Studies on the Specificity of Pepsin*

Ken Inouye and Joseph S. Fruton

ABSTRACT: The synthesis is described of a series of new pepsin substrates of the general type benzyloxycarbonyl-L-histidyl-X-Y-OR, where X = L-phenylalanyl, *p*-nitro-L-phenylalanyl, L-tyrosyl, L-tryptophyl, or L-leucyl, and Y = L-phenylalanyl. The kinetics of pepsin action at the X-Y bond of such substrates at pH 4.0 is characterized by relatively little variation in the values for $K_{M\,\mathrm{app}}$, but considerable differences in the values for k_{cat} are observed with a series of closely related substrates. The data presented in this communication confirm and extend the view that pepsin exhibits preference for hydrophobic L-amino acid residues on both sides of the X-Y bond, and also show that the effect of varying X or Y is not independent of the nature of the other partner in the sensitive bond. A spectrophotometric method is described for following at 310 mμ the cleavage of pepsin substrates having X = *p*-nitro-L-phenylalanyl. This method was used to show that pepsin is an efficient esterase in the cleavage of benzyloxycarbonyl-L-histidyl-*p*-nitro-L-phenylalanyl-(β-phenyl)L-lactic acid methyl ester (Z-His-Phe(NO_2)-Pla-OMe) at the Phe(NO_2)-Pla bond. If either of the L-phenylalanyl residues of the substrate benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-phenylalanine ethyl ester is replaced by a D-phenylalanine residue, the resulting peptide is resistant to cleavage by pepsin. The two diastereoisomeric L-L-D and L-D-L peptides are competitive inhibitors of pepsin, with K_I values near the kinetically determined value of $K_{M\,\mathrm{app}}$ for the corresponding L-L-L substrate. This finding, together with the relative invariance of $K_{M\,\mathrm{app}}$ for different substrates of this type, suggests that for these substrates the Michaelis constant may approximate K_S, the dissociation constant of the enzyme–substrate complex.

The resistance to pepsin of peptides containing D-phenylalanyl residues was used to follow the rate of the base-catalyzed racemization of benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-phenylalanine ethyl ester and related compounds. Evidence is presented for the preferential racemization of the esterified phenylalanyl residue.

In a previous communication from this laboratory (Inouye *et al.*, 1966), the synthesis of a series of new peptide substrates for pepsin was reported. These compounds were of the general type Z-His-X-Y-OEt,[1] where X and Y denote L-phenylalanyl, L-tyrosyl, or L-tryptophyl residues. The site of protonation provided by the imidazolyl group renders the compounds moderately soluble in aqueous buffered media in the pH range 2–5, thus obviating the necessity for the presence of an organic solvent in the assay mixture. An additional advantage of these substrates is the absence of a free carboxylate group in adjacence to the sensitive peptide bond; the presence of such a group was shown to inhibit pepsin action (Inouye *et al.*, 1966).

In what follows, we report the synthesis of additional members of the series Z-His-X-Y-OEt (or OMe), where X and Y include not only the amino acid residues mentioned above, but also those of D-phenylalanine, L-leucine, and *p*-nitro-L-phenylalanine. The use of peptides containing the *p*-nitro-L-phenylalanyl residue has permitted the development of a convenient spectrophotometric method for the measurement of pepsin kinetics. Furthermore, this spectrophotometric method, when applied to the study of the action of pepsin on the depsipeptide Z-His-Phe(NO_2)-Pla-OMe, has shown that pepsin is an efficient esterase.[2] The data accumulated thus far permit tentative conclusions regarding the specificity of pepsin action.

Experimental Section

Enzymic Studies. All experiments were performed with a preparation of twice-crystallized swine pepsin (Worthington Biochemical Corp. lot no. PM 708). The cleavage of synthetic substrates was measured at 37°, and sodium citrate buffers (0.04 M) were used to control the pH. The rate of cleavage was determined either by the ninhydrin method in the manner described previously (Inouye *et al.*, 1966) or by a spectrophotometric method developed for the substrates containing a Phe(NO_2) residue. For the spectrophotometric measurements, a Cary Model 15 recording spectro-

---

* From the Department of Biochemistry, Yale University, New Haven, Connecticut 06520. *Received March 1, 1967.* These studies were aided by grants from the U. S. Public Health Service (GM-06542) and from the National Science Foundation (GB-5212X).

