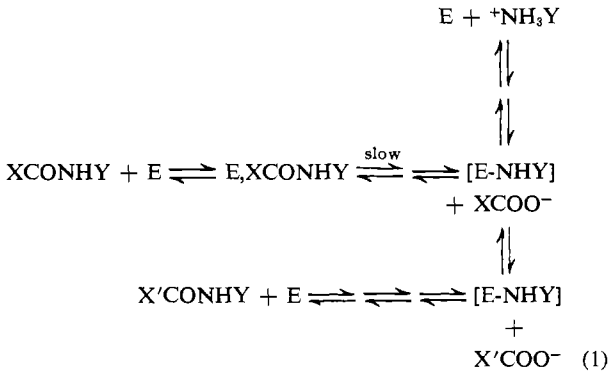
Amino–Enzyme Intermediates in Pepsin-Catalyzed Reactions*

Marc S. Silver† and Mai Stoddard

ABSTRACT: The pepsin-catalyzed hydrolysis of Ac-Phe-Tyr at pH 4.5–4.7 produces an intermediate which reacts with radioactive Ac-Phe (Ac-*Phe*) to generate Ac-*Phe*-Tyr. The intermediate undergoes hydrolysis to Tyr about three times more readily than it reacts with 2.4×10^{-2} M Ac-*Phe*. Ac-Phe-Phe behaves similarly to Ac-Phe-Tyr, but neither Ac-Phe-Tyr-NH_2 nor Ac-Phe-Phe-OEt yields an intermediate which Ac-*Phe* can trap. An amino–enzyme mechanism which postulates that pepsin-catalyzed hydrolyses follow the sequence [enzyme–substrate complex → amino–enzyme + Ac-Phe → final products] appears incapable of explaining these observations in a simple manner. It also cannot account for the quantitative difference in the rate of incorporation of free Ac-Phe into unreacted Ac-Phe-Tyr as measured directly with Ac-*Phe* and indirectly by the Ac-Phe-Tyr-induced exchange of $^{18}OH_2$ with Ac-PheCOOH. Modifications of the simple amino–enzyme mechanism or a mechanism which assumes that hydrolysis proceeds *via* two covalent enzyme–substrate intermediates lead to no really satisfactory rationalization of the experimental data. It seems probable that the transpeptidation reaction is only characteristic of the hydrolysis at high pH of synthetic substrates with a C-terminal carboxyl group near the point of bond cleavage. If so, mechanistic generalizations based upon the transpeptidation reaction are unjustified until the occurrence of amino–enzyme intermediates in the hydrolysis of substrates like Ac-Phe-Tyr-NH_2 (a better model than Ac-Phe-Tyr for a polypeptide) is established.

All efforts to isolate amino–enzymes from the pepsin-catalyzed hydrolysis of peptides have failed (Kitson and Knowles, 1970).[1] The premise that peptic hydrolyses normally produce such intermediates (eq 1) therefore rests upon two kinds of indirect evidence. Transpeptidation experiments, pioneered by Neumann *et al.* (1959) and Fruton *et al.* (1961), suggest that an acceptor $X'COO^-$ can react with the amino–enzyme from XCONHY to produce X'CONHY while kinetic experiments establish that eq 1 accounts for the inhibitor



(1)

action of hydrolysis products and their analogs (Kitson and Knowles, 1971a,b).

Numerous other experimental results are compatible with the amino–enzyme hypothesis but have revealed nothing about the nature of these intermediates (Fruton, 1970).

A fundamental property of each amino–enzyme should be its partitioning ratio, *PR*, the ratio of its rate of transpeptidation to its rate of hydrolysis under specified conditions. We decided to determine some *PR*'s, in the expectation that this knowledge of them should ultimately lead to an understanding of the properties of the amino–enzyme intermediates. The unexpected results obtained have raised serious doubts about the generality of the simple amino–enzyme concept, as exemplified by eq 1.

The following five considerations guided our experimental approach and led us to use Ac-Phe-Tyr[2] and Ac-Phe-Tyr-NH_2 as primary sources of amino–enzymes and Ac-*Phe* (radioactive Ac-Phe) as acceptor: (1) the available detailed kinetic data on the hydrolysis of each substrate (Denburg *et al.*, 1968) would facilitate analysis of the transpeptidation results; (2) the behavior of the amino–enzyme "E-NH-TyrCONH$_2$" from Ac-Phe-Tyr-NH_2 might be more readily interpreted than that of "E-NH-TyrCOOH" from Ac-Phe-Tyr, since the former lacks an ionizable substrate carboxyl group; (3) the choice of Ac-*Phe* as acceptor must further ease analysis of the experimental observations, for transpeptidation generates only radioactive substrate, not a new substance (*cf.* Fruton *et al.*, 1961); (4) the high solubility of Ac-*Phe* permits its use at high concentration, which should enhance the extent of incorporation of radioactivity into unreacted substrate and suppress undesirable transpeptidations in which the substrate also acts as acceptor (Neumann *et al.*, 1959); and (5) we could compare our determination of the extent of exchange between Ac-Phe-Tyr and Ac-*Phe* to that of Kozlov *et al.* (1965) who measured the degree to which Ac-Phe-Tyr increased the rate of exchange of Ac-PheCOOH with $^{18}OH_2$ (each transformation XCONHY → X'CONHY exchanges one oxygen of Ac-PheCOOH with solvent).

* From the Department of Chemistry, Amherst College, Amherst, Massachusetts 01002. *Received August 9, 1971.* Supported by Grant AM-08005 of the U. S. Public Health Service. This research, at its inception, benefited from the advice of members of the Department of Biophysics at the Weizmann Institute, particularly Dr. H. Neumann, and from an NSF Senior Postdoctoral Fellowship to M. S. S.

† To whom to address correspondence.

[1] Ginodman *et al.* (1971) have recently claimed isolation of pepsin with L-tyrosine ethyl ester covalently attached.

[2] Abbreviations are listed in *Biochemistry 5*, 2485 (1966), and all amino acids possess the L configuration unless otherwise specified. A functional group is sometimes added to the abbreviation for an amino acid to clarify a point (*e.g.*, Ac-PheCOOH, $^+NH_3$-Tyr). Italicized Phe (*Phe*) designates ^3H-labeled Phe.

Experimental Section

Thin-Layer Chromatography (Tlc). Tlc analysis established the purity of amino acid derivatives and the qualitative or semiquantitative composition of reaction mixtures from enzymatic hydrolyses. All tlc's utilized silica gel G on glass plates with 4:1:1 (*v*/*v*) 1-butanol–acetic acid–water as the developing solvent. Typical R_F values were: pepsin, 0.0; Tyr, 0.40; Tyr-Tyr, 0.59; Ac-Phe-Tyr, 0.73; Tyr-NH_2, 0.75. We employed the following visualization reagents of Stahl (1962): Pauly (37) or Folin-Ciocalteau (122) for phenols; chlorine–tolidine (32) for peptide bonds; ninhydrin (108) for free amino acids.

High-Voltage Paper Electrophoresis (Hve). A Savant Instruments Inc. FP30A instrument provided the fundamental tool for performing the separations necessary to this investigation. Samples of 10 μl were spotted 3.8 cm apart on a piece of Whatman No. 3MM paper 27 cm wide (six samples per sheet). Electrophoresis proceeded for 2 hr at 4900 V (~150 V/cm) and employed pH 5.3 buffer (20 ml of pyridine plus 8 ml of acetic acid diluted to 3 l). The paper was dried for 2 hr at 100° immediately after completion of the hve run and later cut into six 3.4-cm wide strips, each containing one sample. Strips were either sprayed (as for tlc) or cut at appropriate places into 1.7 × 1.7 cm squares (two squares/1.7 cm running length) for radioactivity determination. Radioactive spots on some strips destined for cutting were first located with a Tracerlab 4π scanner.

Counting Technique. Each scintillation vial was counted for 10 min or longer on a Packard 3314 Tri-Carb scintillation spectrometer and contained 11 ml of Bray's solution (Bray, 1960) plus either 10 μl of sample solution or a 1.7 × 1.7 cm square of unsprayed electrophoresis sheet ("paper sample") lying flat on the bottom of the vial. Controls revealed that allowing the paper samples to soak in the Bray solution for 12 hr prior to counting gave a reproducible, constant counting rate. The dual-channels ratio technique (Bush, 1963) established a counting efficiency of 15% for both liquid and paper samples. Sprayed electrophoreograms showed much lower efficiencies.

The following procedure guaranteed accurate determination of the radioactivity of paper samples. Two 10-μl samples of each solution applied to a given hve sheet were counted directly in Bray, while the total radioactivity of one strip (or two) of the electrophoreogram was measured by cutting all of it into squares. The total counts per minute for the paper samples therefore determined the counting efficiency for paper relative to liquid sample ("relative efficiency"). Calculations assumed all strips of an electrophoreogram possessed identical relative efficiency. Directly measured relative efficiencies for two strips of the same electrophoreogram a ways agreed to 3%. Relative efficiencies for a set of 36 strips from ~30 different sheets averaged 91 ± 3% with a range of 82–99%.

