Soc. 73, 5553.
Wallach, O., and Hoffmann, M. (1877), *Ann. Chem. 184*, 79.
Wieland, T., and Biener, H. (1961), *Tetrahedron 15*, 1.
Wieland, T., Lang, H. U., and Liebsch, D. (1955), *Ann. Chem. 597*, 227.
Wieland, T., and Stimming, D. (1953), *Ann. Chem. 579*, 97.
Wieland, T., and Urbach, H. (1958), *Ann. Chem. 613*, 84.

New Synthetic Substrates for Pepsin*

Ken Inouye, Irene M. Voynick, Georges R. Delpierre, and Joseph S. Fruton

ABSTRACT: The synthesis of several new peptide substrates for crystalline swine pepsin is described. They include benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-phenylalanine ethyl ester and related peptide derivatives in which one or both phenylalanyl residues have been replaced by L-tyrosyl or L-tryptophyl residues. These compounds, as well as glycylglycyl-L-phenylalanyl-L-phenylalanine ethyl ester, are cleaved rapidly at the peptide bond between the two aromatic amino acid residues, the pH optimum being near 4. Benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-phenylalanine is hydrolyzed by pepsin more slowly than the corresponding ethyl ester, with a pH optimum near 3. These differences in pH optima, and the behavior of previously known synthetic substrates such as acetyl-L-phenylalanyl-L-tyrosine, are discussed in terms of the hypothesis that an α-carboxylate group adjacent to the sensitive peptide bond is inhibitory to pepsin action. Several of the new synthetic substrates enhance the rate of inactivation of pepsin by diphenyldiazomethane.

Since the discovery of the first synthetic substrates (*e.g.*, Z-Glu-Tyr[1]) for crystalline swine pepsin (Fruton and Bergmann, 1939), several investigators, notably Baker (1951), have added peptide derivatives that have proved to be valuable for the study of the kinetics of pepsin action. In such studies (Silver *et al.*, 1965; Cornish-Bowden and Knowles, 1965; Jackson *et al.*, 1965), the preferred substrates have been acetyl dipeptides such as Ac-Phe-Tyr and Ac-Phe-Dityr, which are hydrolyzed optimally near pH 2. In connection with our studies on the effect of pepsin substrates on the inactivation of the enzyme by diphenyldiazomethane (Delpierre and Fruton, 1965), it was necessary to prepare synthetic substrates that do not have a carboxyl group, as the reagent readily attacks such groups to form benzhydryl esters (Hiskey and Adams, 1965; Aboderin *et al.*, 1965). Because the conversion of the carboxyl group of an acetyl dipeptide to an ester or amide usually tends to make the resulting compound even less soluble in water than the parent compound, we decided to replace the acetyl group by either benzyloxycarbonylhistidyl or glycylglycyl, thus providing a site of protonation of the acylpeptide ester. Accordingly, the following compounds were prepared: Z-His-Phe-Phe-OEt, Z-His-Phe-Tyr-OEt, Z-His-Phe-Trp-OEt, Z-His-Tyr-Tyr-OEt, and Gly-Gly-Phe-Phe-OEt. All these compounds were found to be good substrates for pepsin, the cleavage occurring between the two aromatic amino acid residues. In the present communication, we report the synthesis of the new substrates and of several compounds related to them, some features of the kinetics of their hydrolysis by pepsin, and their effect on the inactivation of pepsin by DDM.

Experimental Section

Enzymic Studies. All experiments (except when otherwise noted) were performed with a single preparation of twice-crystallized swine pepsin (Worthington Biochemical Corp. Lot No. PM 708), whose specific activity was found to be 2595 ± 115 units/mg (mean of five determinations) when assayed with denatured hemoglobin (1.67%) as substrate at pH 1.8 and 30°. After an incubation period of 10 min, 1.67 volumes of 5% TCA was added to the assay mixture, and the absorbance of the filtrate was determined at 280 mμ (Anson, 1938). One pepsin unit is defined as the amount of enzyme that produces in this assay an increase in absorbance of 0.01 above the blank, using

* From the Department of Biochemistry, Yale University, New Haven, Connecticut. *Received April 22, 1966.* This work was aided by grants from the U. S. Public Health Service (GM-06452) and from the National Science Foundation (G-7451).

[1] Abbreviations used: Gly, glycyl; Phe, L-phenylalanyl; Tyr, L-tyrosyl; Trp, L-tryptophyl; His, L-histidyl; Glu, L-glutamyl; Dityr, diiodo-L-tyrosyl; Ac, acetyl; Bz, benzoyl; Z, benzyloxycarbonyl; OEt, ethoxy; OBu′, *t*-butoxy; Onp, *p*-nitrophenoxy; DCC, dicyclohexylcarbodiimide; DDM, diphenyldiazomethane ; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; DMF, dimethylformamide; THF, tetrahydrofuran. The abbreviated designation of derivatives of amino acids and peptides accords with the proposals of the Joint IUPAC-IUB Commission on Biochemical Nomenclature.

1-cm cells in a Beckman DU spectrophotometer. Within the range of pepsin concentration used in the assay with hemoglobin as the substrate, the increase in absorbance was proportional to the enzyme concentration. In calculating the molar pepsin concentration, a molecular weight of 35,000 was assumed (1 mg = 0.0286 μmole). Enzyme solutions were prepared immediately before use.

A sample of pepsin also was prepared from crystalline swine pepsinogen (Worthington Biochemical Corp. Lot No. PG 114), whose potential pepsin activity was 2680 units/mg (Herriott, 1938). The activation of pepsinogen and the chromatographic isolation of the resulting pepsin was performed in a manner similar to that described by Rajagopalan *et al.* (1966), with a recovery of 93% of the pepsin activity, to give an enzyme preparation having a specific activity of 3180 units/mg.

The cleavage of synthetic substrates was measured at 37°, and sodium citrate buffers (0.04 M) were used to control the pH in the range 1.8–5.0. The rate of cleavage was determined by withdrawing from the incubation mixture 0.5-ml samples, which immediately were mixed with 0.5 ml of ninhydrin solution prepared according to Stein and Moore (1948), and the mixture was then heated in a boiling water bath for 15 min and cooled; after appropriate dilution, the absorbance at 570 mμ was determined in a Coleman Junior spectrophotometer. For each run, control incubation mixtures without substrate or without enzyme were analyzed; within the precision of the analytical method, these control values did not change significantly during the course of a single run. The extent of hydrolysis was estimated by means of standard curves for the ninhydrin reaction of the appropriate split product (L-phenylalanine ethyl ester, L-phenylalanine, L-tyrosine ethyl ester, L-tyrosine, L-tryptophan ethyl ester) under the conditions used for the determination of the ninhydrin color given by samples from the enzyme–substrate mixtures. These curves were linear for amounts of the amino acid or amino acid ester in the ninhydrin reaction mixture up to 0.7 μmole (the highest tested), and were found to be reproducible when repeated at intervals during the course of these studies. In the special case of the cleavage of Gly-Gly-Phe-Phe-OEt, standard curves for the ninhydrin reaction of this compound and of an authentic sample of Gly-Gly-Phe were compared, and found to be identical within the precision of the analytical method employed. This permitted the estimation of the extent of hydrolysis of the tetrapeptide ester from the increase in ninhydrin color arising from the Phe-OEt liberated on hydrolysis.

Inactivation of Pepsin by DDM. DDM was prepared by the method of Smith and Howard (1955), and its concentration in ethanol solution was determined spectrophotometrically at 525 mμ (More O'Ferrall *et al.*, 1964). To test the effect of synthetic peptides on the action of DDM on pepsin, 5-ml incubation mixtures were prepared by the addition of the peptide solution (3.9 ml) in dilute aqueous ethanol to the enzyme solution (1 ml of 0.02 M acetate), followed immediately by the ethanolic DDM solution (0.1 ml). Control experiments without the peptides were prepared in a similar manner. The reaction mixtures were kept at 0, 15, or 30°, and the residual pepsin activity (toward hemoglobin) was determined as described above. All enzyme assay were performed in duplicate on fivefold diluted reaction mixtures.

