Kinetic Studies on the Mechanism of Pepsin Action†

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ABSTRACT: The linear noncompetitive inhibition of the pepsin-catalyzed hydrolysis of Ac-Phe-Phe-Gly at pH 2.1 by L-Ac-Phe, L-Ac-Phe-NH_2, and L-Ac-Phe-OEt has been claimed to substantiate the ordered release of products specified by the amino-enzyme mechanism for pepsin action. According to this interpretation, the binding of inhibitor to free enzyme and the *amino-enzyme intermediate* (Scheme I) generates the observed inhibition pattern. The proposition is valid only if a simple alternative explanation for the kinetic data, Scheme II, can be disproved. Scheme II attributes the inhibition pattern to the binding of inhibitor by free enzyme and the *enzyme–substrate (Michaelis) complex*. The experiments reported here have enabled us to distinguish between the two mechanisms. The pepsin-catalyzed hydrolyses of Ac-Phe-Trp, Z-His-Phe-Trp, Z-Gly-His-Phe-Trp, and Z-Ala-His-Phe-Trp at pH 1.8 occur exclusively at the Phe–Trp bond and must yield the same amino-enzyme, E-Trp, if it is implicated. Under these circumstances, Scheme I requires that a plot of $1/k_c$ *vs.* $[I]_0$ for the four substrates and a given noncompetitive inhibitor provide a set of four parallel lines. Scheme II predicts that the four lines generally will not be parallel. L-Ac-Phe, L-Ac-Phe-NH_2, L-Ac-Phe-OMe, and D-Ac-Phe act as linear noncompetitive inhibitors for the pepsin-catalyzed hydrolysis of the four Trp-containing substrates. The plot of $1/k_c$ *vs.* $[I]_0$ for each inhibitor results in a set of four nonparallel lines. Therefore Scheme II must be correct and the detection of noncompetitive inhibition accompanying the pepsin-catalyzed hydrolysis of peptides offers no insight into the merits of the amino-enzyme hypothesis.

Speculations on the mechanism of pepsin-catalyzed reactions abound in the literature but experiments which convincingly illuminate the mechanism of pepsin action are scarce (for extensive reviews, see Fruton, 1970, 1971; Clement, 1973). For many years the amino-enzyme hypothesis has stood as the most firmly established component of these mechanistic speculations, since it draws support from three independent experimental approaches. Equation 1 captures the essence of this hypothesis and also incorporates the inference, supported by a variety of data, that the rate-limiting step for the peptic hydrolysis of most synthetic substrates immediately succeeds formation of the Michaelis complex (*i.e.*, k_2 is slow).

$$\mathrm{E} + \mathrm{XCONHY} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E,XCONHY} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{(slow)}}{} \mathrm{XCOOH} + \mathrm{E\text{-}NHY} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{E} + \overset{+}{\mathrm{N}}\mathrm{H_3Y} \quad (1)$$

The three categories of experiment which seemingly confirm the intervention of the amino-enzyme, E-NHY, are as follows: (1) transpeptidation reactions, in which a suitable acceptor intercepts the fragment -NHY before it is transferred to water; (2) kinetic inhibition studies (Kitson and Knowles, 1971) and isotopic exchange experiments at stoichiometric equilibrium (Boyer, 1959: Ginodman and Lutsenko, 1972) which are interpreted as verifying the ordered release of products specified by eq 1; (3) isolation of pepsin to which L-tyrosine ethyl ester is attached (Ginodman *et al.*, 1971). The observation that peptic hydrolysis of Ac-Phe-Tyr-NH_2 or Ac-Phe-Phe-OEt[1] does not yield an amino-enzyme which radioactive Ac-Phe can trap, under circumstances where Ac-Phe-Tyr or Ac-Phe-Phe does, has raised serious doubts about the legitimacy of an amino-enzyme hypothesis based on a transpeptidation reaction of such limited generality (Silver and Stoddard, 1972). Hofmann's recent discovery of pepsin-catalyzed transpeptidation reactions which are best explained by postulating the intervention of an acyl-enzyme intermediate has confused the mechanistic situation still more (Takahashi *et al.*, 1974; Wang *et al.*, 1974).

Given these facts, we thought it necessary to determine whether the other evidence for the amino-enzyme hypothesis could withstand close scrutiny. We decided to begin by either verifying or disproving the contention that kinetic inhibition studies corroborate the ordered release of products which eq 1 specifies. Both the experimental and theoretical basis for this proposition resides primarily in two articles by Kitson and Knowles (1971). These authors measured the effect of both enantiomers of Ac-Phe, Ac-Phe-OEt, and Ac-Phe-NH_2 upon the rate of peptic hydrolysis of Ac-Phe-Phe-Gly at pH 2.1 and 4.3. The three L isomers exhibited linear noncompetitive inhibition[2] at pH 2.1 and Kitson and Knowles proposed that this arose because the *amino-enzyme* binds X-COOH or its analogs if they possess the L configuration. Scheme I defines the scheme Kitson and Knowles used in successfully explaining the behavior of the L-inhibitors. Equation 2 diagrams the relevant interactions, eq 3 gives the steady-state expression for the rate of disappearance of substrate in the Lineweaver-Burk format of eq 5 ($K_s = (k_{-1} + k_2)/k_1$) and eq 4 specifies the dependence of

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[1] Abbreviations are listed in *Biochemistry 5*, 2485 (1966) and all amino acids possess the L configuration unless otherwise specified. A functional group may be appended to an abbreviation to clarify a point (*e.g.*, Ac-Phe-COOH) and a slash through a bond introduced to designate the site of peptic cleavage (*e.g.*, Ac-Phe-/-Trp).

[2] The terminology for inhibition is that of Cleland (Cleland, 1963; Hsu *et al.*, 1966). K_4, K_4', K_5, and K_5' define inhibitor dissociation constants.