Materials Purchased. Twice-crystallized pepsin was Worthington Biochemical Corp., lot PM 709. The following chemicals, purchased from Cyclo Chemical Corp. (CCC), Sigma Chemical Corp. (SCC), Fox Chemical Corp. (FCC), Calbiochem (CBC), or Aldrich Chemical Co. (ACC), had physical properties that agreed with literature values and were used as received: Ac-Phe-Tyr (CCC, mp 216–218°), Tyr-Tyr·$2H_2O$ (CCC), Ac-Phe (CCC), Tyr-NH_2·HCl (SCC), D-Tyr-NH_2 (FCC), Tyr-Tyr-Tyr·$2H_2O$ (FCC), Tyr (CBC), and Tyr-OMe (ACC). Ac-Phe-Tyr-NH_2 (mp 249–252°) and Ac-Phe-Tyr-OMe (mp 114–116°; Clement *et al.* (1968) report mp 125–126 and 136–137°), obtained from Cyclo, were recrystallized prior to use. Phe-OMe·HCl, a gift of Dr. M. S. Matta, had mp 160–162° (lit. mp 161–163° for the D isomer (Cohen and Schultz, 1968)). Ac-Phe-Phe-OEt was the gift of Dr. J. R. Knowles (Cornish-Bowden and Knowles, 1969).

Materials Synthesized. Reaction of Ac-DL-Phe (mp 150–153°, lit. mp 152.5–153° (Overby and Ingersoll, 1951)) with Phe (Knowles *et al.*, 1969) and fractional recrystallization of the resultant Ac-DL-Phe-L-Phe, mp 140–175° provided pure Ac-Phe-Phe (mp 245–250° (from methanol), $[\alpha]_D^{23}$ +15.0° (*c* 0.6, pyridine)) and Ac-D-Phe-L-Phe (mp 178–194° (from ethyl acetate)). Knowles *et al.* report mp 245–249° and $[\alpha]_D^{23}$ +14.3° for the former and mp 193–195° for Ac-L-Phe-D-Phe.

Reaction of Z-D-Phe, D-Tyr-NH_2, and isobutyl chloroformate (exp 4 of Anderson *et al.*, 1967) yielded Z-D-Phe-D-Tyr-NH_2, mp 190–195°. Catalytic removal of the Z group, acetylation of the uncharacterized D-Phe-D-Tyr-NH_2, and subsequent alkali treatment (Hofmann *et al.*, 1960) gave Ac-D-Phe-D-Tyr-NH_2, mp 249–252°. The product behaved identically with its enantiomer on tlc and, when treated with pepsin, showed no detectable hydrolysis (ninhydrin reagent) after 19.5 hr under conditions where Ac-Phe-Tyr-NH_2 showed 80% reaction in 2 hr and Ac-Phe-Tyr, 61% in 7 hr.

Radiochemicals. The Radiochemical Centre, Amersham, England, reduced 5 mg of α-acetaminocinnamic acid, mp 190–192° (Herbst and Shemin, 1943), with tritium gas over a 10% palladium-on-charcoal catalyst. They added 400 mg of unlabeled Ac-DL-Phe and supplied the resultant 600-mCi sample as a solution in 10 ml of ethanol, to be referred to as "stock Ac-DL-*Phe*."

Some stock Ac-DL-*Phe* was purified for kinetic and synthetic use. A mixture of 8.0 g of unlabeled Ac-DL-Phe and 2 ml of stock Ac-DL-*Phe* gave 7.0 g of product upon recrystallization from hot water. Further recrystallization from acetone and then again from water gave 5.4 g of Ac-DL-*Phe*, mp 151–153°. This sample was recrystallized from acetone approximately every 6 months and typically had mp 152.5–154°, specific activity 3 × 10^6 cpm/mg. Reaction of Ac-DL-*Phe* with Phe, as described above for the nonradioactive substance, provided Ac-*Phe*-Phe (mp 246–253°) and Ac-D-*Phe*-L-Phe (mp 193–194.5°).

Enzymatic synthesis of Ac-*Phe*-Tyr and Ac-*Phe*-Tyr-OMe employed Ac-*Phe* as starting material. This was obtained from 3.5 g of Ac-Phe plus 0.5 ml of stock Ac-DL-*Phe* and had mp 166–167° and a constant specific activity of ~7 × 10^5 cpm/mg after several recrystallizations from water and acetone. Incubation of 650 mg of Ac-*Phe* and 3.1 g of Tyr-OMe with pepsin at pH 4 (Kozlov *et al.*, 1966) yielded crude Ac-*Phe*-Tyr-OMe which upon recrystallization from ethyl acetate–hexane gave a first crop of 92 mg, mp 131–137°, and a second crop of 9 mg. The first crop plus 93 mg of Ac-Phe-Tyr-OMe was dissolved in ethyl acetate and the solution poured through silica gel layered on a sintered glass funnel. Addition of hexane to the initial eluate gave 11 mg of pure Ac-*Phe*-Tyr-OMe, mp 131–135° (clear melt), which was employed in control 6. Continued elution afforded 120 mg of less pure material, mp 125–134° (unclear melt). Addition of 16 mg of carrier Ac-Phe-Tyr-OMe to the initial 9-mg second crop of Ac-*Phe*-Tyr-OMe gave a sample that provided Ac-*Phe*-Tyr after treatment with hydrochloric acid in acetic acid (Mitz *et al.*, 1950). The final sample for control 2 had mp 218–220° after recrystallization from ethyl acetate–hexane.

The radiochemical and chemical homogeneity of all radiochemicals described was established by the observation that

each was indistinguishable from its nonradioactive counterpart in hve and tlc and, in the former experiments, a single radioactive peak occurred at the expected position. Technical difficulties encountered in applying these criteria to Ac-*Phe*-Phe are discussed later.

General Procedure for Transpeptidation Studies. Incubations employed 0.5 M sodium acetate (pH 4.50 ± 0.02 and 4.70 ± 0.02) and 0.17 M potassium phosphate (pH 1.85 ± 0.05) buffers at 35.0 ± 0.2° and 0.5 M ammonium acetate buffer (pH 4.70 ± 0.02) at 37.0 ± 0.1°. For a typical run in an aqueous solvent, 3.3 mg of Ac-Phe-Tyr was dissolved in 1.1 ml of a warm solution of Ac-DL-*Phe* in pH 4.5 acetate ([Ac-DL-*Phe*] = 10 mg/ml). Dissolution of 2.7 mg of pepsin in 500 μl of the resultant solution, thermostatted at 35°, initiated reaction. The approximate final concentrations of reagents under "usual" conditions were therefore: pepsin = 1.5×10^{-4} M (assuming a molecular weight of 34,200); Ac-Phe-Tyr = 8.1×10^{-3} M; Ac-DL-*Phe* = 4.8×10^{-2} M. The simultaneously performed control lacked Ac-Phe-Tyr. Samples of run and control were removed as desired, quenched with an equal volume of ethanol, and frozen pending subsequent hve, tlc, or ninhydrin analysis.

The low solubility of Ac-Phe-Tyr-NH_2 in water necessitated employing a solvent containing 9% methanol for many runs, which were in all other respects identical to those just described. The methanolic incubation mixture for a run was prepared by dissolving 6.2 mg of Ac-Phe-Tyr-NH_2 in 200 μl of methanol plus 100 μl of buffer and then adding 2.0 ml of a solution of Ac-DL-*Phe* (11.6 mg/ml) in the same buffer. The control lacked Ac-Phe-Tyr-NH_2.

Determination of the amount of radioactivity incorporated into the acetylated dipeptide relied exclusively on hve separations. Run and control samples were spotted alternately on the hve paper, so each run was adjacent to one or more appropriate controls. At least duplicate analyses of each run were made, where one analysis represented the difference in cpm between one run strip and a sample strip. Duplicate runs were often performed. Typically, Ac-Phe-Tyr-NH_2, Ac-Phe-Tyr-OMe, and Ac-Phe-Phe-OMe remained at the original spot, Ac-Phe-Tyr and Ac-Phe-Phe moved to +3.5″, and Ac-DL-*Phe* was centered at +6.5″. In a good control run, the Ac-DL-*Phe* peak measured ~150,000 cpm while either of the other positions of interest showed ≤300 cpm (≤0.2%). Papers whose control runs exhibited >600 cpm at the latter were usually discarded but there was an unavoidable tendency toward increased counts per minute at the origin for samples which had been incubated for ≥24 hr. Figure 1 illustrates the hve separations achieved.

The amount of color produced with ninhydrin reagent in triplicate analyses of run, control, buffer, and solution of Phe,Tyr or Tyr-NH_2 in buffer determined the concentration of unreacted peptide in the run sample. This information and the known difference in cpm between run and control permitted calculation of the percentage of unreacted dipeptide that had undergone exchange. The calculation assumed the exchange specific for Ac-L-*Phe* (see Neumann *et al.*, 1959, and run 24) and that the sole ninhydrin-positive materials from the substrates were the simple hydrolysis products. The correctness of the latter assumption will now be examined.