Chromatography. Examination of the homogeneity of the peptides prepared in this work, and of the cleavage products released by pepsin, was performed by thin layer chromatography, with silica gel G as the supporting phase (Eastman chromatogram sheets K301R). The following solvent systems were used: (A) methanol–ethyl acetate (15:85 by volume); (B) 1-butanol–acetic acid–water (3:1:1 by volume). The following reagents were employed to develop the chromatograms: (a) 0.2% ninhydrin in 1-butanol; (b) 0.5% sulfanilic acid in 0.5 N HCl and 2.5% $NaNO_2$, followed by 10% Na_2CO_3 (Pauly); (c) 0.5% *p*-dimethylaminobenzaldehyde in 95% ethanol, followed by exposure to HCl vapor (Ehrlich); (d) phenol reagent (Folin and Ciocalteu, 1927); (e) iodine vapor.

Synthesis of Peptides[2]

Z-Phe-Phe-OEt. Z-Phe (6.0 g, 0.02 mole) and Phe-OEt, prepared by neutralization of the hydrochloride (5.06 g, 0.022 mole) with cold, aqueous 50% K_2CO_3, were coupled in the usual manner in the presence of DCC (4.13 g, 0.02 mole), with CH_2Cl_2 (50 ml) as the solvent. The filtrate obtained upon removal of dicyclohexylurea was concentrated to dryness and the residue was taken up in ethyl acetate; the solution was washed successively with water, 1 N HCl, and 5% $NaHCO_3$, dried over $MgSO_4$, and concentrated to *ca.* 20 ml. Addition of ether gave 9.1 g (96%) of the product, mp 138–139°. An earlier preparation of this compound, made by coupling Z-Phe-Cl with Phe-EOt, gave a product melting at 140° (Fruton and Bergmann, 1942).

Anal. Calcd for $C_{28}H_{30}N_2O_5$ (474.5): N, 5.9. Found: N, 5.9.

Phe-Phe-OEt. Z-Phe-Phe-OEt (6.0 g, 12.7 mmoles) was suspended in glacial acetic acid (5 ml), a saturated solution of HBr in glacial acetic acid (20 ml) was added, and the resulting solution was kept for 30 min at room temperature. Ether (200 ml) was added to yield 5.2 g (98%) of the dipeptide ester hydrobromide, mp 176–178°. Chromatography in solvent B gave a single spot of R_F 0.68 (ninhydrin).

Anal. Calcd for $C_{20}H_{25}BrN_2O_3$ (421.3): N, 6.65. Found: N, 6.6.

The free dipeptide ester was prepared by neutralization of the hydrobromide with cold, aqueous 50% K_2CO_3 and extraction of the free base with CH_2Cl_2.

[2] All melting points were uncorrected. Microanalyses were performed by Dr. S. M. Nagy, Massachusetts Institute of Technology. Optical rotations were determined with an ETL-NPL automatic polarimeter, Type 143A, using a 0.5-dm tube.

After drying the CH_2Cl_2 solution with $MgSO_4$, the solvent was removed *in vacuo*.

Z-His-Phe-Phe-OEt. A chilled ethyl acetate solution of Z-His-N_3, derived from 1.46 g (4.8 mmoles) of the hydrazide (mp 172–173°; Holley and Sondheimer, 1954), was added to Phe-Phe-OEt, prepared as described above from 1.69 g (4 mmoles) of the hydrobromide. The reaction mixture was kept at 0° for 34 hr. The product separated as a gelatinous precipitate (1.63 g, mp 186–188°). An additional 0.5 g of the product (mp 185–187°) was obtained upon concentration of the filtrate, giving a total yield of 87%. The substance was crystallized from aqueous ethanol with a recovery of 95%; mp 187–188°; $[\alpha]_D^{24}$ −23.4° (*c* 1, 50% aqueous acetic acid). Chromatography in solvent A gave a single spot of R_F 0.48 (Pauly), and in solvent B a single spot of R_F 0.70 (Pauly, iodine).

Anal. Calcd for $C_{34}H_{37}N_5O_6$ (611.7): C, 66.7; H, 6.1; N, 11.45. Found: C, 67.0; H, 6.2; N, 11.3.

An incubation mixture (22 ml) containing Z-His-Phe-Phe-OEt (6.55 mg), pepsin (1 mg), and 0.04 M citrate buffer (pH 4.0) was kept at room temperature overnight (100% hydrolysis), and concentrated to a small volume *in vacuo* at 40°. The crystalline precipitate (*ca.* 1 mg) that separated was collected and washed with water; it melted at 224° with decomposition, in agreement with the melting point of an authentic sample of Z-His-Phe. Samples of the filtrate were examined by thin layer chromatography (solvent B), which showed the presence of a single ninhydrin-positive component of R_F 0.60 (identical with that for an authentic sample of Phe-OEt), and a single Pauly-positive component of R_F 0.59 (identical with that for an authentic sample of Z-His-Phe).

Saponification of Z-His-Phe-Phe-OEt (1.1 equiv of NaOH in aqueous ethanol) for 120 min at room temperature, followed by neutralization with 1 N HCl, gave the benzyloxycarbonyl tripeptide (82%) in the form of a mixture of diastereoisomers, as shown by the fact that although pepsin (0.4 mg/ml, pH 3, 0.04 M citrate buffer) cleaved the compound (0.5 mM) rapidly, the extent of hydrolysis stopped at 80%.

Z-Phe-Phe-OBu^t. Phe-OBu^t hydrochloride (Roeske, 1963) (1.42 g, 5.5 mmoles) was converted to the free base as described above for the dipeptide ethyl ester, and a solution of Z-Phe (1.5 g, 5 mmoles) in CH_2Cl_2 was added, followed by the addition of DCC (1.03 g, 5 mmoles). The reaction mixture (20 ml) was kept overnight at 0° and filtered, and the filtrate was concentrated *in vacuo* to give an oil that was dissolved in ethyl acetate. After successive washing with 1 N HCl and 5% $NaHCO_3$ and being dried over $MgSO_4$, the solution was concentrated *in vacuo*, and the product was crystallized from ether–petroleum ether (bp 30–60°). After recrystallization from ethyl acetate–petroleum ether, the compound melted at 93–94°; yield, 2.2 g (88%).

Anal. Calcd for $C_{30}H_{34}N_2O_5$ (502.6): N, 5.6. Found: N, 5.4.

Phe-Phe-OBu^t Acetate. Z-Phe-Phe-OBu^t (1.26 g, 2.5 mmoles) was subjected to catalytic hydrogenolysis (palladium black) in the presence of *t*-butyl alcohol (20 ml) and glacial acetic acid (0.3 ml) to give the dipeptide ester acetate in quantitative yield, mp 101–103°.

Anal. Calcd for $C_{34}H_{32}N_2O_5$ (428.5): N, 6.5. Found: N, 6.5.

Z-His-Phe-Phe-OBu^t. Phe-Phe-OBu^t acetate (0.71 g, 1.65 mmoles) was converted to the free base (as described above for the ethyl ester), which was mixed with an ethyl acetate solution of Z-His-N_3, derived from 0.61 g (2 mmoles) of the hydrazide. After 24 hr at 0°, the reaction mixture was concentrated *in vacuo* and the resulting oil was treated with ethyl acetate–ether to yield 0.74 g of product. After crystallization from ethanol–water, it melted at 154–156°; yield 0.72 g (68%). Chromatography in solvent A gave a single spot of R_F 0.49 (Pauly, iodine).

Anal. Calcd for $C_{36}H_{41}N_5O_6$ (639.7): N, 10.9. Found: N, 10.9.

Z-His-Phe-Phe. The above protected tripeptide *t*-butyl ester (0.25 g, 0.39 mmole) was treated with TFA (1 ml) for 1 hr at room temperature. The solution was concentrated *in vacuo* at 40° to yield a residue that was triturated with ether and then dissolved in 50% ethanol (20 ml). The solution was shaken for 30 min at room temperature with Amberlite CG-4B (acetate form, 200–400 mesh), warmed to 80° (to redissolve crystalline precipitate), and filtered to remove the resin; the filtrate was evaporated to yield a residue that was crystallized from ethanol–water; yield, 0.165 g (72%); mp 202–204° dec.

Anal. Calcd for $C_{32}H_{33}N_5O_6$ (583.6): C, 65.8; H, 5.7; N, 12.0. Found: C, 66.0; H, 5.8; N, 11.9.