SCHEME I

$$\begin{array}{ccccc} E + XCONHY & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & E,XCONHY & \underset{k_{-2}\ (slow)}{\overset{k_2}{\rightleftharpoons}} & XCOOH \\ \Big\Downarrow K_5 & & & & + \\ E,I & & & & E\text{-}NHY \end{array} \quad \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \quad E + \dot{N}H_3Y \qquad (2)$$

$$\Big\Downarrow K_4'$$

E-NHY, I

$$1/v = \frac{1}{[E]_0}\left\{\frac{k_3 + k_2(1 + [I]_0/K_4')}{k_2k_3}\right\} + \frac{1}{[E]_0[S]}\left\{\frac{K_s(1 + [I]_0/K_5)}{k_2}\right\} \qquad (3)$$

$$1/k_c = 1/k_2 + 1/k_3 + (1/k_3)([I]_0/K_4') \qquad (4)$$

k_c upon $[I]_0$. Scheme I satisfactorily explains all the pH 2.1 data only if the assumption that XCOOH of eq 1 or 2 is liberated as the anion, $XCOO^-$ (*i.e.*, for Ac-Phe-/-Phe-Gly, $XCOO^-$ = Ac-$PheCOO^-$), is also introduced.

$$1/v = (1/[E]_0k_c) + (1/[E]_0[S])(K_m/k_c) \qquad (5)$$

Scheme II, represented by eq 6-8, attributes to L inhibitors the ability to bind to the *enzyme-substrate* complex (K_4). This mechanism also generates noncompetitive inhibition and can be shown to provide a satisfactory rationale for all of Kitson and Knowles' data. Schlamowitz *et al.* (1968) employed Scheme II in analyzing the effect of a variety of inhibitors upon the rate of peptic hydrolysis of Ac-Phe-Tyr at pH 2.

The crucial difference between Scheme I and Scheme II is that the former posits that kinetic-inhibition studies confirm the ordered release of products demanded by the amino-enzyme hypothesis while the latter claims that these studies merely confirm the existence of a Michaelis complex prior to a rate-limiting step.

Two considerations indicate that Scheme II is preferable. First of all, the observation that compounds such as 2,6-dimethoxyphenol function as noncompetitive inhibitors[2] (Schlamowitz *et al.*, 1968) suggests that the observation of noncompetitive inhibition may not be of the profound mechanistic significance which Scheme I implies since this type of inhibition is not exclusively associated with inhibitors closely resembling XCOOH. Secondly, the very fact that $1/k_c$ depends upon $[I]_0$ is not easily reconciled with eq 4 if k_2 is truly rate limiting for peptic hydrolyses; no such problem arises from eq 8. For example, in the Ac-Phe-Phe-Gly experiments, $[I]_0/K_5 \lesssim 1$ (Kitson and Knowles, 1971). Equation 8 predicts a measurable effect of $[I]_0$ upon $1/k_c$ under the readily imagined circumstances that $K_4 \simeq K_5$. Evaluation of the ability of eq 4 to explain noncompetitive inhibition when $[I]_0/K_5 \lesssim 1$ requires an estimate of the relative sizes of k_2 and k_3. For Ac-Phe-Phe-Gly it is likely that k_3 is at least 100 times greater than k_2 ($1/k_2 \geq 10^2(1/k_3)$) since Fruton's data (Fruton, 1970,1971) suggest that k_c for a substrate like Z-Gly-Ala-Phe-Phe-Gly should exceed k_c for Ac-Phe-Phe-Gly by >100 times and the larger k_c sets a *lower* limit to k_3 for the amino-enzyme E-(Phe-Gly). Given these conditions, eq 4 yields noncompetitive inhibition only under the improbable (but not impossible) circumstance that K_4' is at least 100 times smaller than K_5.

A study of the kinetics of the inhibited peptic hydrolysis of the substrates V-Phe-/-Trp, V = Ac-, Z-His, Z-Gly-His- and Z-Ala-His-, should rigorously discriminate between Scheme I and Scheme II. Since each peptide should undergo hydrolysis exclusively at the Phe–Trp bond, each will yield the common amino-enzyme E-Trp according to the amino-enzyme hypothesis. For hydrolysis in the presence of a given inhibitor the four substrates must show identical values for k_3 and K_4' and, in general, different values for k_2 and K_4. Equation 4 predicts that a plot of $1/k_c$ *vs.* $[I]_0$ for the four substrates interacting with a common inhibitor will result in a set of four parallel lines, while eq 8 suggests that the lines will not be parallel.

This paper describes the preparation of the necessary substrates and the kinetics of their peptic hydrolysis at pH 1.8 in the presence of L-Ac-Phe, D-Ac-Phe, L-Ac-Phe-NH_2, and L-Ac-Phe-OMe.

Experimental Section

Thin-Layer Chromatography (tlc). Tlc analysis on glass plates coated with silica gel G enabled us to monitor the course of synthetic reactions, confirm the purity of substrates, and establish the nature of the reaction products from pepsin-catalyzed hydrolyses. The solvent systems employed were: (A) 1-butanol–acetic acid–water (8:1:1, *v*/*v*); and (B) 1-propanol–ammonia (7:3, *v*/*v*). For visualization of spots we used reagents described by Stahl (1962): Pauly (#37) for His- and Trp-containing samples; Folin-Ciocalteau (#122) for Trp-containing materials; ninhydrin (#108) for free amino acids.

Synthesis of Substrates. A sample of Ac-Phe-Trp was available from earlier experiments (Denburg *et al.*, 1968). Methods reported by J. S. Fruton and coworkers enabled us to synthesize the ethyl esters of the remaining three substrates. Hydrolysis with α-chymotrypsin then removed the unwanted ester function.

Inouye *et al.* have described Z-His-Phe-Trp-OEt. Our preparation showed mp 184–186°, $[\alpha]^{25}D$ −22.6° (*c* 1, methanol) (lit. mp 189–190°, $[\alpha]^{24}D$ −24.4°). For the enzymatic hydrolysis, we placed a solution consisting of 150 ml of dilute pH 7 phosphate buffer, 40 ml of methanol, and

SCHEME II

$$\begin{array}{ccccc} E + XCONHY & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & E,XCONHY & \underset{k_{-2}\ (slow)}{\overset{k_2}{\rightleftharpoons}} & E + XCOOH + \dot{N}H_3Y \\ \Big\Downarrow K_5 & & \Big\Downarrow K_4 & & \\ E,I & & E,XCONHY,I & & \end{array} \qquad (6)$$

$$1/v = \frac{1}{[E]_0}\left\{\frac{(1 + [I]_0/K_4)}{k_2}\right\} + \frac{1}{[E]_0[S]}\left\{\frac{K_s(1 + [I]_0/K_5)}{k_2}\right\} \qquad (7)$$