Ac-Phe-Tyr + Ac-DL-*Phe*. Concern about possible complications introduced into the ninhydrin analysis by the formation of Tyr-Tyr (Neumann *et al.*, 1959) led to a semiquantitative tlc investigation of the amounts of Tyr and Tyr-Tyr in the hydrolysis product mixture. This established that: (a) hydrolysis in pH 4.5 acetate under our usual conditions produced 7–10% of the theoretical amount of Tyr-Tyr; (b) omission of Ac-DL-*Phe* from the recipe raised the yield to ~30%, for pH 4.5 acetate or citrate buffer; (c) <1% Tyr-Tyr was formed at pH 1.9 in the absence of added Ac-DL-*Phe*; (d) Tyr-Tyr-Tyr did not separate from Tyr-Tyr and, if present, would have been slowly cleaved to Tyr and, presumably, Tyr-Tyr.

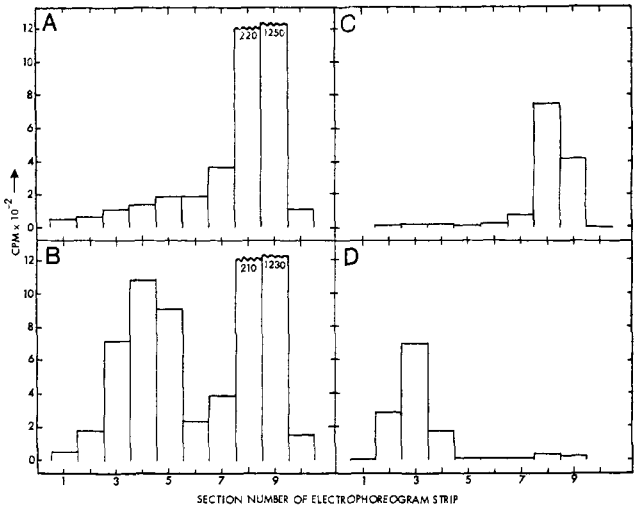


FIGURE 1: Distribution of radioactivity on electrophoreograms as measured by soaking paper samples in Bray's solution. The diagrams represent the following incubations at pH 4.5, 35°: (A) [Ac-DL-*Phe*] = 4.8×10^{-2} M, [E] = 1.5×10^{-4} M, t = 6 hr; (B) same as part A, but also included $[\text{Ac-Phe-Tyr}]_0 = 8 \times 10^{-3}$ M (~50% hydrolysis); (C) Ac-*Phe*-Tyr + E, t = 48 hr (control 2); (D) sample C at t = 0 hr.

Should allowance be made for the appearance of Tyr-Tyr among the reaction products, when calculating the percentage of substrate hydrolyzed from ninhydrin color? The molar ninhydrin color equivalent of Tyr-Tyr was 119% of that of Tyr. If the proportion of Tyr to Tyr-Tyr is assumed to be constant at 90:10 throughout the duration of a run, then percentages of reaction calculated as 25, 50, and 92 ± 4 (average of seven "infinity" determinations) on the basis of Tyr color alone become 26, 52, and 96, respectively, when the correction for Tyr-Tyr is applied. The differences are insignificant for our purposes.

Ac-Phe-Tyr-NH_2 + Ac-DL-*Phe*. No difficulties arose. The sole tyrosine-containing product ever detected was Tyr-NH_2.

Ac-Phe-Phe + Ac-DL-*Phe*. The insolubility of Ac-Phe-Phe (and Ac-Phe-Phe-OEt) forced adoption of a different incubation procedure. Reaction was initiated by the addition of 15.4 mg of pepsin to 3.0 ml of pH 4.7 sodium acetate buffer containing 10.0 mg/ml of Ac-DL-*Phe* and 25 or 50 μl of a *N*,*N*-dimethylformamide solution of Ac-Phe-Phe (30 mg/ml). The dipeptide was omitted from the control. Ninety per cent of the ninhydrin color expected for total hydrolysis to Ac-Phe and Phe appeared after 22 hr. No tlc analysis of the 22-hr hydrolysate was performed.

The concentration of Ac-Phe-Phe in the quenched reaction mixtures was insufficient to give a detectable color with Cl_2–tolidine reagent after hve. At concentrations high enough to give a positive color test, the insolubility of the peptide prevented its migrating as expected during hve. With the aid of synthetic Ac-*Phe*-Phe we finally established that Ac-Phe-Phe, at the low concentrations present in incubation mixtures, behaved just like Ac-Phe-Tyr in hve. Interestingly, the more

soluble stereoisomer Ac-D-Phe-L-Phe moved in the expected manner at concentrations high enough to be detected with the spray reagent.

Equilibration Studies. All equilibrations employed reagent concentrations similar to those in the kinetic runs and followed this basic procedure. The pH of a solution of 0.0866 g of Tyr-NH_2·HCl (final concentration, 0.17 M) in 2.0 ml of pH 4.5 acetate buffer was readjusted to about pH 4.50 by the addition of 0.1 ml of 1 M sodium acetate. The final incubation solution contained 1.05 ml of this, 11.6 mg of Ac-DL-*Phe*, 0.1 ml of methanol, and 6 mg of pepsin. The control lacked the Tyr-NH_2·HCl. Samples taken after 24 and 48 hr were quenched with an equal volume of ethanol and frozen for future hve analyses. These analyses were especially simple, since the reaction product in all cases was a neutral substance which remained near the origin. The difference in counts per minute between runs and controls, obtained as for the transpeptidation reactions, permitted ready calculation of the equilibrium constants of Table III.

Controls. (1) The assumption that Ac-DL-*Phe* contains 50% of its radioactivity in each enantiomer is basic to our data analysis. The method of synthesis certainly suggests the assumption is valid and this control proves it. A mixture of 100 μl of stock Ac-DL-*Phe* solution and 1.00 g of Ac-DL-Phe were recrystallized sequentially from acetone, water, and acetone. The resultant labeled Ac-DL-Phe had an activity of 7.64×10^5 cpm/mg. The experiment was repeated with 1.00 g of Ac-Phe substituted for Ac-DL-Phe. One-half of the radioactivity should be lost, so the final sample should have an activity of 3.82×10^5 cpm/mg; the observed figure was 3.77×10^5 cpm/mg (98.7% of expectation). (2) Calculation of the per cent of unreacted Ac-Phe-Tyr that has undergone exchange with Ac-DL-*Phe* from the observed counts per minute of Ac-*Phe*-Tyr requires knowledge of the counting efficiency for paper samples of Ac-*Phe*-Tyr relative to that for Ac-DL-*Phe*. The dual-channels ratio method suggested these efficiencies were similar. The following experiment provided corroborative evidence both on this point and for our interpretation of the hve analysis of the transpeptidation studies in general. Pepsin (5 mg/ml) was added to a solution of synthetic Ac-*Phe*-Tyr (~3 mg/ml) in methanolic pH 4.5 acetate buffer. A first sample was taken immediately, a second, 48 hr later. Both were quenched. Upon hve analysis, the first gave 1167 ± 35 cpm for the Ac-*Phe*-Tyr spot and 47 ± 8 at the Ac-*Phe* position. The second gave 25 ± 5 and 1213 ± 34 for these respective locations (quadruplicate analyses). Figure 1 illustrates the experimental results. (3) The appearance of radioactivity at the hve spot assigned to Ac Phe-Tyr is an enzyme-dependent process. Incubation of Ac-DL-*Phe* with Ac-Phe-Tyr under the usual conditions in 4.5 acetate for 72 hr, but in the absence of pepsin, gave 45 cpm less at that position than had been true at zero time. (4) The appearance of radioactivity at the hve spot assigned to Ac-Phe-Tyr requires that Ac-Phe-Tyr be present in the original incubation mixture. A control solution that lacked the dipeptide gave, for the Ac-Phe-Tyr location, 471 ± 8 cpm at $t = 0$, 362 ± 104 cpm at $t = 6$ hr, and 386 ± 60 cpm at $t = \infty$. The simultaneous "run" incubation gave cpm essentially identical with those of the control at the Ac-Phe-Tyr spot for the first and last points but >2000 cpm greater for the 6-hr sample. The behavior of this transient peak of radioactivity is indistinguishable from that of synthetic Ac-*Phe*-Tyr described in control 2, but no experiment performed rules out the presence of Ac-*Phe*-Tyr-Tyr or higher peptides. (5) Runs 7–8 established that pepsin from pepsinogen (a gift of Dr. T. P. Stein; see Rajagopalan *et al.*, 1966) behaved similarly to commercial pepsin in our experiment. (6) The failure to detect Ac-*Phe*-Tyr-NH_2 from incubations of Ac-DL-*Phe* with Ac-Phe-Tyr-NH_2 might be an artifact introduced because the counting efficiency for paper samples of Ac-*Phe*-Tyr-NH_2 is unreasonably low. The satisfactory equilibrations run with Tyr-NH_2, control 7, and this control, identical with control 2 except that synthetic Ac-*Phe*-Tyr-OMe was substituted for Ac-*Phe*-Tyr, established the unlikelihood of this possibility. At zero time, Ac-*Phe*-Tyr-OMe had 2017 ± 50 cpm and Ac-*Phe* had 42 ± 19. After treatment with pepsin for 24 hr, the figures were 27 ± 13 and 2078 ± 91 cpm, respectively. (7) A single check on equilibration experiments was provided by the observation that equilibration of Ac-DL-*Phe*, pepsin, and 0.17 M D-Tyr-NH_2 for 48 hr gave only 66 ± 43 cpm more in the run that in the control. The comparable experiment employing L-Tyr-NH_2 gave >8000 cpm excess in the run. See runs 4–6 of Table III.