N^{α},N^{Im}-Z$_2$-His-Phe-Phe-OEt. To a solution of di-(benzyloxycarbonyl)-L-histidine *p*-nitrophenyl ester[3] (mp 110–112°, 4.2 g, 7.7 mmoles) in acetonitrile (50 ml) was added Phe-Phe-OEt, prepared from 3.48 g (8.25 mmoles) of the hydrobromide as described above. After 42 hr at 0°, a crystalline precipitate was collected, and additional product was obtained from the filtrate by the addition of ether; yield, 5.3 g (92%). After recrystallization from acetone, the product melted at 159–160° with decomposition.

Anal. Calcd for $C_{42}H_{43}N_5O_8$ (745.8): N, 9.4. Found: N, 9.3.

Upon treatment of this compound (0.75 g, 1 mmole) in ethanol (10 ml) with 1 M NaOEt in ethanol (1.1 ml) for 40 min at room temperature, neutralization of the reaction mixture with 1 N HCl (1.1 ml) and the addition of water (40 ml) gave a precipitate that was treated with ethanol–water to yield a crystalline product (0.37 g) that melted at 165–169°. After recrystallization from ethanol–water, the compound melted at 172–173°.

Anal. Calcd for $C_{34}H_{37}N_5O_6$ (611.7): N, 11.45. Found: N, 11.4.

This material represents a mixture of diastereoiso-

[3] This compound was prepared in 80% yield by coupling Z$_2$-His with *p*-nitrophenol in the presence of DCC (Inouye, 1965); it was also obtained in 60% yield by the use of *p*-nitrophenyl trifluoroacetate, described by Sakakibara and Inukai (1964).

mers arising from base-catalyzed racemization. When tested with pepsin (0.2 mg/ml; 37°, pH 4.0, 0.04 M citrate buffer) a solution of this product (0.5 mM) was cleaved rapidly but the hydrolysis stopped when 55% of the expected Phe-OEt had been liberated.

N^{α},N^{Im}-Z$_2$-His-Phe-OEt. Z$_2$-His (Inouye and Otsuka, 1962) (10.6 g, 0.025 mole) and Phe-OEt, prepared from 6.32 g (0.0275 mole) of the hydrochloride, were coupled in the usual manner in the presence of DCC (5.16 g, 0.025 mole), with CH_2Cl_2 (60 ml) as the solvent. After the reaction mixture had stood overnight at 0°, CH_2Cl_2 (50 ml) was added, the reaction mixture was warmed to redissolve product that had separated, and the dicyclohexylurea was filtered off. The filtrate was washed successively with 1 N HCl, water, and 5% $NaHCO_3$, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was recrystallized from chloroform–ethyl acetate; yield, 13.8 g (89%); mp 161–162°.

Anal. Calcd for $C_{33}H_{34}N_4O_7$ (598.6): N, 9.4. Found: N, 9.3.

Z-His-Phe-NHNH$_2$. Z$_2$-His-Phe-OEt (6 g, 0.01 mole) was suspended in DMF (5 ml) and ethanol (30 ml), and 85% hydrazine hydrate (2 ml) was added. The reaction mixture was shaken for 20 min to yield a clear solution, kept at room temperature for 22 hr, and chilled. The hydrazide was collected by filtration and washed with cold ethanol and ether; yield, 3.5 g (77%); mp 200–201°.

Anal. Calcd for $C_{23}H_{26}N_6O_4$ (450.5): N, 18.7. Found: N, 18.85.

Z-His-Phe-OEt. The reaction of Z-His-N_3 (from 0.01 mole of the hydrazide) with Phe-OEt in the usual manner gave this product in a yield of 67%, mp 140–141°.

Anal. Calcd for $C_{25}H_{28}N_4O_5$ (464.5): N, 12.1. Found: N, 11.9.

Ammonolysis of this compound (2.43 g, 5.2 mmoles) gave the protected dipeptide amide; yield, 2.0 g (91%); mp 202–203° dec. Davis (1956) has reported mp 203–204° for this product.

His-Phe-NH$_2$ Diacetate. Z-His-Phe-NH_2 (2.07 g, 4.75 mmoles) was subjected to catalytic hydrogenolysis (palladium black) in the presence of 9.5 mmoles of acetic acid, with methanol (20 ml) as the solvent, to yield 1.83 g (92%) of the dipeptide amide diacetate, mp 137–139°.

Anal. Calcd for $C_{19}H_{27}N_5O_6$: C, 54.1; H, 6.5; N, 16.6. Found: C, 53.9; H, 6.4; N, 16.6.

Z-His-Phe. Phe-OBu^t hydrochloride (1.29 g, 5 mmoles) was converted to the free base in CH_2Cl_2, and treated with an ethyl acetate solution of Z-His-N_3, derived from 1.82 g (6 mmoles) of the hydrazide. After standing for 48 hr at 0°, the solution was concentrated *in vacuo* to yield an amorphous solid that resisted crystallization. One-third of this product was treated with TFA (2 ml) at 0°, and the mixture was kept at room temperature for 1 hr. Addition of ether (20 ml) gave a precipitate that was dissolved in warm 50% ethanol and treated with Amberlite CG-4B (acetate form) for 20 min. The mixture was warmed to 80° (to redissolve crystalline precipitate) and filtered to remove the resin, and the filtrate was evaporated to yield a residue that was recrystallized from ethanol–water; yield, 0.37 g (50%); mp 225° dec. Chromatography in solvent B gave a single spot of R_F 0.59 (Pauly).

Anal. Calcd for $C_{23}H_{24}N_4O_5$ (436.5): N, 12.8. Found: N, 12.7.

Z-Phe-Tyr-OEt. Z-Phe (6.0 g, 0.02 mole) and Tyr-OEt (4.19 g, 0.02 mole) were coupled in the usual manner in the presence of DCC (4.13 g, 0.02 mole) with CH_2Cl_2 (55 ml) as the solvent. The precipitate that formed overnight at 0° contained both dicyclohexylurea and the major portion of the coupling product, which dissolved on warming the reaction mixture. The filtrate was evaporated to dryness *in vacuo*, and the residue was taken up in ethyl acetate; the solution was successively washed with 1 N HCl, 5% $NaHCO_3$, saturated NaCl, and water, dried, and concentrated to dryness. The residue was crystallized from CH_2Cl_2 to yield 6.94 g (71%) of product, mp 159–160°. An earlier preparation of this compound, made by coupling Z-Phe-Cl with Tyr-OEt, gave a product melting at 162° (Fruton and Bergmann, 1942).

Anal. Calcd for $C_{29}H_{30}N_2O_6$ (490.5): N, 5.7. Found: N, 5.9.

Phe-Tyr-OEt. Z-Phe-Tyr-OEt (6.0 g, 12.2 mmoles) was converted to the dipeptide ester hydrobromide (4.3 g, 81%, mp 162–164°) in the same manner as described above for Phe-Phe-OEt. Chromatography in solvent B gave a single spot of R_F 0.64 (ninhydrin). The free dipeptide ester was prepared in the same manner as described above for Phe-Phe-OEt.

Anal. Calcd for $C_{20}H_{25}BrN_2O_4$ (437.3): N, 6.2. Found: N, 6.3.

N^{α},N^{Im}Z$_2$-His-Phe-Tyr-OEt. To a solution of di-(benzyloxycarbonyl)-L-histidine *p*-nitrophenyl ester (4.2 g, 7.7 mmoles) in acetonitrile (50 ml) was added Phe-Tyr-OEt, prepared from 3.61 g (8.25 mmoles) of the hydrobromide. After the reaction mixture had been kept for 2 days at 0°, ether (120 ml) was added to yield 5.4 g (92%) of the product. After reprecipitation from ethyl acetate, it melted at 156–158° with decomposition.

Anal. Calcd for $C_{42}H_{43}N_5O_9$ (761.8): N, 9.2. Found: N, 9.4.

Z-His-Phe-Tyr-OEt. The above compound (1.14 g, 1.5 mmoles) was suspended in ethanol (10 ml). After the addition of 1 M NaOEt in ethanol (1.65 ml), the reaction mixture was allowed to stand at room temperature for 30 min, chilled, and neutralized with 1 N HCl (1.65 ml). The addition of ethanol (5 ml) and water (15 ml) gave 0.83 g (89%) of the product, mp 168–170°. After recrystallization from ethanol–water, it melted at 168–169°; $[\alpha]_D^{24}$ −27.5° (*c* 2, methanol). Chromatography in solvent A gave a single spot of R_F 0.45 (Pauly, iodine).

