the carboxylate anion of EH, although eq 12 employs that carboxylate or another in the crucial proton removal. The anion of course could be essential to maintaining the proper reactive conformation of the active site.

Equation 13 offers an alternative to eq 11, a general base mechanism which when paired with eq 10 has most of the virtues and deficiencies of the one just discussed. Mechanism 13 requires only the initial anhydride formation step of eq 10 to achieve exchange of AcPhe



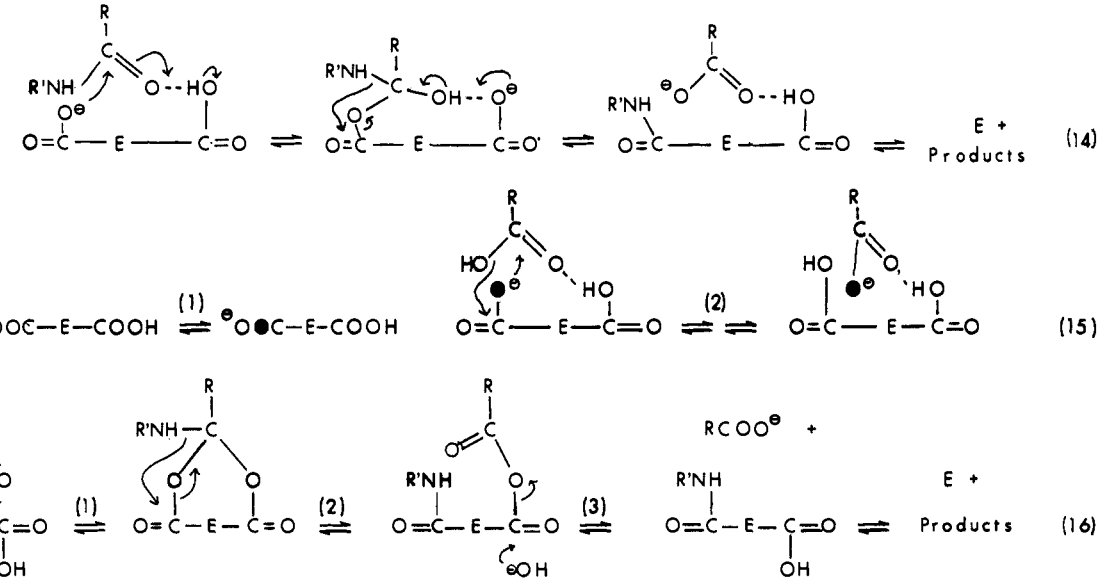
with $H_2{}^{18}O$, not prior exchange of pepsin with solvent. Decomposition of the tetrahedral intermediate of eq 13 to AcPheCOOH + EH must be forbidden, because if permitted, then the reverse reaction, which represents the acyl-enzyme mechanism (and *not* eq 12) with its attendant esterase anomaly, must also be permitted. The arbitrariness of this interdiction causes us to prefer mechanism 11.

Conclusions

The nature of the exchange reaction of pepsin, like everything else involving this enzyme, is unusual. We believe eq 10–11 offer the mechanism for the exchange reaction in best accord with the available experimental data. Our proposal requires a direct transfer of oxygen atoms from substrate to enzyme and demonstration of this would constitute the most direct evidence for a scheme like ours and against the acylenzyme and related hypotheses.

Most desirable is a general mechanism to explain the peptidase, transpeptidation, and exchange reactions of pepsin, all of which appear to be interrelated.[13] For example, our requirement that RCO_2^- or its acid precursor be the sole participant in the exchange reaction offers a simple rationale for the failure[13a] of a carboxylic or thiol ester to function as a transpeptidation acceptor. The three mechanistic proposals discussed below are the best ones currently available, but none is completely satisfactory.

Mechanisms based on a literal interpretation of eq 1 are not readily reconciled to eq 10 for the exchange reaction, since the roles of the amide nitrogen in eq 1 and the oxygen nucleophile in eq 10 appear identical. If eq 1 correctly represents the order of release of products in hydrolysis,[13] the site of attack of the carboxylate anion in the exchange reaction is unavailable to the carboxyl group of a transpeptidation



acceptor since it is already occupied by the amino residue.