Results

Tables I and II present data on the extent to which synthesis of radioactive substrate *via* transpeptidation (eq 1, X′CO = Ac-*Phe*) has accompanied the simultaneous appearance of hydrolysis products under a variety of experimental conditions. Table III describes several determinations of K_{TD}, the equilibrium constant for cleavage of the peptide bond to fully ionized products (eq 2).

$$H_2O + AcPheCONHY \rightleftharpoons AcPheCOO^- + {}^+NH_3Y \quad (2)$$

Reliability of the Experimental Data. The small average errors in the "Δcpm" columns illustrate the excellent reproducibility of the hve analytical procedure under optimum circumstances. These errors usually derive from duplicate or triplicate analyses which employed a single electrophoreogram.

Runs 21 and 30, the only ones which should definitely have Δcpm = 0, define the maximum sensitivity of the hve technique. The small Δcpm's observed in runs 9, 20, 27–29, and 31–33 and equilibration 6 probably reflect a real incorporation of label into substrate for many of them, but Δcpm exceeds 100 for none. This ability to achieve Δcpm <100 for ten runs further establishes the reproducibility of the hve technique.

Accuracy in evaluation of "% of Remaining S Exchanged," the quantity of greatest interest in the kinetic experiments, was probably no better than ±15% (relative), since this calculation accumulated errors from both Δcpm and ninhydrin data. This error accumulation is evidenced by the improved reproducibility in that column when duplicate runs utilized the same ninhydrin analysis (compare the poor agreement between 3, 4 and 5, 6 to the good agreement between 3 and 4, or 5 and 6 or among 12–15).

Accuracy in evaluation of K_{TD} should be high since it is independent of ninhydrin data. The lack of dependence of K_{TD} upon duration of incubation, initial concentration of reagents, nature of Y or direction of approach to equilibrium testifies to this accuracy and offers another proof of the validity of the hve technique. Agreement between our data and the best from the literature is satisfactory. Interestingly, Δcpm for run 27 gives a very reasonable $K_{TD} = 0.5 \pm 0.35$ M for the reaction H_2O + AcPheTyrCOOH ⇌ $AcPheCOO^-$ + ${}^+NH_3$TyrCOOH.

Characteristics of the Amino-Enzymes. The observation

TABLE I: Extent of the Pepsin-Catalyzed Exchange of Ac-*Phe* and Ac-Phe-X at 35°.

Run[a]	X =	Exptl Condn[b]	% of S_0 Hydrolyzed	Δcpm	% of Remaining S Exchanged
1	Tyr	A, H, 1.25, 5	24	834 ± 123	7.2 ± 1.1
2		A, H, 1.25, 3	24[c]	773 ± 111	7.3 ± 1.1
3		A, H, 3, 6	50	1427 ± 132	21 ± 2
4		A, H, 3, 3	50[c]	1413 ± 199	20 ± 3
5		A, H, 3, 3	55	2061 ± 52	33 ± 1
6		A, H, 3, 3	55[c]	1890 ± 47	32 ± 1
7[d]		A, H, 3.25, 2	38	1780 ± 19	17 ± 0
8[d]		A, H, 3.25, 2	40	2843 ± 29	27 ± 0
9		A, H, 26, 4	91	42 ± 32	(100?)[e]
10		A, L, 2.5, 4	50	616 ± 47	20 ± 2
11		B, L, 3, 4	47	914 ± 84	27 ± 2
12		C, L, 3, 3	(47)[f]	706 ± 80	23 ± 3
13		C, L, 3, 4	(47)[f]	550 ± 93	18 ± 3
14		C, L, 3, 3	(47)[f]	662 ± 89	24 ± 3
15		C, L, 3, 3	(47)[f]	706 ± 104	24 ± 4
16	Phe	B, M, 2, 2	31	1307 ± 2	53 ± 0
17		B, N, 2, 1	(31)[g]	627	53
18		B, M, 4, 2	57	1183 ± 24	78 ± 2
19		B, N, 4, 1	(57)[g]	542	75
20	Phe-OEt[h]	B, N, 0.25, 3	50[i]	5 ± 8	0.6 ± 1

[a] All runs had [pepsin] = 1.5×10^{-4} M and [Ac-DL-*Phe*] = 4.8×10^{-2} M. [b] The first letter describes the buffer (A = 4.5 sodium acetate, B = 4.7 sodium acetate, C = 4.7 ammonium acetate at 37°); the second letter gives S_0 (H = 7.2–8.0 $\times 10^{-3}$ M, L = 2.8×10^{-3} M, M = 1.4×10^{-3} M, N = 7.0×10^{-4} M and M and N contain ~1% dimethylformamide); the first number specifies the number of hr of incubation; the second number defines the number of analyses performed. [c] Ninhydrin analyses performed on pooled samples of this run and that directly above. [d] Pepsin from pepsinogen employed. [e] The observed Δcpm implies 99.95% hydrolysis if 100% exchange is assumed. [f] The figure of run 11 was used; tlc analysis gave 40–50% hydrolysis. [g] Assumed. [h] Pepsin = 7.5×10^{-5} M. [i] Tlc estimate.

that a substantial amount of radioactive substrate forms and subsequently disappears during the peptic hydrolysis of Ac-Phe-Tyr and Ac-Phe-Phe establishes that Ac-DL-*Phe* has trapped an intermediate in each of these reactions. The hydrolyses of Ac-Phe-Tyr-NH_2 and Ac-Phe-Phe-OEt are very different. They yield little radioactive substrate. Tables I and II and Figure 2 describe the amount of trapping in similar but not identical terms, while the following quasi-analytical treatment provides a more quantitative measure of the trapping efficiency of Ac-*Phe* (X'). The analysis defines the maximum extent of transpeptidation for Ac-Phe-Tyr-NH_2 and Ac-Phe-Phe-OEt.

Equation 3 is a schematic version of eq 1 and defines the

$$S \xrightarrow{k_s} EY + X,X' \underset{[X']k_b}{\overset{k_s}{\rightleftharpoons}} S^* \qquad PR = \frac{[X']k_b}{k_f} \qquad (3)$$

$$\downarrow k_f$$

$$Y$$

partitioning ratio, *PR*, for the intermediate EY between exchange (transpeptidation) and hydrolysis. When applied to Ac-Phe-Tyr and Ac-Phe-Phe, the equation makes the following reasonably true assumptions: (a) hydrolysis is irreversible; (b) any return of EY to S without equilibration of X and X' can be neglected since it is undetectable by the present experiments; (c) [X'] is constant because dilution of Ac-*Phe* by Ac-Phe from hydrolyzed substrate can be neglected. (No effective value for [X'] is needed; it actually varies at most from 2.4×10^{-2} to 3.2×10^{-2} M during a run); (d) the velocity expression for the enzymatic hydrolysis, $v = k_c[E_0] \times [S]/(K_m + [S])$ is approximately first order during the time of the kinetic experiments ($v = k_c[E_{EFF}][S]/K_m$, $[E_{EFF}] \leq [E_0]$), since $K_m > [S]$ (Denburg *et al.*, 1968; Knowles *et al.*, 1969) and E_{EFF} is relatively static when [Ac-*Phe*] is constant and substrate and hydrolysis products have low and/or similar affinities for pepsin.

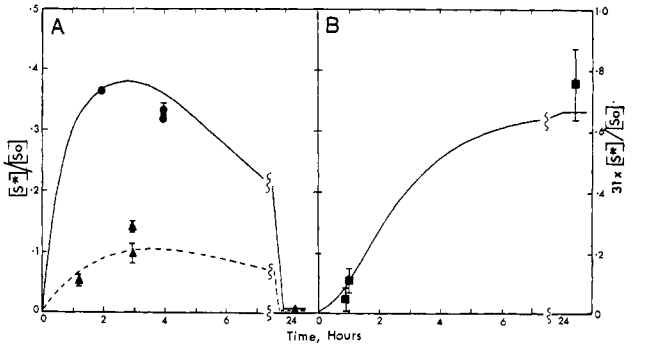


FIGURE 2: Comparison of the predicted and observed time-dependence for exchange of peptide substrates with Ac-DL-*Phe*. The curves in A plot [S*]/[S_0] of eq 6 with the first two sets of values in Table IV: (●) Ac-Phe-Tyr, (▲) Ac-Phe-Phe. Curve B represents 31 × [S*]/[S_0] of eq 8 for Ac-Phe-Tyr-NH_2 if $k_s = 0.7$ hr^{-1}, $k_s/k_p = 1.5$ M, and [X] + [X'] = 3.2×10^{-2} M.