[1] Abbreviations used: Phe(NO_2), *p*-nitro-L-phenylalanyl; Pla, β-phenyl-L-lactyl; D-Pla, β-phenyl-D-lactyl; Pol, L-phenylalaninol; Z, benzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DCHA, dicyclohexylammonium.

[2] A preliminary report of this finding has been presented (Inouye and Fruton, 1967).

photometer, equipped with an automatic sample changer, was employed. By this means, 10–14 points could be recorded during the first 10 min of the incubation, and the initial rate (usually up to 10–20% of hydrolysis) could be determined with considerable precision.

The ultraviolet spectrum of the depsipeptide Z-His-Phe(NO$_2$)-Pla-OMe exhibits a maximum at 277.5 mμ (ϵ 9585) at pH 2.0, and at 278.5 mμ (ϵ 8850) at pH 4.0; the corresponding peptide Z-His-Phe(NO$_2$)-Phe-OMe absorbs maximally at 278.5 mμ (ϵ 9590) in the pH range 2–5. The cleavage product Z-His-Phe(NO$_2$) absorbs maximally at 279 mμ (ϵ 9120) at pH 2, and at 282 mμ (ϵ 9150) at pH 4–5.5. For the kinetic studies at pH 4.0, the wavelength chosen (310 mμ) was that at which the difference spectrum (cleavage products *vs.* substrate) showed maximal change in absorbance; $\Delta\epsilon_{310}$ is 1060 for the cleavage of the Phe(NO$_2$)-Pla bond, and 800 for the cleavage of the Phe(NO$_2$)-Phe bond. At pH 2.0, the reaction was followed at 265 mμ, where the value of $\Delta\epsilon$ is -420 for both substrates. Control experiments, in which either the enzyme or the substrate was omitted, showed no significant change in absorbance during the time period of the kinetic measurements.

In all cases, satisfactory Michaelis–Menten kinetics were observed, and plots of S/v *vs.* S permitted the fitting (method of least squares) of unambiguous straight lines. For each determination of $K_{M\text{app}}$ and $V_{\max}$ derived from such plots, initial rates were measured at five (or more) values of the initial substrate concentration S (usually in the range 0.05–1.0 mM). The estimated precision of the values for $K_{M\text{app}}$ and $V_{\max}$ is in the range ±4–8%. Depending on the rate of enzymic cleavage, the enzyme concentration was in the range 0.0001–0.01 mM; it was assumed that 1 mg of pepsin = 0.0286 μmole (mol wt 35,000). These enzyme concentrations were used to calculate k_{cat} from $V_{\max}$ values.

Enzymic Test for Extent of Racemization. The test compound (10 μmoles) was treated with 0.01 M sodium ethoxide in absolute ethanol (2 ml). This reaction mixture was kept at 30°, and at various time intervals 0.2-ml samples were withdrawn and added to 1.8 ml of 0.04 M citrate buffer (final pH 4.0). An aqueous solution of pepsin (0.2 ml, 1 mg/ml) was added, and the mixture was kept at 30° for 20–40 hr, depending on the time required for the complete hydrolysis of the susceptible substrate. Control solutions (one without enzyme, another without substrate) were kept for the same length of time as the complete mixture. At the end of this time, duplicate samples (0.5 ml) were withdrawn for analysis by the ninhydrin method (Inouye *et al.*, 1966). The extent of racemization was calculated from the difference in the extent of peptide cleavage between the sample not treated with sodium ethoxide and the sample withdrawn from the reaction mixture containing sodium ethoxide. Thus, if a NaOEt-treated sample was cleaved to 75% of the control sample (in all cases, the control sample had been cleaved to 100% for the scission of one peptide bond), the extent of racemization was inferred to have been 50%.

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 Chromagram sheets K301-R2). The following solvent systems were used: (A) methanol–ethyl acetate (1:3, v/v); (B) methanol–benzene (15:85, v/v); (C) 1-butanol–acetic acid–water (4:1:1, v/v); and (D) 1-butanol–acetic acid–pyridine–water (30:6:20:24, v/v). 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% NaCO$_3$ (Pauly); (c) 0.5% *p*-dimethylaminobenzaldehyde in 95% ethanol, followed by exposure to HCl vapor (Ehrlich); (d) phenol reagent (Folin and Ciocalteu, 1927); and (e) iodine vapor.