$$1/k_c = 1/k_2 + (1/k_2)([I]_0/K_4) \qquad (8)$$

21 mg of α-chymotrypsin in a 400-ml beaker. Addition of a few drops of 0.20 M NaOH raised the pH of the solution to 8.2. Addition of 1-ml aliquots of a solution of 580 mg of Z-His-Phe-Trp-OEt in 15 ml of warm methanol was completed in ~45 min; simultaneous addition of NaOH maintained the pH at 8.2; 15 min later, 98% of the expected amount of NaOH had been consumed. Vigorous heating of the solution on the steam bath caused much of the methanol to evaporate. A small portion of Darco was added to the cooled solution and the Darco removed by filtration. Adjustment of the pH of the filtrate to 6 caused light gray Z-His-Phe-Trp to precipitate. The Z-His-Phe-Trp was collected and dissolved in 20 ml of hot methanol. The methanol solution was filtered and the clear filtrate dripped into 40 ml of water at room temperature. The resultant white precipitate of Z-His-Phe-Trp weighed 304 mg after drying at 0.1 mm, 50°. Concentration of the pH 6 filtrate eventually yielded an additional 93 mg of impure Z-His-Phe-Trp. Pure Z-His-Phe-Trp had an unreproducible melting point and $[\alpha]^{25}D$ −12.4°, $[\alpha]^{25}_{365}$ −28.6° (*c* 0.5, methanol). On tlc, it showed a single spot in solvents A (R_F 0.56) and B (R_F 0.75) with Folin reagent and gave no spot with ninhydrin. Z-His-Phe-Trp, Z-Gly-His-Phe-Trp, and Z-Ala-His-Phe-Trp apparently hold solvent tightly and we had some difficulty in obtaining meaningful elemental analyses. Z-His-Phe-Trp lost 2.77% of its weight on drying at 100° prior to analysis. *Anal.* Calcd for $C_{34}H_{34}N_6O_6$: C, 65.61; H, 5.46; N, 13.51; O, 15.42. Found: C, 64.85; H, 5.52; N, 13.37; O, 16.22.

To prepare Z-Gly-His-Phe-Trp-OEt, a solution of Z-Gly-His-N_3, derived from 0.72 g (2 mmol) of the corresponding hydrazide, in 10 ml of ethyl acetate was added to 0.58 g (2 mmol) of oily Phe-Trp-OEt (Inouye *et al.*, 1966) and the resultant solution kept at 4° for 5 days. The resultant gelatinous precipitate was collected, washed with cold ethyl acetate, and dried, weight 0.78 g. Recrystallization of the crude ester from ethanol–water gave 610 mg of material with an ill-defined melting point $[\alpha]^{25}D$ −14.8°, $[\alpha]^{25}_{365}$ −45.5° (*c* 0.5, methanol). On tlc, Z-Gly-His-Phe-Trp-OEt had R_F 0.43 in solvent A and 0.83 in solvent B (Folin), and showed a trace (≤2.5%) of Phe-Trp-OEt (R_F 0.55, solvent A, ninhydrin).

Chymotryptic hydrolysis of 390 mg of Z-Gly-His-Phe-Trp-OEt, in the manner described for Z-His-Phe-Trp-OEt, consumed 92% of the expected amount of NaOH and gave 268 mg of crude Z-Gly-His-Phe-Trp from the precipitation at pH 6. We were unable to satisfactorily recrystallize this material and lost much of the substrate in our attempts. We finally settled for the addition of a methanolic solution of Z-Gly-His-Phe-Trp to water to produce a gummy oil which settled on the walls of the flask. Decantation of the supernatant liquid and drying of the residual oil at 0.1 mm for several days yielded a white brittle material of high purity that could be scraped from the container. The synthesis described and another at one-half the scale yielded a total of 275 mg of Z-Gly-His-Phe-Trp, $[\alpha]^{25}D$ −7.3°, $[\alpha]^{25}_{365}$ −16.9° (*c* 0.5, methanol). On tlc, Z-Gly-His-Phe-Trp showed a single spot in solvent A (R_F 0.31) and B (R_F 0.74) with either Pauly or Folin spray and gave no spot with ninhydrin. A sample lost 3.72% of its weight on drying at 100° prior to analysis. *Anal.* Calcd for $C_{36}H_{37}N_7O_7$: C, 63.61; H, 5.49; N, 14.43; O, 16.47. Found: C, 63.24; H, 5.60; N, 14.37; O, 16.64.

Reaction of Z-Ala-N_3 with His-Phe-Trp-OEt provided the desired Z-Ala-His-Phe-Trp-OEt. The sequence Ala-OEt · HCl → Z-Ala-OEt → Z-Ala-$NHNH_2$ gave hydrazide with mp 132–133°, $[\alpha]^{25}D$ −27.2° (*c* 1, 0.5 M HCl) (lit. mp 138° $[\alpha]^{25}D$ −28.6° (Erlanger and Brand, 1951)). His-Phe-Trp-OEt was obtained by bubbling hydrogen through a suspension of Palladium Black in a solution containing 3.35 g (5.2 mmol) of Z-His-Phe-Trp-OEt, 150 ml of absolute ethanol, and 1.6 g of concentrated HCl. After 3 hr, the catalyst was removed by filtration and 400 mg of $NaHCO_3$ added to the filtrate, which was then concentrated *in vacuo*. The yellow residue so obtained was dissolved in 15 ml of water and a gel formed. The gelatinous suspension was cooled to 0° and its pH adjusted to 8 by the addition of 50% K_2CO_3 solution; the suspension was extracted with 8–10 10-ml portions of cold ethyl acetate while the occasional addition of K_2CO_3 maintained the aqueous phase at pH 8. The pooled ethyl acetate extracts, when dried over Na_2SO_4 and evaporated to dryness *in vacuo*, yielded 1.85 g of yellow semicrystalline His-Phe-Trp-OEt which contained a trace of Z-His-Phe-Trp-OEt. Dissolution of the crude product in a minimum amount of ethyl acetate was followed by addition of enough hexane to produce a few drops of a dark oil. The supernatant, decanted from the oil and evaporated to dryness *in vacuo*, gave a yellow residue of His-Phe-Trp-OEt which showed no detectable impurities on tlc. His-Phe-Trp-OEt has R_F 0.20 in solvent A (Folin, ninhydrin) and 0.68 in solvent B (Folin, ninhydrin).