Anal. Calcd for $C_{34}H_{37}N_5O_7$ (627.7): C, 65.1; H, 5.9; N, 11.15. Found: C, 64.9; H, 6.1; N, 11.0.

An incubation mixture (10 ml) containing Z-His-Phe-Tyr-OEt (0.5 mM), pepsin (0.4 mg), and 0.04 M citrate (pH 4.0) was kept at 37° overnight (100% hydrolysis), and samples were examined by thin layer

chromatography (solvent B). One ninhydrin-positive component was noted with R_F 0.55 (identical with that of an authentic sample of Tyr-OEt) and one Pauly-positive component was found with R_F 0.63 (identical with that of an authentic sample of Z-His-Phe). No ninhydrin-positive spots were noted at 0.40 or 0.64, corresponding to those given by authentic samples of tyrosine or of Phe-Try-OEt, respectively.

An attempt to prepare Z-His-Phe-Tyr-OEt by coupling Z-His-Phe-N_3 with Tyr-OEt did not yield a satisfactory product.

Trp-OEt. L-Tryptophan (10.2 g, 0.05 mole) was esterified in ethanol (100 ml) by the addition of $SOCl_2$ (3.65 ml, 0.05 mole) to yield 13.1 g (98%) of the ethyl ester hydrochloride, mp 221–222° dec. The hydrochloride (4.0 g, 15 mmoles) was neutralized with 50% K_2CO_3, and the free base was extracted with ether. After drying the solution over $MgSO_4$ and evaporation *in vacuo*, addition of petroleum ether gave 3.4 g (98%) of the product, mp 67–69°.

Anal. Calcd for $C_{13}H_{16}N_2O_2$ (232.3): N, 12.1. Found: N, 11.9.

Z-Phe-Trp-OEt. Z-Phe (3.0 g, 0.01 mole) and Trp-OEt (2.3 g, 0.01 mole) were coupled in the usual manner in the presence of DCC (2.06 g, 0.01 mole) with CH_2Cl_2 as the solvent. The filtrate obtained upon removal of the dicyclohexylurea was concentrated to dryness and the residue was dissolved in ethyl acetate; the solution was washed successively with 1 N HCl, 5% $NaHCO_3$, saturated NaCl, and water, dried, and concentrated to dryness. The residue was crystallized with ethyl acetate–petroleum ether; yield, 4.5 g (88%); mp 123–125°.

Anal. Calcd for $C_{30}H_{31}N_3O_5$ (513.6): N, 8.2. Found: N, 8.1.

Z-His-Phe-Trp-OEt. Z-Phe-Trp-OEt (1.23 g, 2.4 mmoles) was subjected to catalytic hydrogenolysis (palladium black) in the presence of ethanol (20 ml) and glacial acetic acid (0.3 ml). The reaction mixture was warmed to dissolve the product, and the solution was filtered and concentrated *in vacuo* to give a residue which was treated with ether (10 ml) to yield 0.92 g (87%) of a product that did not melt below 235°. It gave a positive reaction with ninhydrin reagent. To a chilled suspension of Phe-Trp-OEt acetate (0.725 g, 1.65 mmoles) in CH_2Cl_2 (15 ml) and water (5 ml), a solution of 50% K_2CO_3 (5 ml) was added, and the mixture was shaken. Insoluble material (0.23 g) was filtered off and washed thoroughly with CH_2Cl_2 and water, and the organic phase of the filtrate and washings was dried and concentrated *in vacuo* to yield an oil. This product was treated with an ethyl acetate solution of Z-His-N_3, derived from 0.61 g (2 mmoles) of the hydrazide, and the mixture was kept at 0° for 50 hr. A gelatinous precipitate separated, and additional product was obtained by evaporation of the filtrate. The product was washed with ethyl acetate and crystallized from ethanol–water; yield, 0.54 g (51%); mp 189–190°; $[\alpha]_D^{24}$ −24.4° (*c* 1, methanol). Chromatography in solvent A gave a single spot of R_F 0.46 (Pauly, Ehrlich, iodine).

Anal. Calcd for $C_{36}H_{38}N_6O_6$ (650.7): C, 66.4; H, 5.9; N, 12.9. Found: C, 66.4; H, 6.0; N, 13.0.

An incubation mixture (10 ml) containing Z-His-Phe-Trp-OEt (0.5 mM), pepsin (0.4 mg), and 0.04 M citrate buffer (pH 4.0) was kept at 37° overnight (100% hydrolysis), and samples were examined by thin layer chromatography (solvent B). One ninhydrin-positive component was noted with R_F 0.61 (identical with that given by an authentic sample of Trp-OEt) and one Pauly-positive component was found with R_F 0.63 (identical with that given by an authentic sample of Z-His-Phe).

N^{α},N^{im}-Z$_2$-His-Tyr-OEt. Z_2-His (10.6 g, 0.025 mole) and Tyr-OEt (mp 103–104°, 5.23 g, 0.025 mole) were coupled in the usual manner in the presence of DCC (5.16 g, 0.025 mole), with CH_2Cl_2 (180 ml) as the solvent. The DCC must be added immediately after the ester to prevent the separation of the insoluble amine salt. After standing overnight at 0°, the reaction mixture was filtered, and the filtrate was washed successively with 1 N HCl, water, and 5% $NaHCO_3$, dried over $MgSO_4$, and concentrated *in vacuo* to a syrup that crystallized upon the addition of ethyl acetate; yield, 14 g (91%). After recrystallization from ethyl acetate, the product melted at 148–149° with decomposition.

Anal. Calcd for $C_{33}H_{34}N_4O_5$ (614.6): N, 9.1. Found: N, 8.9.

Z-His-Tyr-NHNH$_2$. Z_2-His-Tyr-OEt (12.3 g, 0.02 mole) was dissolved in DMF (10 ml) and ethanol (50 ml), and 85% hydrazine hydrate (4 ml) was added. After standing for 24 hr at room temperature, the reaction mixture was chilled, and the resulting crystalline product was collected; yield, 8.4 g (90%); mp 210–212° dec.

Anal. Calcd for $C_{23}H_{26}N_6O_5$ (466.5): N, 18.0. Found: N, 17.8.

The same compound (mp 208–209° dec) was obtained by treatment of Z-His-Tyr-OEt with hydrazine hydrate. However, the reaction of Z-His-N_3 with Tyr-OEt did not give the protected dipeptide ester in satisfactory yield. After recrystallization from ethanol, this preparation of Z-His-Tyr-OEt melted at 145–148° after preliminary softening at 100°. Davis (1956) reported mp 94–95° for this product.

Anal. Calcd for $C_{25}H_{28}N_4O_6$ (480.5): N, 11.7. Found: N, 11.5.

Ammonolysis of the above ester (2.15 g, 4.5 mmoles) gave 1.8 g (90%) of the corresponding amide (Davis, 1956), which upon catalytic hydrogenolysis in the presence of acetic acid gave His-Tyr-NH_2 acetate (mp 158–160°) in quantitative yield.

Anal. Calcd for $C_{18}H_{23}N_5O_5$ (377.4): C, 54.1; H, 6.1; N, 18.6. Found: C, 54.2; H, 6.2; N, 18.3.

Z-His-Tyr-Tyr-OEt. Z-His-Tyr-$NHNH_2$ (4.2 g, 9 mmoles) was dissolved in 2 N HCl (25 ml), DMF (25 ml) was added, and to the chilled solution was added 2 M $NaNO_2$ (5 ml). After 3 min, ice-cold 50% K_2CO_3 (15 ml) and water (125 ml) were added. The precipitated azide was filtered, washed with cold water, dried *in vacuo* over P_2O_5 for 2 hr, and added to a chilled solution of

Tyr-OEt (1.57 g, 7.4 mmoles) in DMF (20 ml). After 66 hr at 0°, water (100 ml) was added to the reaction mixture to yield 3.3 g (68%) of product, which was washed with 5% $NaHCO_3$ and water and crystallized from ethanol–water mp 110–114°; $[\alpha]_D^{24}$ −24.3° (*c* 2, methanol). Chromatography in solvent A gave a single spot of R_F 0.41 (Pauly, iodine).

Anal. Calcd for $C_{34}H_{37}N_5O_8 \cdot H_2O$ (661.7): C, 61.7; H, 5.9; N, 10.6. Found: C, 61.6; H, 6.1; N, 10.5.