Synthesis of Peptides[3]

Z-Phe-Phe-OMe. This compound was prepared in the manner described previously (Inouye *et al.*, 1966) for the ethyl ester. After recrystallization from ethyl acetate–ether, it melted at 147–149°, yield 95%. *Anal.* Calcd for $C_{27}H_{28}N_2O_5$ (460.5): N, 6.1. Found: N, 6.1. The dipeptide methyl ester hydrobromide was obtained in 98% yield by the procedure described previously (Inouye *et al.*, 1966) for the ethyl ester, mp 198–200°. *Anal.* Calcd for $C_{19}H_{23}BrN_2O_3$ (407.3): N, 6.9. Found: N, 6.8.

Z-His-Phe-Phe-OMe. This compound was obtained in the manner described previously (Inouye *et al.*, 1966) for the ethyl ester. From 2.18 g (7.2 mmoles) of Z-His-NHNH$_2$ (mp 172–173°) and 2.44 (6.0 mmoles) of Phe-Phe-OMe hydrobromide, the yield was 3.2 g (89%), mp 194–196°, $[\alpha]_D^{23}$ $-26.3°$ (*c* 1, 50% aqueous acetic acid). Chromatography (solvent B) gave a single spot of R_F 0.54 (Pauly, iodine). *Anal.* Calcd for $C_{33}H_{35}N_5O_6$ (597.7): C, 66.4; H, 5.9; N, 11.7. Found: C, 66.8; H, 6.0; N, 11.7.

Chromatographic examination (solvent C) of a peptic hydrolysate of this compound (2 mM substrate, 0.04 mg of pepsin/ml, pH 4.0, 37°, 24 hr) showed the presence of a single Pauly-positive component of R_F 0.70 (identical with that for an authentic sample of Z-His-Phe) and a single ninhydrin-positive component of R_F 0.64 (identical with that for an authentic sample of Phe-OMe). The ninhydrin-positive spot was well separated from those given by L-phenylalanine (R_F 0.58), Phe-Phe (R_F 0.72), and Phe-Phe-OMe (R_F 0.73). A portion of the Z-His-Phe was isolated by filtration of the peptic hydrolysate, mp 224–225° dec (melting point of authentic sample 225° dec).

Catalytic hydrogenolysis (palladium black) of Z-His-Phe-Phe-OMe (0.60 g, 1 mmole) in the presence of 3 equiv of HCl, with methanol (20 ml) as the solvent,

---

[3] 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.

gave the tripeptide ester dihydrochloride in quantitative yield, mp 215–216° dec. Chromatography (solvent A) gave a single spot of R_F 0.43 (ninhydrin, Pauly); the corresponding ethyl ester gave the same R_F value.

*Z-Phe-*D*-Phe-OEt.* This compound was prepared from Z-Phe and D-Phe-OEt in the manner described previously (Inouye *et al.*, 1966) for the L-L isomer, mp 118–119°. *Anal.* Calcd for $C_{28}H_{30}N_2O_5$ (474.5): N, 5.9. Found: N, 5.8. Catalytic hydrogenolysis (palladium black) of 1.2 g (2.5 mmoles) of this product gave 0.96 g (100%) of the dipeptide ester hydrochloride.

*Z-His-Phe-*D*-Phe-OEt.* The free base derived from 0.47 g (1.25 mmoles) of the above hydrochloride was coupled with Z-His-N_3, derived from 0.46 g (1.5 mmoles) of the hydrazide, in the manner described previously (Inouye *et al.*, 1966) for the L-L-L isomer, yield 0.65 g (85%), mp 187–188°, $[\alpha]^{24}_{D}$ −13.4° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent B) gave a single spot of R_F 0.59 (Pauly, iodine). *Anal.* Calcd for $C_{34}H_{37}N_5O_6$ (611.7): C, 66.8; H, 6.1; N, 11.45. Found: C, 66.8; H, 6.2; N, 11.6.