An ethyl acetate solution (6 ml) containing 0.8 g (1.5 mmol) of pure His-Phe-Trp-OEt was added to the oily Z-Ala-N_3 derived from 0.66 g (2.5 mmol) of Z-Ala-$NHNH_2$ (Erlanger and Brand, 1951). The reaction mixture was kept at 4° for 68 hr and the resultant gelatinous precipitate collected and dried, weight 0.89 g. The crude Z-Ala-His-Phe-Trp-OEt was dissolved in DMF and reprecipitated by the addition of water, yield 0.62 g, one spot on tlc in solvent A (R_F 0.50) with Folin, and no spots with ninhydrin reagent. Chymotryptic hydrolysis of 520 mg of Z-Ala-His-Phe-Trp-OEt (dissolved in DMF prior to addition to the enzymatic solution) resulted in the consumption of 96% of the expected amount of NaOH and in the isolation of 266 mg of crude Z-Ala-His-Phe-Trp from the pH 6 precipitation. The peptide was dissolved in 25 ml of methanol, a small amount of insoluble material was removed by filtration, and the peptide was reisolated by evaporating the solvent, yield 242 mg. The purified substrate had $[\alpha]^{25}D$ −16.8°, $[\alpha]^{25}_{365}$ −40° (*c* 0.5, methanol); on tlc it exhibited a single spot in solvent A (R_F 0.32) and B (R_F 0.70) (Folin) and gave no spot with ninhydrin spray. The peptide lost 1.86% of its weight prior to analysis; the total of all elements present was 98.97% and the missing 1% could not be accounted for. *Anal.* Calcd for $C_{37}H_{39}N_7O_3$: C, 64.06; H, 5.67; N, 14.13; O, 16.14. Found: C, 63.11, 63.07; H, 5.73, 5.66; N, 14.06; O, 16.07.

Inhibitors. Conventional synthetic methods yielded the desired inhibitors. L-Ac-Phe, recrystallized from acetone, had mp 165–168°, $[\alpha]^{25}D$ +46.2° (*c* 1, 95% ethanol) (lit. mp 168–171°, $[\alpha]^{20}D$ +46.1° (Kitson and Knowles, 1971)) while D-Ac-Phe showed mp 162–166°, $[\alpha]^{25}D$ −45.0°. Recrystallization of L-Ac-Phe-OMe from ether–hexane afforded material of mp 86–87°, $[\alpha]^{25}D$ +16.0° (*c* 2, methanol) (lit. mp 90°, $[\alpha]^{25}D$ +17.8° (Jones and Niemann, 1963)). Recrystallized L-Ac-Phe-NH_2 (from ethyl acetate) had mp 174–176.5°, $[\alpha]^{25}D$ +26.9° (*c* 1, methanol) (lit. mp 180–182°, $[\alpha]^{20}D$ +27.2° (Kitson and Knowles, 1971)).

Miscellaneous Details. Starting materials for peptide synthesis were obtained from Cyclo Chemical Corp., Los Angeles, Calif., or Bachem Inc., Marina Del Rey, Calif. All

Table I: Kinetics of the Pepsin-Catalyzed Hydrolysis of Four Substrates at pH 1.8, 35° in 3% Methanol.[a]

Substrate[b]	Runs[c]	$10^5[S]_0$ (M)[d]	$10^6[E]_0$ (M)[d,e]	$Av^f k_c$ (sec^{-1})	$Av^f 10^4 K_m$ (M)	$Av^f k_c/K_m$ (M^{-1} sec^{-1})
Ac-Phe/Trp	5	13–66	18	0.0424 ± 0.0026	6.94 ± 0.60	61 ± 2
Z-His-Phe/Trp	6	10–50	19	0.0411 ± 0.0010	3.59 ± 0.30	115 ± 8
	1[g]			0.0360 ± 0.0007[h]	3.51 ± 0.12[h]	103
Z-Gly-His-Phe/Trp	5	8.4–51	7.7	0.140 ± 0.011	6.39 ± 0.80	220 ± 10
Z-Ala-His-Phe/Trp	4	8.0–40	1.8	0.595 ± 0.030	5.22 ± 0.29	1140 ± 20
	1[g]			0.441 ± 0.014[h]	4.17 ± 0.22[h]	1060

[a] Except where specified, runs employed Worthington lot PM OEA pepsin. [b] Cleavage occurs exclusively at the Phe/Trp bond in all examples. [c] The number of independent Lineweaver–Burk plots from which the average values for k_c, K_m, and k_c/K_m have been derived. Each plot contained ten or more points. [d] Typical values. [e] Based on the assumption that ϵ_{280} = 51,500 for pepsin. [f] The errors represent standard deviations from the mean except where noted. [g] This run employed pepsin derived from the activation of pepsinogen. [h] Standard error (Wilkinson, 1961).

but two kinetic runs utilized commercial pepsin, lot PMOEA from Worthington Biochemical Corp., Freehold, N.J. The two exceptional runs employed "pure" pepsin derived from the activation of Worthington pepsinogen, lots PG 1GB and PG 1IA (Rajogopalan *et al.*, 1966; Trujillo and Schlamowitz, 1969). Reagent grade inorganic materials, redistilled water, and Spectroquality methanol were used for all kinetic runs.

Elemental analyses were by Micro-Tech Laboratories, Inc., Skokie, Ill. Optical rotations were measured in a Perkin-Elmer Model 141 polarimeter. Melting points were determined with a Mel-Temp apparatus and are uncorrected. Calculations were performed on an IBM 1130 computer at Amherst College or on the time-sharing system of the University of Massachusetts. All errors specified in this paper represent standard deviations.

Kinetic Methods. The rates of hydrolysis of all substrates were determined spectrophotometrically (Silver *et al.*, 1965; Denburg *et al.*, 1968) at 295 nm, slit 0.1 mm, on a Cary 16 instrument equipped with a Model 1626 interface, Sargent SR recorder, and cell holder and compartment thermostated at 35.0 ± 0.3°. A reaction mixture contained 3.0 ml of aqueous 0.4 M phosphate buffer (μ = 0.5, pH 1.82 ± 0.03) which held any necessary inhibitor, I, 0.100 ml of aqueous enzyme solution, and 0.100 ml of methanolic substrate solution. Introduction of inhibitor in no instance altered the pH of the phosphate buffer.

A "set" of kinetic runs consisted of 40 recorder tracings obtained on the same day. These 40 tracings represented duplicate measurements of the rate of hydrolysis of each of five different substrate concentrations, $[S]_0$, in each of four different buffer solutions ($[I]_0$ = 0 and three others). Table I specifies typical substrate and enzyme concentrations; $[I]_0$ was approximately 0–21 × 10^{-3} M for D- or L-Ac-Phe, 0–14 × 10^{-3} M for L-Ac-Phe-OMe, and 0–41 × 10^{-3} M for L-Ac-Phe-NH_2. Visual estimation of v, the initial velocity of each run, gave the data necessary for a weighted least-squares analysis (Wilkinson, 1961) of the Lineweaver–Burk plots, eq 5.