Knowles[13] has formulated an attractive mechanism for hydrolysis and transpeptidation (eq 14) and exchange (eq 15). Equation 16 coupled with eq 10 and 11 offers an alternative in which the electrophilic carboxyl group of the enzyme enters into covalent bonding with the substrate rather than acting as a

philic reaction. See the discussion and references of ref 53 about models used here.

(53) T. C. Bruice and S. Benkovic, "Biorganic Mechanisms," Vol. I, W. A. Benjamin, Inc., New York, N. Y., 1966, pp 107, 108, 177, 178.

(54) By analogy to methyl hydrogen phthalate and related model systems discussed in ref 53, pp 173–186.

(55) J. Tang, *J. Biol. Chem.*, 240, 3810 (1965).

proton donor. The merits of eq 10 and 11 have been enumerated; let us evaluate eq 14 and 15 *vs.* eq 10 and 16.

Two unsatisfactory characteristics of both mechanisms are: (a) reaction proceeds *via* a four-membered ring; (b) neither mechanism is reconcilable to oxygen tracer experiments on transpeptidation[36,50] unless some further modification is introduced. Equation 16 probably requires more profound adjustment.

Two aspects of eq 10 and 16 which are less favorable than corresponding parts of eq 14 and 15 are: (c) departure or entrance of OH^- in eq 10, 12, and 16 (steps 1 and 3) would benefit from participation by a third, unspecified functional group of the enzyme. This group, if capable of undergoing dissociation, either must have a pK_a outside the pH regions investigated in kinetic studies (here and in ref 4, 13, etc.) or its state of ionization does not affect the rate-determining step of those kinetic studies. (d) The driving force for the reverse of step 2 of eq 16 is not apparent.

There are three features of eq 14 and 15 that are particularly unsatisfactory: (e) the Knowles' mechanism invokes enzyme specificity to explain the esterase anomaly yet this specificity must be low enough to permit both (EH_2 + S) and (EH + SH) ambiguously to enter into exchange reaction 15, which corresponds to the low pD case. (f) For the exchange at high pD (EH + S or E + SH), another proton must be removed from eq 15. This reaction could represent nucleophilic attack on the carboxylate anion of the substrate, as in the system of Hegarty and Bruice,[26] but we would expect its rate constant to be considerably smaller than that for eq 15, and experimentally it is not (Table II). (g) How mechanism 15 explains[13] the near equality of the second-order rate constants for loss of label from pepsin and for incorporation of label into AcPhe is unclear. This equality is calculated on the assumption that the rate of exchange of pepsin with $^{18}OH_2$ depends upon [AcPhe], for which eq 15 makes no provision. If in eq 15 step 1 is fast and 2 slow, the rate of loss of label from enzyme is governed by the fast step and exceeds the rate of formation of labeled AcPhe. If step 1 of eq 15 is slow and 2 fast, the first step controls the rate of introduction of label into both enzyme and substrate, since the exchangeable oxygen atoms of the two undergo rapid equilibration. Experimentally, the half-life for exchange of AcPhe far exceeds that for pepsin exchange under comparable experimental conditions.[9]

Equation 14 provides a simpler explanation for the peptidase and transpeptidation reactions than does 16. When the exchange reaction is required to be explicable by the same mechanism, we believe the balance lies in favor of eq 10 and 16. If there is some merit in that mechanism, then the objections to it suggest that at least one other functional group may be present at the active site of pepsin, the properties of which are indicated in c above.

Experimental Section

General. Pepsin was Worthington lot PM709 or prepared[35] (pepsin-P) from Worthington lot PG 6LC pepsinogen. Either synthetic[56] or commercial (Mann Research Laboratories) DL-AcPhe, recrystallized from acetone, mp 150–153° (lit.[56] mp 150–151°), was used. AcPheTyr, AcPheTrp, and phenyl tetrahydrofurfuryl sulfite have been described.[4,57] Inorganic compounds and organic solvents were reagent grade, and distilled water was redistilled before use. Several lots of $^{18}OD_2$ (Biorad, containing 1.5 atom-% ^{18}O) were employed, and nmr analysis indicated the aqueous buffers contained >98% deuterium solvent protons. The runs in ethanol–water utilized ethanol–OH and contained less deuterium. The pH meter readings (Radiometer TTTl) of aqueous buffers containing 10 mg/ml of DL-AcPhe were determined; addition[58] of 0.4 to the meter reading defined pD's for solutions in $^{18}OD_2$. Because of the method by which the solvent correction was calculated for the runs in ethanol–water (see below) the pD's listed for these runs in Table I refer to the pD of the aqueous component of the buffer. Meter readings of the ethanolic buffers exceeded those of the aqueous components by ~0.2 unit. All reactions were carried out at 35.4 ± 0.2°.