TABLE II: Extent of the Pepsin-Catalyzed Exchange of Ac-*Phe* and Ac-Phe-X in 9% Methanol at 35°.

Run[a]	X =	Exptl Details[b,c]	% of S_0 Hydrolyzed	Δcpm	% of Remaining S Exchanged
21	Tyr	0, 2	0	−15 ± 32	
22[d]		7, 3	44	1778 ± 74	22 ± 1
23[e]		7, 3	47	1861 ± 119	25 ± 2
24[f]		6, 3	50–60[g]	385 ± 25	27–33[h]
25		6, 4	51	2266 ± 142	27 ± 2
26		6, 4	57	2195 ± 160	30 ± 2
27		48, 4	112	65 ± 50	(100?)
28[i]		0.75, 1	20	80	0.7
29[i]		1.25, 2	35	46 ± 39	0.5 ± 0.4
30	Tyr-NH_2	0, 3	0	−8 ± 9	
31[f]		1, 3	42	14 ± 1	0.9 ± 0.1
32		1, 6	50	22 ± 20	0.3 ± 0.3
33		1, 6	57	59 ± 15	0.8 ± 0.3
34		24, 3	106	388 ± 67	(100?)

[a] As for Table I. [b] $[S_0] = 7.2–8.0 \times 10^{-3}$ M in pH 4.5 sodium acetate buffer. [c] The first number specifies the hr of incubation and the second, the number of analyses. [d] Also contained [Ac-D-Phe-D-Tyr-NH_2] $= 8 \times 10^{-3}$ M. [e] Also contained [Ac-Phe-OMe] $= 1.2 \times 10^{-2}$ M. [f] 2.4×10^{-2} M Ac-L-Phe replaced the usual Ac-DL-*Phe*. [g] Tlc estimate, since the ninhydrin samples were lost. [h] 27% exchange if 50% hydrolysis, 33% exchange if 60% hydrolysis. [i] pH 1.9 phosphate buffer, $[E_0] = 7.5 \times 10^{-5}$ M.

Solution of eq 3 by a steady-state treatment of [EY] and insertion of the initial conditions [S] = $[S_0]$, [Y] = [S*] = 0 provides eq 4–6. First-order plots for the disappearance of unlabeled substrate (eq 4) and total substrate (eq 5) determine k_s and *PR*. A single run, when combined with the point for $t = 0$, is sufficient to define the two parameters. Table IV gives the results of several calculations and Figure 2A compares graphs of eq 6 to experimental data for two examples. The fact that k_s for Ac-Phe-Phe is about twice as large as k_s for Ac-Phe-Tyr accords well with the report that k_c/K_m for the former is twice that for the latter at pH 2 (Jackson *et al.*, 1966) and strengthens our confidence in this analysis.

$$d[S]/dt = -k_s[S] \quad (4)$$

$$d([S] + [S^*])/dt = -([S] + [S^*])k_s/(1 + PR) \quad (5)$$

$$[S^*]/[S_0] = e^{-k_s t/(1+PR)} - e^{-k_s t} \quad (6)$$

The approximations of eq 3 are not so good when the hydrolysis of Ac-Phe-Tyr-NH_2 or Ac-Phe-Phe-OEt is treated. For these cases substrate affinity for pepsin is higher (Denburg *et al.*, 1968; Knowles *et al.*, 1969) and reversal of products to substrate cannot be ignored (run 34). If equilibration contributes appreciably to [S*], eq 3–6 overestimate *PR* since they attribute *all* S* to the trapping of intermediate EY. A correction for the contribution from equilibration must be made and eq 7 enables us to do it. Equation 7 approximately

$$S \xrightarrow{k_s} X,X' + Y \underset{([X]+[X'])k_p}{\overset{k_s}{\rightleftharpoons}} S^* \quad (7)$$

represents the product equilibration phenomenon and possesses the great virtue that the differential equation describing its time dependence is readily integrated if ([X] + [X']) is constant (assumed to be 3.2×10^{-2} M) and [S*] ≪ ([S] + [Y]). Equation 4 again defines the rate of disappearance of substrate and eq 8 predicts the time dependence of [S*]. The curve for Ac-Phe-Tyr-NH_2 in Figure 2B results when $k_s = 0.7$ hr^{-1} and $k_s/k_p = K_{exp} = 1.5$ M (Table III). The plot reveals that substrate–product equilibration can account for all the Ac-*Phe*-Tyr-NH_2 detected. The true partitioning

$$[S^*]/[S_0] = (1 - e^{-k_s t} - k_s t e^{-k_s t})([X] + [X'])/(k_s/k_p) \quad (8)$$

ratios for Ac-Phe-Tyr-NH_2 and Ac-Phe-Phe-OEt may be considerably less than the 0.01 deduced from eq 3 to 6.

Seven features, A–G, of the intermediates which may or may *not* accompany the hydrolyses studied can be deduced from the tabulated data. An extended discussion of E and G, the features of greatest significance, will follow a brief commentary on all seven points.

(A) The intermediate from Ac-Phe-Phe undergoes transpeptidation with Ac-DL-*Phe* more readily than does that from Ac-Phe-Tyr (Figure 2A and Table IV). In contrast, Ac-Phe-Phe appeared to give less transpeptidation product than Ac-Phe-Tyr with Z-Phe as acceptor (Mal'tsev *et al.*, 1966). The specificity of the amino–enzymes toward acceptors could cause different results for the two experiments but no definite conclusion is possible since the behavior of the transpeptidation products Z-Phe-Phe and Z-Phe-Tyr in the earlier work is unknown. (B) Partitioning of the intermediate from Ac-Phe-Tyr is identical in water and 9% methanol (runs 3–6, 10 *vs.* 25, 26). It presumably is the same in 3% methanol, the solvent Denburg *et al.* (1968) employed in kinetic studies. (C) Two- to threefold changes in $[S_0]$ fail to affect partitioning of the intermediate from Ac-Phe-Tyr or Ac-Phe-Phe (runs 3–6 *vs.* 10, 16 *vs.* 17, 18 *vs.* 19). This observation and the absence of much Tyr-Tyr among the reaction products from (Ac-Phe-Tyr + Ac-*Phe*) makes improbable any mechanism for the Ac-*Phe*-transpeptidation reaction which requires interaction between amino–enzyme intermediate and substrate. (D) Ac-Phe-Tyr yields no trappable intermediate at pH 2, where substrate, acceptor, and enzyme-

TABLE III: Equilibrium Constant for the Hydrolysis of the Peptide Bond as Determined by Equilibrating Ac-DL-*Phe* and X.[a]

Run[b]	X	$[X_0]$, M	t (hr)	Δcpm	K_{exp}[c,d] (M)	K_{TD}[d,e] (M)
1[f]	Tyr-NH_2		24	388 ± 67	1.3 ± 0.2	1.2
2		0.087	24	3354 ± 590	1.8 ± 0.3	1.6
3			49	3672 ± 193	1.8 ± 0.1	1.6
4		0.174	24	8490 ± 150	1.27 ± 0.02	1.1
5			48	9238 ± 97	1.24 ± 0.01	1.1
6	D-Tyr-NH_2	0.174	48	66 ± 43		
7	*g*					2.0
8	Phe-OMe	0.088	23	2472 ± 51	0.96 ± 0.02	0.85
9			44	2373 ± 37	1.03 ± 0.02	0.92
10[h]	Tyr-OEt					0.71

[a] All our experiments refer to pH 4.5 sodium acetate buffer, 8.7% methanol at 35°, with [pepsin] = 1.5×10^{-4} M and [Ac-DL-*Phe*$_0$] = 4.8×10^{-2} or 2.4×10^{-2} M (runs 8 and 9). [b] Three analyses of all runs except six for runs 2 and 4. [c] The stoichiometric equilibrium constant. [d] Activity of water was taken as 1.0. [e] For the pH-independent cleavage of the peptide bond to fully ionized products, assuming the pK of Ac-Phe is 3.60 and that X is completely protonated at pH 4.5. The corresponding values for $-\Delta F°$ (cal/mole) are: 180 (average of 1–5), 420 (7); −75 (8,9), and −210 (10). [f] From the hydrolysis of Ac-Phe-Tyr-NH_2, run 34. [g] The hydrolysis of Bz-Tyr–Gly-NH_2 in water at 25° (Dobry *et al.*, 1952). [h] In water at 25° (Kozlov *et al.*, 1966).

TABLE IV: Kinetic Constants Characterizing Eq 3–6 and Figure 2.