An incubation mixture (10 ml) containing Z-His-Tyr-Tyr-OEt (0.5 mM), pepsin (2 mg), and 0.04 M citrate buffer (pH 4.0) was kept at 37° overnight (100% hydrolysis), and samples were examined by thin layer chromatography (solvent B). One ninhydrin-positive spot was noted with R_F 0.55 (identical with that given by an authentic sample of Tyr-OEt), and a component was noted with R_F 0.67 that was reactive toward both the Pauly and phenol reagents, and may be assigned to Z-His-Tyr.

Z-Gly-Gly-Phe-Phe-OEt. Z-Phe-Phe-OEt (2.37 g, 5 mmoles) was subjected to catalytic hydrogenolysis (palladium black) in the usual manner to yield the dipeptide ester, which was coupled with Z-Gly-Gly (mp 178°, 1.33 g, 5 mmoles) in the presence of DCC (1.04 g, 5 mmoles), with THF (150 ml) as the solvent. After standing overnight, the reaction mixture was evaporated *in vacuo* to dryness and the residue was extracted with chloroform (50 ml). The suspension was filtered, and the filtrate was washed successively with 1 N HCl, 5% $NaHCO_3$, and water, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was treated with ethyl acetate to yield a crystalline product, which was recrystallized from ethyl acetate; yield, 2.07 g (70%); mp 166–168°.

Anal. Calcd for $C_{32}H_{36}N_4O_4$ (588.7); N, 9.5. Found: N, 9.5.

Gly-Gly-Phe-Phe-OEt Acetate. The protected tetrapeptide ester (0.71 g, 1.2 mmoles) was subjected to catalytic hydrogenolysis (palladium black) in the presence of glacial acetic acid (1.3 mmoles), with ethanol (20 ml) as the solvent, to yield 0.56 g (90%) of the tetrapeptide ester acetate, mp 153–155°; $[\alpha]_D^{24}$ −8.0° (*c* 2, methanol). Chromatography in solvent B gave a single spot of R_F 0.49 (ninhydrin).

Anal. Calcd for $C_{26}H_{34}N_4O_7$ (514.6): C, 60.7; H, 6.7; N, 10.9. Found: C, 60.8; H, 6.7; N, 10.9.

An incubation mixture (10 ml) containing Gly-Gly-Phe-Phe-OEt (1.0 mM), pepsin (2 mg), and 0.04 M citrate buffer (pH 4.0) was kept at 37° for 6 hr (100% hydrolysis), and samples were examined by thin layer chromatography (solvent B). Two ninhydrin-positive components were noted with R_F values of 0.46 (identical with that of an authentic sample of Gly-Gly-Phe) and of 0.64 (identical with that of an authentic sample of Phe-OEt). No spot was noted at R_F 0.32 or 0.71, corresponding to those given by authentic samples of Gly-Gly and Phe-Phe-OEt, respectively, on the same chromatogram.

Z-Gly-Phe-Phe-OEt. Z-Gly (1.46 g, 7 mmoles) and Phe-Phe-OEt hydrobromide (2.95 g, 7 mmoles) were coupled in the usual manner in the presence of DCC (1.44 g, 7 mmoles) and of tri-*n*-butylamine (1.67 ml, 7 mmoles), with CH_2Cl_2 (20 ml) as the solvent. After standing at 0° overnight, the reaction mixture was filtered and the filtrate was concentrated *in vacuo* to yield a residue that was extracted with acetonitrile (90 ml). From the filtrate, after removal of dicyclohexylurea, were obtained 3.2 g of product, mp 143–145°, which was recrystallized from ethyl acetate–ether to yield 2.9 g (78%) of product, mp 144–146°.

Anal. Calcd for $C_{30}H_{33}N_3O_6$ (531.7): N, 7.9. Found: N, 7.8.

Gly-Phe-Phe-OEt Acetate. The protected tripeptide ester (2.66 g, 5 mmoles) was subjected to catalytic hydrogenolysis (palladium black) in the presence of glacial acetic acid (5.5 mmoles), with ethanol (20 ml) as the solvent, to yield 1.7 g (74%) of the tripeptide ester acetate, mp 97–98.5°; $[\alpha]_D^{24}$ −1.6° (*c* 2, methanol). Chromatography in solvent B gave a single spot of R_F 0.54 (ninhydrin).

Anal. Calcd for $C_{24}H_{31}N_3O_6$ (457.5): C, 63.0; H, 6.8; N, 9.2. Found: 63.05; H, 7.0; N, 9.3.

An incubation mixture (2.5 ml) containing Gly-Phe-Phe-OEt (1.0 mM), pepsin (5 mg), and 0.04 M citrate buffer (pH 4.0) was kept at 37° overnight (100% hydrolysis), and samples were examined by thin layer chromatography (solvent B). Two ninhydrin-positive components were noted with R_F values of 0.52 (identical with that of an authentic sample of Gly-Phe) and of 0.64 (identical with that of an authentic sample of Phe-OEt).

Gly-Phe-Phe-NH_2 Acetate. Z-Gly-Phe-Phe-OEt (0.5 g, 0.94 mmole) was treated for 3 days with methanol (20 ml) previously saturated with NH_3 at 0°. On evaporation *in vacuo*, a crystalline product was obtained. After recrystallization from ethanol–ether, it melted at 166–168°; yield, 0.34 g (73%). This material was subjected to catalytic hydrogenolysis (palladium black) in the usual manner in the presence of acetic acid (0.67 mmole) to yield 0.27 g (93%) of the tripeptide amide acetate, mp 146–148°.

Anal. Calcd for $C_{22}H_{28}N_4O_5$ (428.5): N, 13.1. Found: N, 12.8.

Gly-Gly-Phe. This compound was prepared in a manner similar to that described by Ben-Ishai (1954). Chromatography in solvent B gave a single spot of R_F 0.46 (solvent B, ninhydrin).

Ac-Phe-Tyr. This compound was prepared in the manner described by Baker (1951). After four recrystallizations from aqueous methanol, it melted at 221–222°. Baker (1951) reported mp 230°, and Silver *et al.* (1965) reported mp 220–222°.

Results

In Table I are given representative data for the hydrolysis of a series of new synthetic substrates for swine pepsin; a value for the rate of hydrolysis of Ac-Phe-Tyr (*cf.* Silver *et al.*, 1965) is given for comparison. It will be noted that the compounds Z-His-Phe-Trp-OEt, Z-His-Phe-Phe-OEt, Z-His-Phe-Tyr-OEt, Gly-Gly-Phe-Phe-OEt, and Z-His-Tyr-Tyr-OEt are

TABLE I: Action of Pepsin on Synthetic Substrates.[a]

Substrate	Pepsin (mg/ml)	Hydrolysis[b] Time (min)	Hydrolysis[b] %
Z-His-Phe-Trp-OEt	0.04	10	53
Z-His-Phe-Phe-OEt	0.04	20	56
Z-His-Phe-Phe-OBu^t	0.04	20	56
Z-His-Phe-Tyr-OEt	0.04	20	29
Gly-Gly-Phe-Phe-OEt	0.05	20	38
Z-His-Tyr-Tyr-OEt	0.20	90	41
Z-His-Phe-Phe[c]	0.40	20	46
Gly-Phe-Phe-OEt	0.50	20	26
Ac-Phe-Tyr[d]	0.20	90	58

[a] Except where otherwise noted, tested at pH 4.0 (0.04 M citrate) and 37°; initial substrate concentration, 0.5 mM. [b] The extent of hydrolysis is given in per cent of that expected for the cleavage of one peptide bond. [c] pH 3.2. [d] pH 2.1. In a separate experiment, 6.4% methanol was present, and the extent of hydrolysis in 90 min was 28%.

all hydrolyzed relatively rapidly under the conditions given, the apparent susceptibility of the substrates decreasing roughly in the order listed above. Four of these five compounds are hydrolyzed more rapidly than is Ac-Phe-Tyr, under the conditions given in Table I. With the exception of Z-His-Phe-Trp-OEt and Z-His-Phe-Tyr-OEt, all these compounds are soluble in 0.04 M citrate buffer (pH range 2–5) up to a concentration of 1 mM (the highest tested); the solubility of Z-His-Phe-Trp-OEt and of Z-His-Phe-Tyr-OEt at pH values near 2 is satisfactory, but at pH 4 the limit of solubility is near 0.5 mM. It should be emphasized that, except in the case of these two compounds, the addition of an organic solvent (*e.g.*, methanol) is not necessary to obtain 1 mM solutions. It has been shown by Tang (1965) that aliphatic alcohols inhibit the action of pepsin on Z-Glu-Tyr; we confirm this finding for the hydrolysis of Ac-Phe-Tyr at pH 2, and of Z-His-Phe-Tyr-OEt in the pH range 2–4.