Kinetics of Exchange Reactions.[59] DL-AcPhe (0.1171 g) was dissolved in 12.0 ml of pD 5.42 citrate buffer at room temperature, and 0.1211 g of pepsin added. Gentle swirling caused the enzyme to dissolve, and the bottle containing the resultant solution was stoppered and placed in the constant temperature bath. The solution (6 ml) was removed 2 hr after the addition of enzyme, the pH of the sample was adjusted to 1–2 with concentrated hydrochloric acid, and three extractions with 15-ml portions of ether performed. The combined ether extracts were dried over calcium chloride, filtered into a small flask, and concentrated by passing a stream of dry nitrogen over the surface of the ether. When no liquid remained, the flask was placed in a desiccator which was evacuated to 1-mm pressure overnight. The flask was removed and 20 ml of toluene added to it. The toluene was briefly heated to boiling on a hot plate, filtered, and cooled in the freezer. The resultant precipitate of DL-AcPhe was collected by filtration, dried at reduced pressure (mp 152–154.5°), and analyzed for ^{18}O content. Control runs omitted either enzyme or DL-AcPhe.

In a typical run at pD <4.0, to 0.1338 g of DL-AcPhe dissolved in 1.5 ml of absolute ethanol was added 12.0 ml of pD 2.3 phosphate. The resultant homogeneous solution was swirled briefly, and 0.1414 g of pepsin added to provide the final reaction mixture which was treated as described above. Several hours often elapsed between addition of ethanol and buffer, but addition of enzyme always rapidly followed that of buffer.

Hydrolysis Kinetics. These were run to determine (a) the effect of ethanol present in runs at pD <4.0; (b) the inhibition caused by acetate as compared to citrate; (c) the activity of pepsin *vs.* pepsin-P. They were used to give the corrected (parenthesized) values of Table I.

The initial rate[4] of peptic hydrolysis of AcPheTrp in a pH 1.9 phosphate buffer exceeded that in a solution which was 8:1, phosphate buffer–ethanol, by a factor of 7.4. A similar experiment with phenyl tetrahydrofurfuryl sulfite[57] in pH 2.35 glycine perchlorate buffer gave a factor of 5.5. Multiplication of the exchange rates observed in ethanol–water by 6.5 gave the corrected values.

At pH 4.90, [DL-AcPhe] = 10 mg/ml, the rate of hydrolysis of AcPheTyr (ninhydrin method) in 0.5 *M* citrate was 1.25 times faster than in 0.5 *M* acetate. Division of the experimental k_1 for exchange in pD 5.42C by 1.25 gave the corrected value. Similar experiments with AcPheTrp at pH 3.9 showed that the rate of hydrolysis in 0.2 *M* tartrate or 0.1 *M* acetate surpassed that in 0.5 *M* acetate by a factor of 1.5, so the experimental k_1 for exchange in pD 4.42C was corrected by division by 1.5.

Pepsin-P was only 0.7 times as effective as pepsin in catalyzing the sulfite ester hydrolysis described above, but appeared somewhat more effective in the exchange reaction (Table I). Since the pepsin-P available for the sulfite assay was scarce and scraped from the walls of the container, the difference in specificity between the two pepsins in the two reactions may not be significant.

Method of Oxygen-18 Analysis. A variation of the guanidine hydrochloride (GCl) method for the analysis of phosphate converted the oxygen atoms of DL-AcPhe to carbon dioxide.[60] Mann Research Ultrapure GCl, lot T-3548, was oven dried at 110° for 12 hr prior to use. A mixture of 5 mg of AcPhe and a 2–3-fold excess of GCl (by weight) in a breakseal tube was dried at room temperature under vacuum (50 μ) over phosphorus pentoxide overnight,

(56) H. B. Gillespie and H. R. Snyder, "Organic Syntheses," Coll. Vol. II, John Wiley & Sons, Inc., New York, N. Y., 1943 p 489.

(57) T. W. Reid, T. P. Stein, and D. Fahrney, *J. Amer. Chem. Soc.*, 89, 7125 (1967).