Substrate	Runs[a]	k_s (hr^{-1})	$k_s/(1 + PR)$ (hr^{-1})	PR[b]	pH
Ac-Phe-Tyr	1–6	0.32	0.24	0.33	4.5
Ac-Phe-Phe	16–19	0.58	0.21	1.8	4.7
Ac-Phe-Tyr	10	0.37	0.28	0.33	4.5
Ac-Phe-Tyr	11	0.32	0.21	0.49	4.7
Ac-Phe-Tyr-NH_2	30, 32–34	0.70	≤0.70	≤0.01	4.5
Ac-Phe-Phe-OEt	20	2.8	≤2.8	≤0.01	4.7

[a] Tables I and II. [b] $PR = [X']k_b/k_f$ and expresses the partitioning of the enzyme intermediate between exchange and hydrolysis.

bound tyrosine carboxyl groups are almost certainly undissociated (runs 28,29). (E) Neither Ac-Phe-Tyr-NH_2 nor Ac-Phe-Phe-OEt produces a trappable intermediate at pH 4.5–4.7 (runs 20, 32, 33) although the acceptor is in the properly ionized form, Ac-*Phe*COO^- (Kitson and Knowles, 1971b). The unlikelihood that the failure to detect radioactive substrate is an experimental artifact was discussed under control 6. Runs 9, 21, and 27–30 further testify to the validity of a small observed Δcpm, for with each of these six runs prior knowledge made Δcpm ≈ 0 the anticipated result. Runs 28 and 29 (*cf.* D) are particularly noteworthy; they revealed few counts per minute in a run strip at a spot where a great many appeared with other kinetic samples. Runs 24 and 31 establish the identical behavior of Ac-DL-*Phe* and Ac-L-*Phe* as acceptors and eliminate the possibility that Ac-D-Phe in racemic acceptor is the trivial cause of the absence of transpeptidation with Ac-Phe-Tyr-NH_2 and Ac-Phe-Phe-OEt. (F) Runs 10 (pH 4.5) and 11 (pH 4.7), performed simultaneously, suggest that a preferential increase in transpeptidation accompanies a pH rise in a region where substrate and acceptor are entirely in the anionic form (see Table IV). Further experiments will check this conclusion. (G) Runs 12–15 should reproduce the conditions under which Kozlov *et al.* (1965) measured the Ac-Phe-Tyr-promoted exchange of Ac-PheCOOH with $[^{18}O]OH_2$. Only 25% of the residual substrate has exchanged with Ac-*Phe*.

Do Transpeptidation and Hydrolysis Share a Common Rate-Determining Step? The values of k_s for Ac-Phe-Tyr and Ac-Phe-Phe derived from eq 3 to 6 depend upon both ninhydrin and counting data since they define the total rate constant for production of EY no matter whether Y or S* is ultimately formed. If eq 3 is correct and transpeptidation is a consequence of, and not competitive with, the rate-determining step for hydrolysis, k_s should approximate the rate constant for hydrolysis observed in the absence of transpeptidation. Under the conditions specified in developing eq 3–6, $k_s = k_c[E_0]/K_m$.

For Ac-Phe-Tyr, the data and equations of Denburg *et al.* (1968) predict $k_c/K_m = 3600\ M^{-1}\ sec^{-1}$ at pH 4.5, 35°, 3% methanol. The presence of 4.8×10^{-2} M Ac-DL-Phe should decrease the rate of hydrolysis by ~1.5× (Kitson and Knowles, 1971b) and the absence of methanol should increase it ~1.5× (Inouye *et al.*, 1966). If $E_0 = 1.5 \times 10^{-4}$ M, the calculated k_s of 0.5 hr^{-1} compares favorably to the 0.32 hr^{-1} observed (Table IV).

A similar calculation for Ac-Phe-Phe, employing pH 2 kinetic data (Jackson *et al.*, 1966) and assuming the pH dependencies of the kinetic parameters for Ac-Phe-Tyr and Ac-Phe-Phe are identical, gives $k_s = 0.6\ hr^{-1}$ at pH 4.7. Table IV shows the identical value.

Since k_s for Ac-Phe-Tyr-NH_2 is strictly a hydrolytic rate constant, the agreement between it and the value calculated

from the data of Denburg *et al.*, after allowance is made for the Ac-DL-Phe and methanol effects, merely confirms that spectrophotometric and ninhydrin kinetic methods give comparable results.

The demonstrated equivalence of the values for k_s derived from the present experiments and from hydrolyses is consistent with the hypothesis, which underlies eq 3–6, that hydrolysis and transpeptidation share a common rate-determining step. The parallelism between rates of hydrolysis and of transpeptidation for three acetylated dipeptides have led Mal'tsev *et al.* (1966) to the same conclusion.

Discussion

Peptic hydrolysis of Ac-Phe-Tyr-NH_2 or Ac-Phe-Phe-OEt at pH 4.5 surprisingly yields no intermediate which Ac-DL-*Phe* can trap. The partitioning ratio for Ac-Phe-Tyr, PR_{Tyr}, is at least 100 times greater than that for Ac-Phe-Tyr-NH_2, PR_{TyrAm}.

We are aware of no other attempt to react a known acceptor with a donor whose amino portion bears a nonionizable C-terminal carboxyl function. The experiment of closest resemblance is that of Kitson and Knowles (1971b), who reported that incubation of Ac-*Phe*, Ac-Phe-Phe-Gly, and pepsin at pH 4.7 yields Ac-*Phe*-Phe-Gly. These authors assumed but never unambiguously established that a trapped intermediate and not substrate–product equilibration was the source of labeled substrate. Probably both factors contribute appreciably to formation of Ac-*Phe*-Phe-Gly, for the amount of Ac-*Phe*-Phe-Gly detected somewhat surpasses that expected from equilibration alone. Ac-Phe-Phe-Gly consequently resembles Ac-Phe-Tyr, not Ac-Phe-Tyr-NH_2, in behavior. It yields a trappable intermediate and the pH dependence of its rate of hydrolysis is identical with that for Ac-Phe-Phe (Knowles *et al.*, 1969).

Mechanistic Implications of the Partitioning Ratios. Discussion of the significance of the partitioning ratios utilizes eq 9, an expanded version of eq 1. Equation 9 has the following three properties: (a) it designates dissociation constants as K's and employs the notation of Denburg *et al.* (1968); (b) it explicitly recognizes the ionization of the carboxyl group of Tyr derived from Ac-Phe-Tyr; and (c) it makes no provision for conversion of E-TyrCOO$^-$ to product (k_5' step) since such a pathway would tend to make PR_{Tyr} less than PR_{TyrAm} while we seek to explain why PR_{Tyr} exceeds PR_{TyrAm}. The top row of eq 9 completely describes the hydrolysis of Ac-Phe-Tyr-NH_2 if $CONH_2$ is substituted for COOH. Equation 9 is also properly compatible with the equilibration data. It defines $(K_{exp})_{Am}$, the experimental stoichiometric equilibrium constant for hydrolysis of Ac-Phe-Tyr-NH_2 at pH 4.5, as $k_3k_5/k_4k_6k_{ESH}K_p$. $(K_{exp})_{Tyr}$, the corresponding quantity for Ac-Phe-Tyr, is approximately $(K_T/K_S)(K_{exp})_{Am} \approx 10\ (K_{exp})_{Am}$, since p$K_T$ = 2.2, pK_S = 3.5, and $^+NH_3$-TyrCOO$^-$ and Ac-Phe-TyrCOO$^-$ are the predominant species in solution at pH 4.5. Run 27 gives $(K_{exp})_{Tyr} = 7.7 \pm 5.8$ M (*cf.* Table III).

$$
\begin{array}{ccccc}
E + SH & \overset{K_{ESH}}{\rightleftharpoons} & E,SH & \underset{k_4[X]}{\overset{k_3}{\rightleftharpoons}} & X + E\text{-TyrCOOH} \\
K_S \downarrow\uparrow & & & & \downarrow\uparrow K_{EI} \\
E + S & \overset{K_{ES}}{\rightleftharpoons} & E,S & \underset{k_4'[X]}{\overset{k_3'}{\rightleftharpoons}} & X + E\text{-TyrCOO}^-
\end{array}
\qquad
\begin{array}{ccc}
\overset{k_5}{\underset{k_6}{\rightleftharpoons}} & E + NH_2\text{-TyrCOOH} & \\
 & K_P \downarrow\uparrow & \\
 & {}^+NH_3TyrCOOH & \\
 & K_T \downarrow\uparrow & \\
 & {}^+NH_3TyrCOO^- & (9)
\end{array}
$$

The observation $PR_{Tyr} \gg PR_{TyrAm}$ requires either that the ratio $k_4[X]/k_5$ for E-TyrCOOH is at least 100 times greater than for E-TyrCONH$_2$ (and the same must hold for E-PheCOOH *vs.* E-PheCOOEt) or that eq 9 is wrong. Let us consider the likelihood of the first alternative.