Data are included in Table I for the hydrolysis by pepsin of Z-His-Phe-Phe-OBu^t, which appears to be cleaved as rapidly as the corresponding ethyl ester, and for Z-His-Phe-Phe and Gly-Phe-Phe-OEt, whose hydrolysis at a rapid rate requires a relatively large enzyme concentration.

When crystalline pepsinogen was activated rapidly at pH 2, and the resultant pepsin solution, after chromatographic purification (see Experimental Section), was tested at a level of 108 (hemoglobin) units/ml of incubation mixture for its action on Z-His-Phe-Phe-OEt (0.5 mM) at pH 2.0 or at pH 4.0 (0.4 M citrate) at 37°, the rate of cleavage was found to be identical with that in a parallel experiment in which the commercial pepsin preparation (0.04 mg/ml, 108 units/ml) was tested under the same conditions. It may be concluded therefore that the cleavage of Z-His-Phe-Phe-OEt (and of closely related peptide substrates) by the preparation of crystalline pepsin used in our studies is a property of the authentic enzyme, and not caused by enzymic impurities in the commercial pepsin preparation.

Chromatographic examination of the incubation mixtures showed that all the new substrates are cleaved by pepsin (both the commercial crystalline material and the enzyme prepared from pepsinogen) at the peptide bond linking the two aromatic amino acid residues. This finding is in accord with earlier results (Baker, 1951) showing that such bonds are preferentially attacked by the enzyme. Furthermore, under the conditions of these studies, the hydrolysis ceased when one peptide bond had been cleaved completely, as judged both by the ninhydrin assay method and by chromatographic examination of hydrolysates. Thus, the cleavage of substrates of the type Z-His-Phe-X-OEt gave rise to the appearance of Z-His-Phe and the appropriate amino acid ester, whereas the cleavage of Gly-Gly-Phe-Phe-OEt gave Gly-Gly-Phe and Phe-OEt as the only products. No other chromatographic components were seen, and the absence of a terminal α-carboxyl group precludes the possibility of transpeptidation (Neumann *et al.*, 1959; Fruton *et al.*, 1961).

Synthesis of Peptides. As indicated in the Experimental Section, the synthesis of Z-His-Phe-Phe-OEt and of Z-His-Phe-Trp-OEt involved the coupling of the azide of Z-His with the appropriate dipeptide ester, and Z-His-Phe-Tyr-OEt was made by coupling N^{α},-N^{Im}-Z_2-His-Onp with Phe-Tyr-OEt, followed by removal of the N^{Im}-benzyloxycarbonyl group with sodium ethoxide. Z-His-Tyr-Tyr-OEt was prepared by coupling the azide of Z-His-Tyr with Tyr-OEt. The DCC method was used to join single protected amino acid residues, or to effect coupling involving Z-Gly or Z-Gly-Gly.

It is worthy of mention that attempts to prepare Z-His-Phe-Phe-OEt from the N^{α},N^{Im}-di(benzyloxycarbonyl) compound by treatment with sodium ethoxide, in ethanolic solution, gave evidence of an unexpectedly facile base-catalyzed racemization, as shown by the fact that the cleavage of the product by pepsin proceeded only to 50% of the theory. This interesting racemization is under further investigation; it should be noted, however, that treatment of N^{α},N^{Im}-Z_2-His-Phe-Tyr-OEt with sodium ethoxide gave a product that was cleaved by pepsin to 100%. Apparently, under the conditions of this treatment, the L-phenylalanyl residue is more susceptible to racemization than is the L-tyrosyl residue.

pH Dependence of Cleavage of New Substrates. Examination of the pH dependence of pepsin action on the new substrates lacking a carboxyl group showed that these compounds are cleaved preferentially at pH values near 4 (Figure 1). In view of the widely accepted value of about 2 for the pH optimum of pepsin action on substrates such as Ac-Phe-Tyr (Baker,

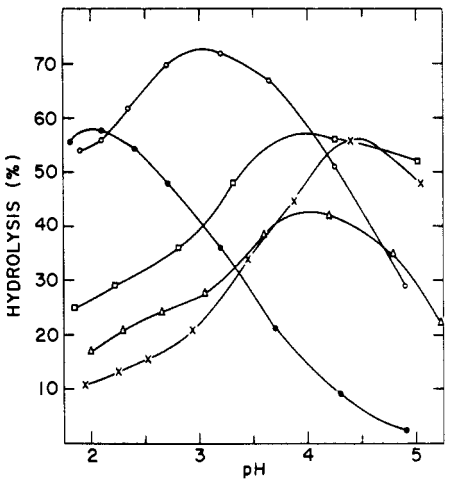

FIGURE 1: pH optima for the hydrolysis of synthetic substrates by pepsin. Substrate concentration, 0.5 mM; temperature, 37°. ●, Ac-Phe-Tyr, 0.2 mg of pepsin/ml, 90 min; ○, Z-His-Phe-Phe, 0.4 mg of pepsin/ml, 40 min; □, Gly-Gly-Phe-Phe-OEt, 0.05 mg of pepsin/ml, 40 min; △, Z-His-Tyr-Tyr-OEt, 0.2 mg of pepsin/ml, 90 min; ×, Z-His-Phe-Phe-OEt, 0.04 mg of pepsin/ml, 15 min.

1951) and Ac-Phe-Dityr (Jackson *et al.*, 1965), it seemed possible that the difference lay in the presence of an α-carboxyl group in the latter substrates. Accordingly, Z-His-Phe-Phe was prepared (by acidolysis of the corresponding *t*-butyl ester), and found to be cleaved completely at the Phe-Phe linkage by pepsin. It was of interest to find that whereas the pH optimum for Z-His-Phe-Phe-OEt is near 4.5, the pH optimum for Z-His-Phe-Phe is near 3.0 (Figure 1), and that the latter compound is cleaved much more slowly than is the corresponding ethyl ester. This finding suggests that the apparent pH optima for the action of pepsin on carboxylic acids such as Ac-Phe-Tyr or Ac-Phe-Dityr depend, at least in part, on the deprotonation of the COOH group, and that the α-COO^- group of a substrate molecule is inhibitory to enzyme action.

It may be noted here that one of the problems encountered in the early work with synthetic substrates for pepsin (Fruton and Bergmann, 1939; Baker, 1951) was the limited solubility of the compounds at acid pH values. Nearly all the susceptible peptide derivatives were carboxylic acids whose solubility decreases as the pH is lowered, and some of them (*e.g.*, Z-Glu-Tyr) were very sparingly soluble at pH 2. As regards compounds not having a carboxyl group, the situation may be exemplified by the report of Baker (1951) that Z-Phe-Phe-NH_2 is resistant to pepsin action; she noted, however, that the extreme insolubility of the compound rendered the interpretation of this negative result uncertain in relation to the question of the necessity of a free carboxyl group in the substrate. The compounds Z-Glu-Tyr-Gly-NH_2, Z-Glu-Tyr-NH_2, and Z-Glu-Tyr-OEt, by virtue of their side-chain carboxyl group, exhibit some solubility in acidic buffer solutions and are cleaved slowly with a pH optimum near 4 (Bergmann and Fruton, 1941; Casey and Laidler, 1950).

In view of the possible inhibitory effect of carboxylate groups in pepsin substrates on the action of the enzyme, the effect of a comparable concentration of carboxylate ions in the buffer system was examined. The rate of cleavage of Z-His-Phe-Phe-OEt (0.5 mM) at pH 3.75 by pepsin (0.04 mg/ml) was measured in the presence of 0.01 M buffers prepared from acetic acid ($pK' = 4.7$), formic acid ($pK' = 3.8$), and chloroacetic acid ($pK' = 2.9$) and the corresponding sodium salt; 0.1 M NaCl was present to maintain a relatively constant ionic strength. In spite of the large differences in the concentration of carboxylate ion in the three buffer systems, the rate of hydrolysis of the substrate was the same (2.7%/min) in all three cases; this rate was close to that observed in 0.04 M citrate buffer (no NaCl added). Although, at the buffer concentrations used, no inhibitory effect of carboxylate ion was noted, it may be expected that in the pH range 2–4, such inhibition will be evident at higher concentrations of buffers such as acetate buffer.