Calculation of k_c from the Lineweaver–Burk plots required knowing $[E]_0$, the stoichiometric enzyme concentration, and $\Delta\epsilon_{295}$, the difference between the molar extinction coefficient, ϵ, of substrate and hydrolysis products at 295 nm. The optical density (OD) of a pepsin-containing solution at 280 nm defined $[E]_0$, with ϵ_{280} (pepsin) assumed to be 51,500. We determined $\Delta\epsilon_{295}$ by allowing some reaction mixtures to reach "infinity" (constant OD); for these reactions, $\Delta\epsilon_{295} = (OD_{t=0} - OD_{t=\infty})/[S]_0$. Average values for $\Delta\epsilon_{295}$ were: Ac-Phe-Trp, 566 ± 17 (5,11); Z-His-Phe-Trp, 615 ± 10 (4,10); Z-Gly-His-Phe-Trp, 617 ± 10 (5,10); and Z-Ala-His-Phe-Trp, 600 ± 23 (3,6), where the first figure in each parentheses represents the number of independent sets of runs for which $\Delta\epsilon_{295}$ was obtained and the second defines the number of "infinity" reaction mixtures contributing to the average $\Delta\epsilon_{295}$.

Both spectroscopic and tlc data confirm that each of the four substrates undergoes peptic hydrolysis at pH 1.8 exclusively and completely at the Phe-/-Trp bond. Spectroscopically, the OD of an "infinity" mixture invariably agreed (±5%) with that calculated for a solution of Trp, at concentration $[S]_0$, under the same experimental conditions (ϵ_{295} for Ac-Phe, Z-His-Phe, Z-Gly-His-Phe, or Z-Ala-His-Phe is negligibly small). Peptic hydrolysis of Ac-Phe-Trp at pH 2 has already been shown to yield only Ac-Phe and Trp, with a trace of Trp-Trp (Denburg *et al.*, 1968; Jackson *et al.*, 1969). Finally, tlc examination of "infinity" mixtures obtained from the other three substrates revealed the following: (a) no unreacted substrate in either solvent A or B (Folin); (b) a single ninhydrin-positive spot (also Folin, Pauly) that had the same R_F as Trp in solvent A (0.40) and B (0.53); (c) a Pauly-positive, Folin-negative spot which traveled slightly behind Trp in solvent A and with substrate in solvent B; this behavior is characteristic of genuine Z-His-Phe and, presumably, of Z-Gly-His-Phe and Z-Ala-His-Phe, samples of which were not at hand.

Results

The experiments cited above establish that pepsin, at pH 1.8, 35°, hydrolyzes the peptides V-Phe-Trp, V = Ac-, Z-His-, Z-Gly-His- and Z-Ala-His-, quantitatively at the Phe–Trp bond and that the rates of these hydrolyses can be determined spectrophotometrically at 295 nm. The primary kinetic data consisted of 18 "sets" of runs, where each set represented four ten-point Lineweaver–Burk plots of $1/v$ against [S] (eq 5), one for each of four different $[I]_0$. Figure 1 displays the results from one set of runs.

Plots of K_m/k_c and $1/k_c$ *vs.* $[I]_0$ were constructed for each set of runs. Figure 2 illustrates the excellent linearity of the K_m/k_c–$[I]_0$ plots; the average correlation coefficient was 0.996 ± 0.005 for the 18 sets of data listed in Table II. The intersection point of these K_m/k_c *vs.* $[I]_0$ plots with the horizontal axis is at $K_i = -[I]$ and it determines the values

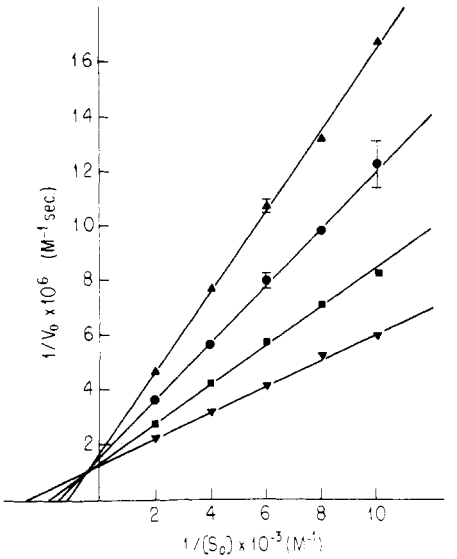

FIGURE 1: Lineweaver-Burk plot (eq 5) for the peptic hydrolysis of Z-His-Phe-Trp at pH 1.8, 35°, in the presence of L-Ac-Phe-NH_2. Each point represents the average of two runs. The enzyme concentration was 1.94×10^{-5} M and $[I]_0$ was nil (▼), 1.24×10^{-2} M (■), 2.68×10^{-2} M (●), or 4.13×10^{-2} M (▲).

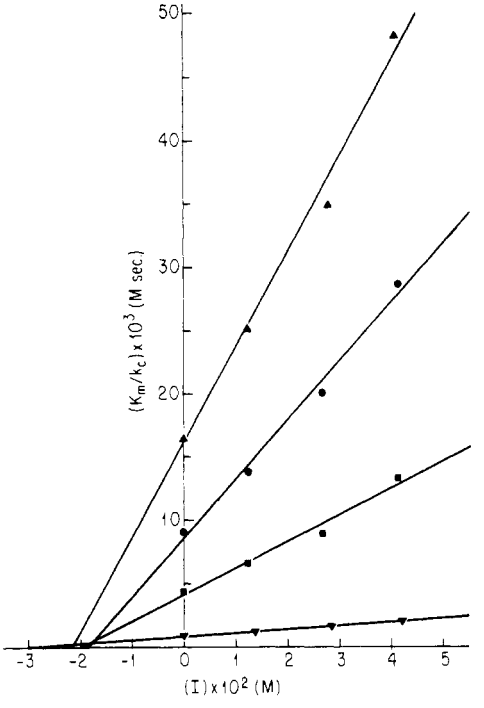

FIGURE 2: Demonstration that a plot of K_m/k_c *vs.* [I] is linear for I = L-Ac-Phe-NH_2. The substrates are: Ac-Phe-Trp (▲), Z-His-Phe-Trp (●), Z-Gly-His-Phe-Trp (■), and Z-Ala-His-Phe-Trp (▼).

for K_i listed in column 3 of Table II ($K_{i\,slope}$ of Cleland). Because eq 9 is valid for both mechanism I and mechanism II (compare eq 3 and 7 to 5), K_i is equivalent to K_5 in either mechanism. Figures 3 and 4 and the data in the last column

$$(K_m/k_c) = (K_s/k_2) + (K_s/k_2K_5)[I]_0 \qquad (9)$$

of Table II reveal that plots of $1/k_c$ *vs.* $[I]_0$ were reasonably linear and displayed positive slopes.[3] We thus have observed linear noncompetitive inhibition[3] in all cases (Cleland, 1963). The intersection point of the $1/k_c$ *vs.* $[I]_0$ plots with the horizontal axis is at $K_{i2} = -[I]$ and defines the values for K_{i2} listed in column 4 of Table II ($K_{i\,intercept}$ of Cleland). Available data on the hydrolysis of synthetic substrates by pepsin (Fruton, 1970,1971) indicate that the approximation $k_3 \gg k_2$ is a good one for the substrates here employed, as well as for Ac-Phe-Phe-Gly. In this approximation, Scheme I equates K_{i2} to k_3K_4'/k_2 (*cf.* eq 4) while Scheme II states $K_{i2} = K_4$ (*cf.* eq 8).