We believe it improbable that enzymatic discrimination between E-TyrCOOH and E-TyrCONH$_2$ is sufficient to explain why $PR_{Tyr} \gg PR_{TyrAm}$, but have been unable to design an experiment to either prove or disprove this opinion. The inability of pepsin to cleave the peptide analog Z-His-Phe-L-phenylalaninol (Inouye and Fruton, 1968) or the anions Ac-Phe-TyrCOO$^-$, Ac-Phe-PheCOO$^-$, etc. (Denburg *et al.*, 1968; Cornish-Bowden and Knowles, 1969), undeniably establishes pepsin's sensitivity to the nature of the functional group at the position in question. Intuition suggests that the differences between these unreactive materials and hydrolyzable substrates are more profound than those between TyrCOOH and TyrCONH$_2$, where stereochemical, electronic, and hydrogen-bonding properties are so similar. The acid is less basic than the amide but Ac-Phe-Phe-OEt should be about as basic as the acid and it also fails to exchange with Ac-DL-*Phe*. More persuasive than intuition are experimental results at low pH, where TyrCOOH is undissociated. The kinetics of hydrolysis of Ac-Phe-Tyr-NH_2 and Ac-Phe-Tyr (Denburg *et al.*, 1968) or Ac-Phe-Phe-NH_2 and Ac-Phe-Phe (Cornish-Bowden and Knowles, 1969) are identical there, and Ac-Phe-Tyr at pH 2, like Ac-Phe-Tyr-NH_2 at pH 4.5, fails to exchange with Ac-DL-*Phe*. Consequently there is neither theoretical or experimental support for the enzyme-specificity argument.

Amino–Enzyme Disaster. I. The assumption that $k_4[X]/k_5$ of eq 9 should be essentially the same for E-TyrCOOH and E-TyrCONH$_2$ seems to us much more reasonable than the enzyme-specificity argument. Enzymatic distinctions between COOH and CONH$_2$ in transfer to Ac-*Phe* (k_4) should operate to a similar extent in transfer to H_2O (k_5). As the analysis below shows, the postulated identity of $k_4[X]/k_5$ is irreconcilable with the observation $PR_{Tyr} \gg PR_{TyrAm}$ and hence with the amino–enzyme mechanism as exemplified by eq 9.

The ratio $k_4[X]/k_5$ determines PR_{TyrAm} but its relationship to PR_{Tyr} depends upon the importance of the k_4' route of eq 9. If Ac-Phe-TyrCOO$^-$ completely resists hydrolysis ($k_3'/K_{ES} = 0$), the reverse k_4' process is also disallowed and eq 9 falsely demands $PR_{TyrAm} = PR_{Tyr}$.

Introduction of a sufficiently rapid k_4' pathway could in principle make PR_{Tyr} large enough. Actually, the kinetics of the hydrolysis of Ac-Phe-Tyr (Denburg *et al.*, 1968) set an upper limit to the contribution from k_4' that is grossly inadequate. Calculation of this limit proceeds as follows. For the reversible reaction of eq 9, requirement that at equilibrium [E-TyrCOOH]/[E][SH] be independent of pathway defines the relationship $k_3/k_4K_{ESH} = k_3'Ks/k_4'K_{ES}K_{EI}$. The data of Denburg *et al.* give $k_3/K_{ESH} = 30\ M^{-1}\ sec^{-1}$ and pK_S = 3.5. The rate of reaction of the species Ac-Phe-TyrCOO$^-$ was immeasurably slow but the assumption that the rate of hydrolysis of Ac-Phe-Tyr at pH 4.5 could be as fast as that observed at pH 4.0 (it surely is much less) defines $k_3'/K_{ES} \leq 5\ M^{-1}\ sec^{-1}$ (eq 15 of Denburg *et al.*). Consequently the kinetic data specify $k_4'K_{EI}/k_4 \leq 1.7 \times 10^{-4.5}$ M.

$$
\left.
\begin{aligned}
PR_{Tyr} &= (k_4[X][\text{E-TyrCOOH}] + \\
&\qquad k_4'[X][\text{E-TyrCOO}^-])/k_5[\text{E-TyrCOOH}] \\
&= (k_4[X] + k_4'[X]K_{EI}/[H^+])/k_5 \\
&= PR_{TyrAm}(1 + k_4'K_{EI}/k_4[H^+])
\end{aligned}
\right\} \quad (10)
$$

Equation 10 expresses PR_{Tyr} for mechanism 9 in terms of PR_{TyrAm} and other known quantities. It falsely predicts that PR_{Tyr} will be no more than $(1 + \leq 1.7) \approx 3$ times larger than PR_{TyrAm} at pH 4.5.

Amino–Enzyme Disaster. II. The previous argument for the inadequacy of mechanism 9 rests upon the unproven assumption that enzyme specificity is not responsible for $PR_{Tyr} \gg PR_{TyrAm}$. Comparison of our data for Ac-Phe-Tyr–Ac-*Phe* exchange to those of Kozlov *et al.* (1965) for an analogous experiment provides independent evidence for the failure of eq 9 (see G in Results).

Kozlov *et al.* measured the amount of exchange between $^{18}OH_2$ and Ac-PheCOOH (as Ac-DL-Phe) in the absence and presence of Ac-Phe-Tyr under standard conditions. The added Ac-Phe-Tyr had no effect at pH 2.4 but at pH 4.7 it increased the extent of exchange by 7%, from 34.0 ± 1.0 to 41.3 ± 1.1%. Control experiments indicated that the portion of eq 9 rewritten as eq 11 best accounted for this enhanced exchange and eq 11 can also describe our transpeptidation experiment. Each cycle of reaction 11, when X = Ac-Phe and X′ = Ac-*Phe*, results in the exchange of one oxygen atom of Ac-PheCOOH with $^{18}OH_2$ and the conversion of one molecule of Ac-Phe-Tyr into Ac-*Phe*-Tyr. If eq 11 accounts for both series of experiments, the amount of enhanced $^{18}OH_2$ exchange observed by Kozlov *et al.* should be calculable from the data for runs 12–15 and the known concentrations of reactants. This calculation yields an upper limit, since Ac-*Phe* counts every cycle of eq 11 but ^{18}O counts only those cycles where X′COOH has not previously undergone the relatively fast Ac-Phe-Tyr-independent exchange with $^{18}OH_2$.

$$XCONHY + X'COOH + {}^{18}OH_2 \longrightarrow X'CONHY + XCO^{18}OH + OH_2 \quad (11)$$

For runs 12–15, $[S_0] = 2.8 \times 10^{-3}$ M, ~50% hydrolysis has occurred and ~25% of the residual substrate has undergone exchange with Ac-*Phe*. These numbers give [X′CONHY] $= (2.8 \times 10^{-3}\ M)(0.5)(0.25) = 3.5 \times 10^{-4}$ M. For [X′COOH] $= 2.4 \times 10^{-2}$ M the concentration of exchangeable oxygen atoms is 4.8×10^{-2} M and the added Ac-Phe-Tyr should have increased the exchange of Ac-PheCOOH with $^{18}OH_2$ by only $(100)(3.5 \times 10^{-4})/(4.8 \times 10^{-2}\ M) = 0.75\%$. The simple amino-enzyme mechanism cannot explain why this figure is only one-tenth that observed. The supposed enzyme–tyrosine intermediate seemingly catalyzes multiple exchanges between Ac-PheCOOH and $^{18}OH_2$ before reverting to Ac-*Phe*-Tyr or proceeding to Tyr.

The difference between the Ac-*Phe*- and $^{18}OH_2$ measurements for the rate of exchange between Ac-Phe-Tyr and Ac-Phe is undoubtedly real. Neither we nor Kozlov *et al.* detected transpeptidation at low pH and the calculation above requires that their less sensitive technique should have given the same negative result at pH 4.7. The cited incompatibility between eq 11 and the Ac-*Phe*- and $^{18}OH_2$ observations thus relies on a comparison of two pieces of data from Kozlov *et al.* and not on a more questionable comparison of numerical data from two different laboratories.[3]