(58) P. K. Glascoe and F. A. Long, *J. Phys. Chem.*, 64, 188 (1960).

(59) Typical procedures are given.

(60) P. D. Boyer, D. J. Graves, C. H. Suelter, and M. E. Dempsey, *Anal. Chem.*, 33, 1906 (1961).

and the tube sealed under vacuum and then placed in a 290° oven for 12 hr. The cooled tube was opened in an evacuated vacuum system and its contents frozen with liquid nitrogen. After noncondensable gases had been pumped off, a Dry Ice–acetone bath replaced the liquid nitrogen and the liberated CO_2 was permitted to contact 0.75 ml of previously degassed sulfuric acid. A liquid nitrogen bath caused condensation of the CO_2 in a gas-sample tube which was transferred to a modified CEC-21-401 mass spectrometer that measures the mass ratio 46:44. One analysis required 1 μmol of CO_2 and 5 mg of AcPhe generally provided more than 20 μmol, but the yield of CO_2 was somewhat variable and not quantitative.[61]

The following observations established the validity of the analytical method. (a) Although the purity of the CO_2 is not critical, impurities of mass 44 and 46 must be absent. Two likely contaminants are nitrous oxide (mass 44) and ethanol (mass 46). Exposure of the CO_2 to P_2O_5, which is highly effective in removing the last traces of ethanol, resulted in no significant change in the 46:44 ratio. (b) Several analyses of natural abundance DL-AcPhe gave 0.202 ± 0.001 for the atom-% ^{18}O content. (c) Extensive scrambling of all the oxygens of DL-AcPhe occurred in the analysis,[32] so the percentage exchange was based on analyses of samples which had achieved complete equilibration with $^{18}OD_2$. The criterion for complete exchange was identical ^{18}O content of recovered DL-AcPhe for runs incubated for 48 and 72 hr in pD 4.82A (nonenzymatic exchange is negligible). Total exchange was determined for each batch of $^{18}OD_2$ employed. The internal consistency and reproducibility of the data therefore further verifies the experimental procedures. For example, runs 1–4 and 7–10 of Table I used one sample of $^{18}OD_2$, and runs 6 and 12, another, while run 9 was performed months after run 8, and by a different worker. (d) As a final check of our analytical procedures, a sample of DL-AcPhe was incubated in pD 3.20P for 5 days, during which time it was calculated from runs 25–27 that complete enzymatic exchange of the L-isomer plus 20% spontaneous exchange of the D-isomer should have taken place. The observed atom-% ^{18}O was 97% of that predicted.

Miscellaneous Details of the Analysis of pH Dependence. A fit of the data for pD >4.0 was first made, as discussed in the Results. Only $k_{-3}E_0/K_{ES}$ was varied in this treatment, in order to emphasize that the pK's determined from peptidase reactions[4] could explain the exchange experiments. Equation 8, with $k_{-6}E_0/K_{ESH} = 8.9 \times 10^{-5}$ sec^{-1}, will make a slight contribution to the total expected k_1 at pD 4.0. The dotted curve of Figure 3 represents the total calculated k_1 when allowance for this contribution is made.

Because phosphate, like citrate but unlike acetate, dues not appreciably inhibit the peptidase action of pepsin,[4,31a] a buffer correction term is actually needed to make the eq 8 term (phosphate) entirely consistent with the data for pD >4.0 (acetate chosen as standard). Since utilization of this minor correction affects none of our arguments significantly and is difficult to justify, given the gross solvent corrections already applied to the ethanol–water runs, it has been ignored. If applied, the correction would improve the agreement between expected and observed k_1's for pD 3.20 and diminish the difference between solid and dotted theoretical curves in Figure 3.

The rate constant for nonenzymatic (spontaneous) exchange (k_s) was significant only at pD 2.13. The desired k_1 for this pD was, obtained as follows. The k_s was calculated from runs 30 and 31 and an assumed infinity point where both D and L isomers had undergone complete exchange. Subtraction of the contribution of the spontaneous reaction of D-AcPhe to the excess ^{18}O-content of runs 28 and 29 provided corrected ^{18}O values. The first-order plot of these corrected values gave a first-order rate constant which was the sum of the unknown k_1 plus the known k_s.

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(61) The procedure described gave the best yields.