Two comments about the preceding arguments should be made. First, the consistency obtained in both our kinetic analysis and that of Kitson and Knowles (1971) justifies treating L-Ac-Phe as a dead-end inhibitor, similar to the other three, although it alone, as an alternative product XCOOH, can enter into the reaction. Presumably the k_{-2} step is negligibly slow at pH 2; the failure to detect transpeptidation at pH 2 independently confirms this supposition (Silver and Stoddard, 1972). Second, the assertion $K_i = K_5$ requires that K_i for a given inhibitor be the same no matter which substrate is utilized in evaluating K_i. Table II contains several exceptions to this rule. Possible reasons for these exceptions are discussed at the end of the paper.

Table II: The Inhibition of Pepsin-Catalyzed Hydrolyses at pH 1.8, 35°.

Set	Reactants[a]	$10^3 \cdot K_i$ (M)[b]	$10^3 \cdot K_{i2}$ (M)[c]	Slope of $1/k_c$–$[I]_0$ plot (M^{-1} sec)[d]
1	L-Ac-Phe + Ac-Phe-Trp	14	81	310 ± 5
2	+ Z-Q	18	49	460 ± 70
3	+ Z-Q[e]	17	67	400 ± 150
4	+ Z-Gly-Q	15	67	110 ± 60
5	+ Z-Ala-Q	26	58	31 ± 5
6	+ Z-Ala-Q[e]	19	120	19 ± 7
7	D-Ac-Phe + Ac-Phe-Trp	19	70	340 ± 50
8	+ Z-Q	16	180	140 ± 20
9	+ Z-Gly-Q	25	39	150 ± 50
10	+ Z-Ala-Q	25	60	29 ± 7
11	L-Ac-Phe-NH_2 + Ac-Phe-Trp	21	36	560 ± 70
12	+ Z-Q	18	120	190 ± 30
13	+ Z-Gly-Q	20	35	190 ± 40
14	+ Z-Ala-Q	31	180	8.5 ± 7.3
15	L-Ac-Phe-OMe + Ac-Phe-Trp	16	34	680 ± 150
16	+ Z-Q	18	53	460 ± 80
17	+ Z-Gly-Q	18	34	220 ± 20
18	+ Z-Ala-Q	28	38	40 ± 13

[a] The substrates are listed after the inhibitors; Q represents the -His-Phe-Trp fragment. [b] Calculated from the K_m/k_c–$[I]_0$ plot as described in the text. [c] Calculated from the $1/k_c$ –$[I]_0$ plot as described in the text. [d] Standard errors are given. [e] Employed "pure" pepsin derived from the activation of pensinogen.

Discussion

Figures 3 and 4 display a series of plots of $1/k_c$ against $[I]_0$. Not one of the four inhibitors has yielded the set of four parallel lines which eq 4 demands. The broken lines in Figures 3 and 4 graphically illustrate the shortcomings of eq 4. These lines represent what eq 4 predicts the dependence of $1/k_c$ on $[I]_0$ for the hydrolysis of Z-Ala-His-Phe-Trp to be, given the experimental data for Z-His-Phe-Trp.

[3] In set 14 of Table II, the inhibition is nearly linear competitive.

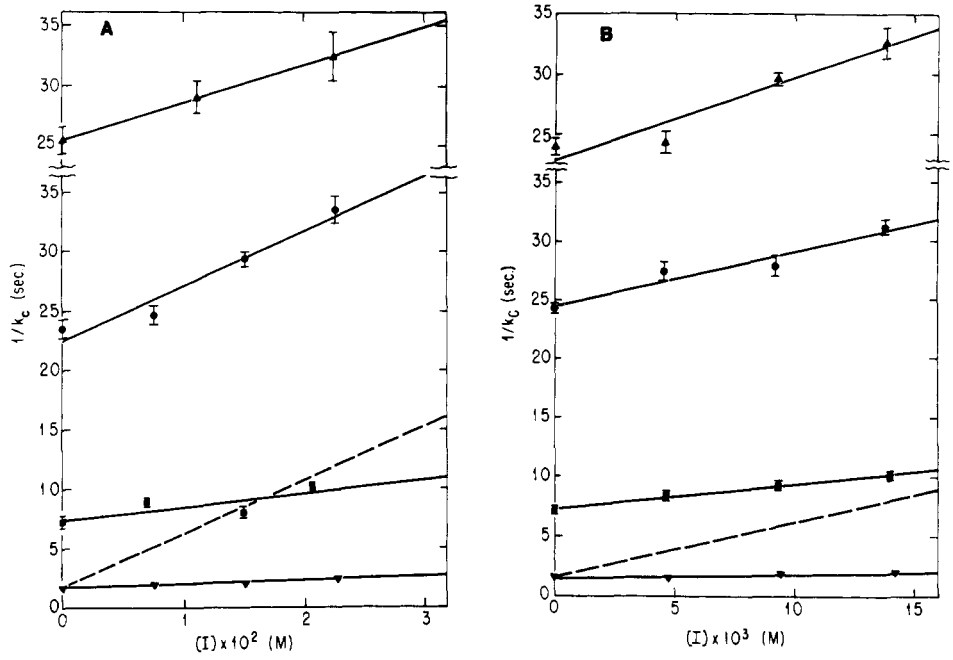

FIGURE 3: Plot of $1/k_c$ *vs.* [I] to distinguish between mechanisms I and II. The broken line in Figures 3 and 4 is parallel to the line for Z-His-Phe-Trp and represents what eq 4 predicts for the dependence of $1/k_c$ on [I] for Z-Ala-His-Phe-Trp. The substrates are: Ac-Phe-Trp (▲), Z-His-Phe-Trp (●), Z-Gly-His-Phe-Trp (■), and Z-Ala-His-Phe-Trp (▼). In A, I = L-Ac-Phe; in B, I = L-Ac-Phe-OMe.