Kinetics of Pepsin Action on New Substrates. Since the demonstration by Baker (1951) that Ac-Phe-Tyr is cleaved rapidly by pepsin, there has been recurrent discussion of the apparent first-order kinetics of this hydrolysis. Baker (1954) initially attributed this behavior to the slow release of Ac-Phe from the enzyme, but Green (1956) pointed out that this interpretation cannot be correct, and Baker (1956) offered experimental data (correcting earlier published results) in favor of the view that Ac-Phe is a competitive inhibitor of pepsin, with an affinity for the enzyme similar to that of Ac-Phe-Tyr. The apparent first-order kinetics of the peptic cleavage of Ac-Phe-Tyr has been confirmed recently by Silver *et al.* (1965), and our data for this substrate also agree with this conclusion. The question of the relative affinity of Ac-Phe and of Ac-Phe-Tyr for pepsin is still unclear, however, in view of the report of Jackson *et al.* (1965) that they were unable to determine K_i for Ac-Phe (with Ac-Phe-Dityr as substrate), as the value appeared to be greater than 0.01 M.

In the present study it was found that apparent zero-order kinetics applied to the peptic hydrolysis up to about 50% for Z-His-Phe-Phe-OEt, Z-His-Phe-Tyr-OEt, Z-His-Phe-Trp-OEt, and Gly-Gly-Phe-Phe-OEt (all near 0.5 mM, pH 4). The kinetic data for the new substrates permitted satisfactory estimation of initial velocities for use in the graphical determination of the Michaelis constant and of maximal velocity. It was found that the entire course of the hydrolysis of Z-His-Phe-Phe (5 mM) at pH 3 accords with first-order kinetics, suggesting a relation between the presence of an α-carboxyl group adjacent to the sensitive peptide bond (as in Ac-Phe-Tyr) and the first-order character of the cleavage of this bond by pepsin. Further studies, now in progress, on the kinetics of

TABLE II: Kinetics of Pepsin Action on Synthetic Substrates.[a]

Substrate	$[S]_0 \times 10^4$ (M)	$[E]_0 \times 10^6$ (M)	$K_m \times 10^4$ (M)	$k_3 \times 10^2$ sec^{-1}
Z-His-Phe-Phe-OEt	1.0–10.0	1.14	1.8	31
Z-His-Phe-Tyr-OEt	0.9–4.8	1.14	2.3	16
Z-His-Phe-Trp-OEt	2.0–6.0	0.57	2.3	51
Z-His-Tyr-Tyr-OEt	2.0–10.0	5.72	2.4	0.94
Gly-Gly-Phe-Phe-OEt	2.0–10.0	1.43	2.8	18

[a] pH 4.0 (0.04 M citrate), 37°. $[S]_0$ = initial concentration of substrate; $[E]_0$ = initial concentration of enzyme (assuming that 1 mg of pepsin = 0.0286 μmole).

the hydrolysis of this and other substrates having an α-carboxyl group may clarify this relationship.

Examination of the effect of changes in substrate concentration gave data for S/v *vs.* S plots and for the determination of K_m and k_3, where S is the initial substrate concentration, and the other terms are defined by the expression $v = k_3ES/(K_m + S)$; v is the initial velocity and E is the enzyme concentration. Representative plots are shown in Figure 2, and the results are summarized in Table II. It will be noted that, for the substrates examined, the values of K_m do not differ greatly from each other, the differences in the observed rates of hydrolysis being largely reflected in the variation in the values of k_3. Of the hitherto-known synthetic substrates for pepsin, Ac-Phe-Dityr appears to be the one hydrolyzed most rapidly; Jackson *et al.* (1965) have reported values of $K_m = 7.5 \times 10^{-5}$ M and $k_3 = 0.2$ sec^{-1} (pH 2.0, 37°) and $K_m = 8.4 \times 10^{-4}$ M and $k_3 = 0.07$ sec^{-1} (pH 4.5, 37°). Silver *et al.* (1965) have reported the following data for Ac-Phe-Tyr (3.4% methanol, pH 2, phosphate buffer, 35°): K_m = *ca.* 2×10^{-3} M, k_3 = *ca.* 0.04 sec^{-1}. Comparison of these values with those given in Table II indicates that the best of the new substrates described in the present communication compare favorably in their kinetic behavior with Ac-Phe-Dityr, whose low solubility limits its utility (Jackson *et al.*, 1965).

Effect of Synthetic Substrates on Inactivation of Pepsin by DDM. As shown in Table III, pepsin is rapidly inactivated by DDM (14-fold molar excess) at pH 5, and the synthetic substrates Z-His-Phe-Phe-OEt, Z-His-Phe-Tyr-OEt, and Z-His-Tyr-Tyr-OEt cause an increase in the rate of this inactivation. It is noteworthy that the best of these three substrates (Z-His-Phe-Phe-OEt) also is most effective in promoting the inactivation by DDM. In the case of Z-His-Tyr-Tyr-OEt, whose solubility allowed experiments below room temperature, the effect of the peptide on inactivation by DDM was tested at 0°, as well as at 15 and 30°. It will be noted from Table III that, in the absence of added peptide, the rate of pepsin inactivation is slower at 0° than at the higher temperatures, but that the presence of the peptide causes a significantly greater enhancement of inactivation at 0 than at 30°. This result may be a consequence of tighter binding of the substrate to the enzyme at the lower temperature.

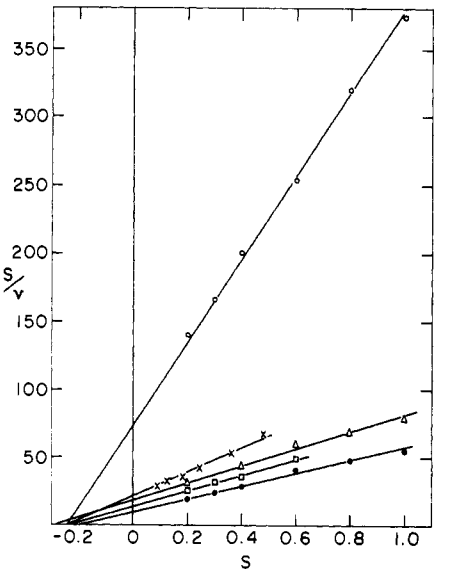

FIGURE 2: S/v *vs.* S plots for the hydrolysis of synthetic substrates by pepsin at pH 4.0; temperature, 37°; substrate concentration, mM; initial velocity, mM × min^{-1}. The straight lines were fitted to the points by the method of least squares. ○, Z-His-Tyr-Tyr-OEt, 0.2 mg of pepsin/ml; ×, Z-His-Phe-Tyr-OEt, 0.04 mg of pepsin/ml; △, Gly-Gly-Phe-Phe-OEt, 0.05 mg of pepsin/ml; □, Z-His-Phe-Trp-OEt, 0.02 mg of pepsin/ml; ●, Z-His-Phe-Phe-OEt, 0.04 mg of pepsin/ml.

That the effect observed was not due to some intrinsic property of either the phenolic or imidazole group was shown by the failure of Ac-Tyr-OEt, Bz-His-NH_2, or imidazole to affect the course of the inactivation. Further, it was found that under the conditions of the hemoglobin assay for the enzyme activity, the presence of Z-His-Tyr-Tyr-OEt had no effect on the assay values obtained. It will be seen from Table III that Gly-Gly-Phe-Phe-OEt, an excellent substrate for pepsin, had little effect on the course of the inactivation of pepsin by DDM.

TABLE III: Effect of Synthetic Substrates on Inactivation of Pepsin by Diphenyldiazomethane.[a]

	Pepsin Activity[b]					
	0°		15°		30°	
Compd Added	15 min	60 min	10 min	30 min	10 min	20 min
None	2400	1900	1950	1525	1340	1265
Z-His-Phe-Phe-OEt					525	475
Z-His-Phe-Tyr-OEt					825	850
Z-His-Tyr-Tyr-OEt	1750	700	1250	600	875	800
Gly-Gly-Phe-Phe-OEt[c]			1930	1425		
Ac-Tyr-OEt					1375	1325
Bz-His-NH_2			2000	1525		
Imidazole			2050	1475		

[a] Concentration in reaction mixture (5 ml): pepsin, 5.7 μM; added compound, 0.82 mM; DDM, 0.088 mM; ethanol, 10%; acetate buffer (0.004 M), pH 5.1. The reaction mixture was kept at the indicated temperature for the time period noted. [b] Given as units per milligram, with hemoglobin as substrate, to permit comparison with activity of untreated pepsin (2595 ± 115 units/mg). [c] To avoid the introduction of added acetate, this compound was used as the hydrochloride, prepared by catalytic hydrogenolysis (palladium black) of Z-Gly-Gly-Phe-Phe-OEt in the presence of HCl.