Attempts to Salvage the Amino–Enzyme. We have found no modification of eq 9 which would explain both the difference in behavior of Ac-Phe-Tyr and Ac-Phe-Tyr-NH_2 and the disparity between the Ac-*Phe*- and $^{18}OH_2$ data. Described below are three of our less grotesque efforts. (1) Postulation that Ac-Phe-Tyr-Tyr is an obligatory intermediate in the exchange between Ac-Phe-Tyr and Ac-*Phe* or that Ac-Phe-Tyr-NH_2 but not Ac-Phe-TyrCOO$^-$ complexes strongly to amino–enzyme and this complex undergoes hydrolysis but not transpeptidation could lead to a resolution of the $PR_{Tyr} - PR_{TyrAm}$ dilemma. Point C of the Results and the failure of Ac-D-Phe-D-Tyr-NH_2 or Ac-Phe-OMe to affect PR_{Tyr} (runs 22 and 23; probably only the ester binds to the intermediate (Kitson and Knowles, 1971a,b) renders either idea unlikely. (2) A "sticky-enzyme" hypothesis is harder to disprove. It postulates: (a) no Ac-*Phe*-Tyr-NH_2 is produced because the E-TyrCONH$_2$,Ac-Phe (or E-TyrCOOH,Ac-Phe) complex first formed in the k_3 step reverts to substrate or proceeds to E + Ac-Phe + Tyr-NH_2 before it exchanges Ac-Phe for Ac-*Phe;* (b) relatively rapid ionization of E-TyrCOOH, Ac-Phe to E-TyrCOO$^-$, Ac-Phe permits exchange of Ac-Phe for Ac-*Phe* in the anionic complex to be competitive with k_4[X] and k_5 and to ultimately yield Ac-*Phe*-Tyr; and (c) because of microscopic reversibility, the ternary complex E,Ac-Phe,Tyr-NH_2 is the direct precursor of amino–enzyme going from right to left in eq 9. A demonstration that Ac-Phe-OEt and Ac-Phe-NH_2, noncompetitive inhibitors for Ac-Phe-Phe-Gly (Kitson and Knowles, 1971b) exhibit competitive inhibition toward Ac-Phe-Tyr-NH_2 at pH 4.5 might lend some credence to the hypothesis. (3) The "two-intermediate" mechanism is also unsatisfactory. Silver *et al.* (1970) have presented the latest version of this mechanism in which a dicovalent enzyme–substrate intermediate, X-E-BH, precedes the amino–enzyme, E-BH. Equation 12, an

$$\begin{array}{ccccccc} E + SH & \overset{K_{ESH}}{\rightleftharpoons} & E,SH & \underset{k_4}{\overset{k_3}{\rightleftharpoons}} & X\text{-}E\text{-}BH & \underset{k_6[X]}{\overset{k_5}{\rightleftharpoons}} & E\text{-}BH + X \\ \Big\updownarrow K_S & & & & \Big\updownarrow K_1 & & \Big\updownarrow K \\ E + S & & & & X\text{-}E\text{-}B & \underset{k_6'[X]}{\overset{k_5'}{\rightleftharpoons}} & E\text{-}B \end{array} \quad \begin{array}{c} \overset{k_7}{\rightleftharpoons} \\ \underset{k_8}{} \end{array} E + BH \qquad (12)$$

abbreviated representation of the scheme, defines the essential mechanism. Compared to eq 9 (BH = TyrCOOH or TyrCONH$_2$), eq 12 ignores transformation of E + S into X-E-B and the reverse process, shown earlier to be of negligible importance, and omits K_P and K_T.

We discovered no version of eq 12 which explains why $PR_{Tyr} \gg PR_{TyrAm}$ and also satisfies microscopic reversibility if the behavior of TyrCOOH- and TyrCONH$_2$-containing species are assumed to be identical (*i.e.*, that the enzyme-specificity argument is wrong). Schemes fail no matter whether: (a) k_3 or k_5 is rate limiting in kinetic experiments where $[Ac\text{-}Phe]_0 = 0$. Definitive evidence on which step is slow is lacking (Fruton, 1970). Rate-limiting k_3 has the virtue of bringing the Ac-*Phe* and ^{18}O data into concordance, since if k_3 is slow, $k_5 \gg k_4$. This implies that (E-BH, E-B) can catalyze exchange between Ac-PheCOOH and $^{18}OH_2$ without concomitant reversion to SH (formation of Ac-*Phe*-Tyr) *if* the k_5,k_6 step breaks and makes the carbonyl–oxygen bond of X = AcPheCOOH. The proviso that this interconversion break the carbonyl–oxygen bond contradicts the mechanism formulated by Silver *et al.* (1970) and the proposal of Kitson and Knowles (1971a,b) that Ac-Phe is released as the anion, Ac-PheCOO$^-$. Both of these possible objections rely on the interpretation of kinetic data and a way to bring the kinetic

[3] Repetition of the $^{18}OH_2$-experiments in collaboration with Dr. T. P. Stein has yielded preliminary results in accord with the earlier work. The effect of added Ac-Phe-Tyr-NH_2 on the $^{18}OH_2$–Ac-PheCOOH exchange will also be determined.

observations into agreement with an eq 12 like mechanism might be found. (b) X-E-B and E-B interconvert more or less rapidly than X-E-BH and E-BH. (c) pK_1 does or does not equal pK_2. Seemingly a large pK_1 plus small pK_2 could raise the effective concentration of X-E-BH relative to E-BH and enable Ac-Phe-Tyr to give more transpeptidation than Ac-Phe-Tyr-NH_2. However, these species enter into both forward and backward reactions. Manipulation of the pK's can be shown to leave PR_{Tyr} unaltered.

Conclusion

Mechanistic generalizations about pepsin founded on the transpeptidation reaction certainly require more experimental justification than is now available. The reaction is of extremely limited scope, characterizing only the hydrolysis at pH $\geq$ 4 of substrates like Ac-Phe-Tyr or Ac-Phe-Phe which possess a C-terminal carboxyl group near the point of bond rupture. A shift to pH 2, the pH of optimum peptic activity, or to Ac-Phe-Tyr-NH_2 as substrate, a better model for the normal protein substrates of the enzyme, abolishes it. Furthermore, the amino–enzyme mechanism, the prime evidence for which is the transpeptidation reaction, quantitatively fails to accommodate either the *PR* data or the $^{18}OH_2$–Ac-*Phe* discrepancy in a simple manner. It best rationalizes these observations if an unlikely enzyme-specificity argument is grafted to a variant of eq 12 that meets the conditions specified above. The result is not a very attractive mechanism.

Dropping the amino–enzyme as an intermediate directly on the hydrolysis pathway has some appeal. One known pepsin preparation shows no transpeptidolytic activity (Neumann and Sharon, 1960), and mechanisms for transpeptidation invoking nucleophilic participation or other eccentric behavior of the carboxylate anion derived from the amino portion of the substrate can be imagined.

Acknowledgments

The criticisms and unpublished data of Drs. T. M. Kitson and J. R. Knowles aided preparation of the manuscript for publication.

References

Anderson, G. W., Zimmerman, J. E., and Callahan, F. M. (1967), *J. Amer. Chem. Soc. 89*, 5012.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Bush, E. T. (1963), *Anal. Chem. 35*, 1024.

Clement, G. E., Snyder, S. L., Price, H., and Cartmell, R. (1968), *J. Amer. Chem. Soc. 90*, 5603.

Cornish-Bowden, A. J., and Knowles, J. R. (1969), *Biochem. J. 113*, 353.

Cohen, S. G., and Schultz, R. M. (1968), *J. Biol. Chem. 243*, 2607.

Denburg, J. L., Nelson, R., and Silver, M. S. (1968), *J. Amer. Chem. Soc. 90*, 479.

Dobry, A., Fruton, J. S., and Sturtevant, J. M. (1952), *J. Biol. Chem. 195*, 149.

Fruton, J. S. (1970), *Advan. Enzymol. 33*, 401.

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

Ginodman, L. M., Lutsenko, N. G., Barshevskaya, T. N., and Somova, V. V. (1971), *Biokhimiya 36*, 604.

Herbst, R. M., and Shemin, D. (1943), *in* Organic Synthesis, Collect. Vol. II, New York, N. Y., Academic Press, p 1.

Hofmann, K., Thompson, T. A., Yajima, H., Schwartz, E. T., and Inouye, H. (1960), *J. Amer. Chem. Soc. 82*, 3715.

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

Inouye, K., Voynick, I. M., Delpierre, G. R., and Fruton, J. S. (1966), *Biochemistry 5*, 2473.

Jackson, W. T., Schlamowitz, M., and Shaw, A. (1966), *Biochemistry 5*, 4105.

Kitson, T. M., and Knowles, J. R. (1970), *Chem. Commun.*, 361.

Kitson, T. M., and Knowles, J. R. (1971a), *Biochem. J. 122*, 241.

Kitson, T. M., and Knowles, J. R. (1971b), *Biochem. J. 122*, 249.

Knowles, J. R., Sharp, H., and Greenwell, P. (1969), *Biochem. J. 113*, 343.

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

Kozlov, L. V., Ginodman, L. M., Orekhovich, V. N., and Valueva, T. A. (1966), *Biokhimiya 31*, 315.

Mal'tsev, N. I., Ginodman, L. M., Orekhovich, V. N., Valueva, T. A., and Akimova, L. N. (1966), *Biokhimiya 31*, 983.

Mitz, M. A., Axelrod, A. E., and Hofmann, K. (1950), *J. Amer. Chem. Soc. 72*, 1231.

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

Neumann, H., and Sharon, N. (1960), *Biochim. Biophys. Acta 41*, 370.

Overby, L. R., and Ingersoll, A. W. (1951), *J. Amer. Chem. Soc. 73*, 3363.

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

Silver, M. S., Stoddard, M., and Stein, T. P. (1970), *J. Amer. Chem. Soc. 92*, 2883.

Stahl, E. (1962), Thin-Layer Chromatography, New York, N. Y., Academic Press.