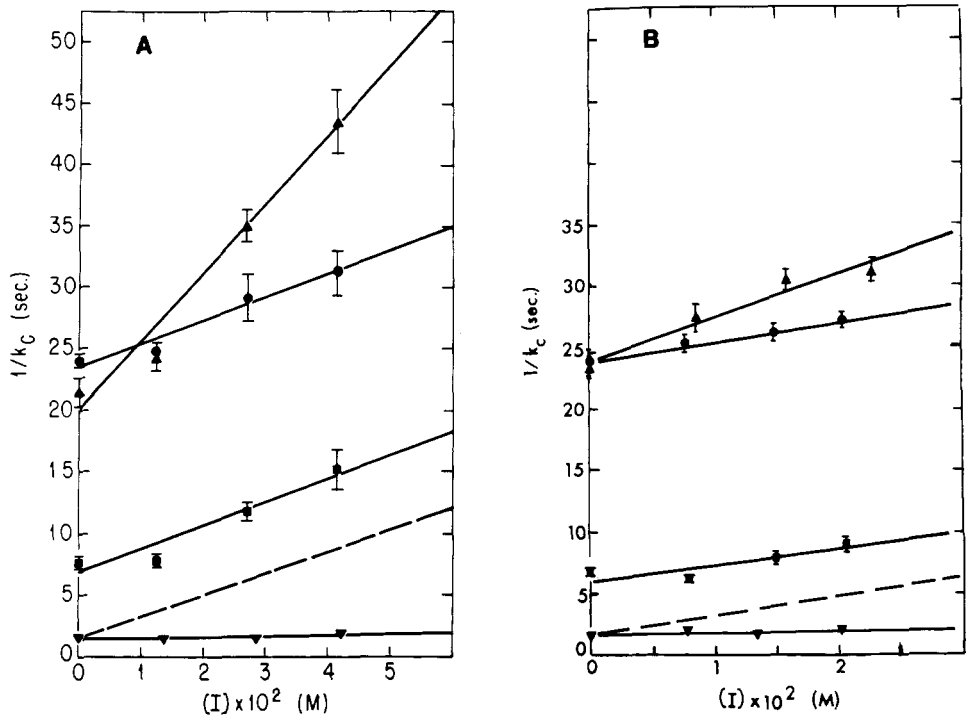

FIGURE 4: Plot of $1/k_c$ *vs.* [I]. See the caption for Figure 3. The substrates are: Ac-Phe-Trp (▲), Z-His-Phe-Trp (●), Z-Gly-His-Phe-Trp (■), and Z-Ala-His-Phe-Trp (▼). In A, I = L-Ac-Phe-NH_2; in B, I = D-Ac-Phe.

The gaps between the lines and the data points are obvious. Three different considerations establish that these gaps are real, and that Scheme I *cannot* be responsible for the observed dependence of $1/k_c$ upon $[I]_0$: (1) the error bars on the data points, which represent the standard error assigned to $1/k_c$ by the weighted least-squares analysis of the Lineweaver–Burk plots (Wilkinson, 1961), fail to overlap the broken lines; (2) the reproducibility in the determination of k_c for uninhibited runs in a series of independent experiments performed months apart (Table I) reaffirms that the errors in $1/k_c$ are customarily less than 10%, *i.e.*, much less than the gap between data points and broken line; (3) the differences between the slopes of the $1/k_c$ *vs.* $[I]_0$ plots for Z-Ala-His-Phe-Trp and the slopes of the other three plots for the same inhibitor were statistically significant by the *t*-test criterion (Brownlee, 1965). Of a total of 13 such comparisons (including the "pure" enzyme pair 3 *vs.* 6) one was significant at the 0.975 confidence level, two, at the 0.995 level, and the rest, at the 0.999 level.

Scheme I also has the defect that it equates K_{i2} with k_3K_4'/k_2. For any particular inhibitor and the amino-enzyme E-Trp, k_3 and K_4' are invariant. If the trend in k_2

approximates that of k_c in Table I, Scheme I predicts that K_{i2} will decrease in the series Ac-Phe-Trp ≃ Z-His-Phe-Trp > Z-Gly-His-Phe-Trp > Z-Ala-His-Phe-Trp as k_2 rises. Table II fails to reveal such a trend in K_{i2}.

Scheme II, by contrast, readily accommodates both our and others' experimental observations very well. It postulates the existence of a ternary complex, pepsin–substrate–inhibitor, a requirement compatible with the suggestion that pepsin has an extended binding site (Fruton, 1970,1971; Wang *et al.*, 1974). The slopes of the $1/k_c$–$[I]_0$ plots for mechanism II are $1/k_2K_4$ (eq 8). If k_2 approximates k_c and k_2 primarily influences the slopes of these plots, the slopes should decrease in the order Ac-Phe-Trp ≃ Z-His-Phe-Trp > Z-Gly-His-Phe-Trp > Z-Ala-His-Phe-Trp. All four plots conform to this expectation. Those of Figure 3 do so especially well, since K_{i2} for L-Ac-Phe and Ac-Phe-OMe is nearly independent of the substrate employed.

Finally, mechanism II rationalizes all of Kitson and Knowles' data. Presumably the linear competitive inhibition by D-Ac-Phe they encountered stems from the inability of the pepsin, Ac-Phe-Phe-Gly complex to bind D-Ac-Phe effectively. While an explanation for all the Ac-Phe-Phe-Gly data at pH 2.1 based on Scheme I necessitates introduction of the *ad hoc* assumption that XCOOH in eq 1 is liberated as the anion, Scheme II accounts for these data without such an assumption.

Mechanistic Summary. Our results establish that currently available kinetic data for the noncompetitive inhibition of pepsin-catalyzed hydrolyses at pH 2, the pH-optimum for the enzyme, merely confirm the existence of a pepsin–substrate–inhibitor complex. Since these data reveal nothing about events occurring subsequent to the rate-limiting decomposition of the Michaelis complex, they provide no insight into the merits of the amino-enzyme hypothesis or of the suggestion that XCOOH in eq 1 is liberated as the anion $XCOO^-$.

The arguments we have presented render it likely that Scheme II offers the preferred explanation for the noncompetitive inhibition by L-Ac-Phe-OEt and L-Ac-Phe-NH_2 in the hydrolysis of Ac-Phe-Phe-Gly at pH 4.3 (Kitson and Knowles, 1971). Unfortunately our Trp-containing substrates are not suitable for rigorously demonstrating this contention. The kinetic data at high pH are especially important since Ginodman and Lutsenko (1972) report that their isotope-exchange data establish that product release is completely ordered *only* at pH 4.7 or higher.