Discussion

The results presented above show that a free carboxyl group is not needed in synthetic substrates of pepsin, and a comparison of the rate of cleavage of Z-His-Phe-Phe-OEt and Z-His-Phe-Phe indicates that substitution of the terminal α-carboxyl group favors peptic cleavage of the Phe-Phe linkage. Herriott (1962) suggested that the α-carboxyl group of Ac-Phe-Phe may be involved in hydrogen bonding with a carboxyl group of pepsin; although our data do not contradict this hypothesis, they indicate that a COOH group is not essential in the substrate. Furthermore, the pH dependence of the cleavage of the new synthetic substrates suggests that the presence of an α-carboxylate group adjacent to the sensitive peptide bond may be inhibitory to the peptic cleavage of that bond. Such inhibition would help to explain the difference in the pH optima for acyl peptides (pH 2–3) and for acyl peptide esters (*ca.* pH 4), as the apparent p*K* values of the α-carboxyl groups of acyl peptides may be expected to be near pH 3.

The pH optima for the hydrolysis of the new synthetic substrates are of special interest, in view of the opinion (Baker, 1951; Herriott, 1962) that the optimal cleavage of Ac-Phe-Tyr near pH 2 approximates the situation in the action of pepsin on protein substrates. This opinion must be revised, however, in the light of studies of Schlamowitz and Peterson (1959), who showed that, before denaturation by acid, alkali, or urea, proteins such as bovine serum albumin or hemoglobin are cleaved optimally by pepsin at pH values near 2, whereas after denaturation the pH optimum shifts to about 3.5. Clearly, simple synthetic substrates must be considered to resemble more closely the structural situation in the sensitive portions of denatured proteins, rather than native proteins. If the hydrophobic groups of a native protein are in the interior of the molecule, with the charged groups near the surface, it may be that protonation of the carboxyl groups is required for optimal attack by pepsin, because the carboxylate groups inhibit enzymic attack.

A comparison of Gly-Gly-Phe-Phe-OEt and Gly-Phe-Phe-OEt as substrates for pepsin suggests that a positive charge becomes more inhibitory as it approaches the sensitive Phe-Phe linkage. Further kinetic studies are needed to examine the effect of charged groups in relation to their proximity to the sensitive peptide bond. Studies are also in progress on the possible inhibitory action of split products such as Z-His-Phe, as well as on the effect of diastereoisomeric peptides such as Z-His-D-Phe-Phe-OEt and Z-His-Phe-D-Phe-OEt.

The available data indicate that in substrates such as Z-His-Phe-X-OEt, the rate of peptic cleavage of the Phe-X bond decreases in the order X = Trp, Phe, Tyr. Furthermore, in the compound Z-His-Phe-Tyr-OEt, replacement of Phe by Tyr causes a marked decrease in the rate of cleavage of the sensitive bond. It may be added that the apparent lack of effect of the replacement of the ethyl group in Z-His-Phe-Phe-OEt by a *t*-butyl group (Table I) suggests that the larger group does not introduce steric hindrance in the enzyme–substrate interaction.

The finding that substrates such as Z-His-Phe-Phe-OEt or Z-His-Tyr-Tyr-OEt greatly enhance the rate of inactivation of pepsin by DDM raises the question whether the enzyme–substrate interaction leads to a change in the conformation of the protein so as to render some of the side-chain carboxyl groups more accessible to attack by the reagent. There are numerous

instances in which enzymes, far from being protected by their substrates, are inactivated more rapidly (for a review, see Grisolia, 1964), and it has been suggested that substrate-induced conformational changes may be involved. In the present case, however, the failure of Gly-Gly-Phe-Phe-OEt to cause significant change in the extent of inactivation of pepsin by DDM is difficult to explain on these grounds. Further studies are clearly needed, and work is in progress in attempts to identify the peptide regions of the protein substituted by DDM; a possibility that cannot be excluded at present is that the result with Gly-Gly-Phe-Phe-OEt is a reflection of a compensation of two effects, protection of the active site and the unmasking of protein carboxyl groups. The latter process, assumed above to be the main effect of substrates such as Z-His-Phe-Phe-OEt, may be related to the presence of an additional apolar group in the form of the benzyloxycarbonyl group in the histidine-containing substrates.

Acknowledgment

We wish to thank Warren A. Carlson for his valuable technical assistance.

References

Aboderin, A. A., Delpierre, G. R., and Fruton, J. S. (1965), *J. Am. Chem. Soc. 87*, 5469.
Anson, M. L. (1938), *J. Gen. Physiol. 22*, 79.
Baker, L. E. (1951), *J. Biol. Chem. 193*, 809.
Baker, L. E. (1954), *J. Biol. Chem. 211*, 701.
Baker, L. E. (1956), *Nature 178*, 145.
Ben-Ishai, D. (1954), *J. Org. Chem. 19*, 62.
Bergmann, M., and Fruton, J. S. (1941), *Advan. Enzymol. 1*, 63.
Casey, E. J., and Laidler, K. J. (1950), *J. Am. Chem. Soc. 72*, 2159.
Cornish-Bowden, A. J., and Knowles, J. R. (1965), *Biochem. J. 95*, 71P.
Davis, N. C. (1956), *J. Biol. Chem. 223*, 935.
Delpierre, G. R., and Fruton, J. S. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1161.
Folin, O., and Ciocalteu, V. (1927), *J. Biol. Chem. 73*, 627.
Fruton, J. S., and Bergmann, M. (1939), *J. Biol. Chem. 127*, 627.
Fruton, J. S., and Bergmann, M. (1942), *J. Biol. Chem. 145*, 253.
Fruton, J. S., Fujii, S., and Knappenberger, M. H. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 759.
Green, N. M. (1956), *Nature 178*, 145.
Grisolia, S. (1964), *Physiol. Rev. 44*, 657.
Herriott, R. M. (1938), *J. Gen. Physiol. 21*, 501.
Herriott, R. M. (1962), *J. Gen. Physiol. 45*, 57.
Hiskey, R. G., and Adams, J. B. (1965), *J. Am. Chem. Soc. 87*, 3969.
Holley, R. W., and Sondheimer, E. (1954), *J. Am. Chem. Soc. 76*, 1326.
Inouye, K. (1965), *Bull. Chem. Soc. Japan 38*, 1148.
Inouye, K., and Otsuka, H. (1962), *J. Org. Chem. 27*, 4236.
Jackson, W. T., Schlamowitz, M., and Shaw, A. (1965), *Biochemistry 4*, 1537.
More O'Ferrall, R. A., Kwok, W. K., and Miller, S. I. (1964), *J. Am. Chem. Soc. 86*, 5553.
Neumann, H., Levin, Y., Berger, A., and Katchalski, E. (1959), *Biochem. J. 73*, 33.
Rajagopalan, T. G., Moore, S., and Stein, W. H. (1966), *Federation Proc. 25*, 589.
Roeske, R. (1963), *J. Org. Chem. 28*, 1251.
Sakakibara, S., and Inukai, N. (1964), *Bull. Chem. Soc. Japan 37*, 1231.
Schlamowitz, M., and Peterson, L. U. (1959), *J. Biol. Chem. 234*, 3137.
Silver, M. S., Denburg, J. L., and Steffens, J. J. (1965), *J. Am. Chem. Soc. 87*, 886.
Smith, L. I., and Howard, K. L. (1955), Organic Synthesis, Coll. Vol. 3, New York, N. Y., Wiley, p 351.
Stein, W. H., and Moore, S. (1948), *J. Biol. Chem. 176*, 367.
Tang, J. (1965), *J. Biol. Chem. 240*, 3810.

CORRECTION

In the paper by James W. Prahl and Hans Neurath in Volume 5, No. 6, June 1966, on p 2131, the reference Putnam and Neurath (1946) should read *J. Biol. Chem. 166*, 603, rather than *J. Biol. Chem. 223*, 457.