It seems fair to conclude that the results from kinetic inhibition and transpeptidation studies at present offer little if any support to the amino-enzyme hypothesis for pepsin action. Reexamination of the other evidence upon which the hypothesis rests is obviously desirable. Meanwhile, alternative mechanisms should be seriously evaluated (Takahashi *et al.*, 1974; Wang *et al.*, 1974).

Is Scheme II Complete? Since Scheme II equates K_5 to K_i, it requires that all four substrates yield the same value of K_i for a given inhibitor. The data generally conform to this expectation, but Z-Ala-His-Phe-Trp invariably affords K_i's which are too large (runs 5, 10, 14, and 18 of Table II) while Z-Gly-His-Phe-Trp does so in one instance (run 9). A detailed analysis of the source[4] of these discrepant values for K_i has been performed; we report here a brief summary of that analysis.

$$K_i' = [I]_0/[((k_c/K_m)_{I=0}/(k_c/K_m)_{I_0}) - 1] \quad (10)$$

Equation 10 aided evaluation of various extensions of Scheme II. It defines K_i', closely analogous to K_i, but calculable for each $[I]_0$ in a set of runs. The values for K_i' so obtained either are independent of $[I]_0$ or decrease slightly (15–20%) as $[I]_0$ increases from 0 to K_i. The simplest modification of Scheme II which quantitatively accounts for the discrepant K_i phenomenon and also reproduces the dependence of K_i' on $[I]_0$ resembles a scheme developed by Schlamowitz *et al.* (1968). It meets all the conditions specified earlier in distinguishing Scheme II from I. This modified form of Scheme II postulates the existence of an EI_2 complex

$$EI_2 \overset{K_5'}{\rightleftharpoons} EI + I$$

($K_5' \simeq 5K_5$) and the conversion of the E,XCONHY,I complex of eq 6 into hydrolysis products for runs 5, 9, 10, 14, and 18

$$E,XCONHY,I \xrightarrow{\beta k_2} E + I + \text{products}$$

($\beta k_2/K_4)/k_2 \simeq 0.01\ M^{-1}$). Neither suggestion is incompatible with the picture of an extended active site for pepsin which emerges from the recent experiments performed in Fruton's and Hofmann's laboratories.

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[4] Runs 3 and 6, which utilized pure pepsin, gave nearly identical values for K_i. This suggests that the phenomenon may be uniquely associated with kinetics employing commercial pepsin, but the data are obviously inadequate to prove this point.

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The Action of Bacterial Cytidine Deaminase on 5,6-Dihydrocytidine†

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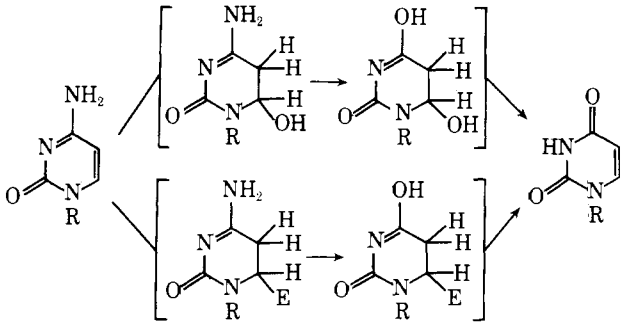
ABSTRACT: Cytidine deaminase from *Escherichia coli* was found to catalyze the hydrolytic deamination of 5,6-dihydrocytidine, at a rate slightly lower than its rate of action on the normal substrate. The results suggest that nucleophilic addition by the enzyme at the 5,6 position of the substrate is not an essential part of catalysis, unless the active site is so flexible that deamination can occur with addition in one case (cytidine) and without addition in another case (5,6-dihydrocytidine). 3,4,5,6-Tetrahydrouridine bears a close structural resemblance to a hypothetical "tetrahedral" intermediate formed by direct water addition to 5,6-dihydrocytidine. The hydrolytic activity of the enzyme toward 5,6-dihydrocytidine and its potent inhibition by 3,4,5,6-tetrahydrouridine are presumably related by the ability of the active site to stabilize structures of this kind by tight binding. Cytidine deaminase shows no detectable activity as a catalyst for the dehydration of 6-hydroxy-5,6-dihydrouridine.

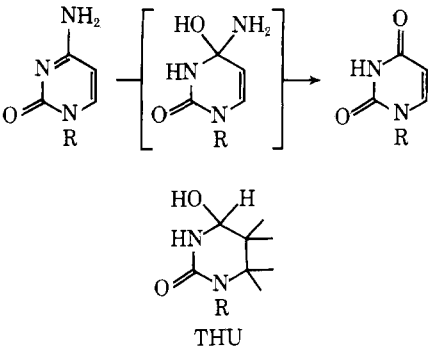
Recent studies have shown that a variety of nucleophiles catalyze the deamination of cytidine by adding to the 5,6-double bond (Shapiro and Klein, 1966, 1967; Notari, 1967; Wechter, 1970; Wechter and Kelly, 1970). These models suggest that the action of cytidine deaminase (cytidine aminohydrolase, EC 2.5.4.5) might similarly involve addition of the enzyme (or of enzyme-bound water) at the 5,6-double bond of cytidine as part of the catalytic mechanism (Scheme I).

SCHEME I



An alternative mechanism would involve direct addition of water at the 3,4-double bond, followed by elimination of ammonia as in the enzymatic deamination of adenosine (Evans and Wolfenden, 1970, 1972, 1973). This might explain the potent inhibition of cytidine deaminase by 3,4,5,6-tetrahydrouridine (THU), which resembles a possible intermediate which might be formed by addition of water to cytidine (Scheme II) (Cohen and Wolfenden, 1971).

SCHEME II



A distinction between THU and the intermediate enclosed in brackets in Scheme II is in the saturation of the 5,6 bond. If this interpretation of the inhibition by THU is correct, THU might actually be considered an analog of an intermediate in the deamination of 5,6-dihydrocytidine rather than cytidine itself, and cytidine deaminase might catalyze deamination of 5,6-dihydrocytidine. If, on the other hand, the reaction proceeds through addition of enzyme or water at the 5,6 position (Scheme I), catalysis should be effectively *blocked* in the case of 5,6-dihydrocytidine. In this communication, we describe experiments undertaken in an attempt to decide between these alternatives.

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