*Z-*D*-Phe-Phe-OEt.* This compound was prepared from Z-D-Phe and Phe-OEt in the manner described previously (Inouye *et al.*, 1966) for the L-L isomer, mp 118–119°. *Anal.* Calcd for $C_{28}H_{30}N_2O_5$ (474.5): N, 5.9. Found: N, 5.7. Catalytic hydrogenolysis (palladium black) of 1.2 g (2.5 mmoles) of this product gave 0.94 g (99%) of the dipeptide ester hydrochloride.

*Z-His-*D*-Phe-Phe-OEt.* The free base derived from 0.47 g (1.25 mmoles) of the above hydrochloride was coupled with Z-His-N_3, derived from 0.46 g (1.5 mmoles) of the hydrazide, in the manner described previously for the L-L-L isomer, yield 0.61 (80%); after recrystallization from ethyl acetate, the product melted at 137–139°, $[\alpha]^{24}_{D}$ −8.5° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent B) gave a single spot of R_F 0.59 (Pauly, iodine). *Anal.* Calcd for $C_{34}H_{37}N_5O_6$ (611.7): C, 66.8; H, 6.1; N, 11.45. Found: C, 66.9; H, 6.1; N, 11.7.

Action of Pepsin on Mixture of Z-His-Phe-Phe-OEt and Its Diastereoisomers. N^{α},N^{Im}-Z_2-His-Phe-Phe-OEt was treated with NaOEt in the manner described previously (Inouye *et al.*, 1966), and a solution of the resulting product (mp 172–173°, 122 mg, 0.2 mmole) in 20 ml of 0.1 N HCl, containing pepsin (4 mg), was kept at 25° for 18 hr. The solution was heated at 100° for 15 min, the coagulated protein was filtered off, and $NaHCO_3$, (1.0 g) was added to the chilled filtrate. The resulting precipitate was dissolved in ethyl acetate, and the solution was washed with 5% $NaHCO_3$, dried over $MgSO_4$, and evaporated *in vacuo* to yield 45 mg (74%, assuming complete racemization of one phenylalanyl residue) of product. After recrystallization from ethanol–water, it melted at 187–188°, $[\alpha]^{24}_{D}$ −14.0° (*c* 0.5, 50% aqueous acetic acid). Upon admixture with an authentic sample of the L-L-D compound, no depression of the melting point was observed. The mixture melting point with a sample of the L-L-L compound was 180–183° (sintering at 160°). The bicarbonate-insoluble product is, therefore, Z-His-Phe-D-Phe-OEt. Neutralization of the aqueous basic reaction mixture (after extraction with ethyl acetate) to pH 4 gave a product (35 mg, 80%) that melted at 223° dec, and that was identical in properties with an authentic sample of Z-His-Phe.

Z-Phe-Phe-NH_2. Z-Phe (1.2 g, 4 mmoles) and Phe-NH_2 (mp 94–95°, 0.66 g, 4 mmoles) were coupled in the usual manner in the presence of DCC (0.83 g, 4 mmoles), with dimethylformamide as the solvent. After removal of the dicyclohexylurea, the product (1.8 g, 100%) was precipitated by the addition of water; after recrystallization from ethanol it melted at 231°. *Anal.* Calcd for $C_{26}H_{27}N_3O_4$ (445.5:) N, 9.4. Found: N, 9.3.

For the preparation of the dipeptide amide hydrobromide, the above product (1.34 g, 3 mmoles) was suspended in glacial acetic acid (2 ml), a saturated solution of HBr in acetic acid (5 ml) was added, and the reaction mixture was kept at room temperature for 30 min. Ether (50 ml) was added to yield 1.0 g (83%) of the product, mp 236–237°. *Anal.* Calcd for $C_{18}H_{22}BrN_3O_2$ (392.3): N, 10.7. Found: N, 10.5. The free dipeptide amide was obtained in 81% yield (mp 136–138°) upon neutralization of the hydrobromide with cold 50% K_2CO_3, and extraction with CH_2Cl_2 in the usual manner.

Z-His-Phe-Phe-NH_2. To an ethyl acetate solution of Z-His-N_3, derived from 0.55 g (1.8 mmoles) of the hydrazide, was added a solution of Phe-Phe-NH_2 (0.47 g, 1.5 mmoles) in tetrahydrofuran (15 ml). The mixture was kept at 0° for 2 days; the product (0.78 g, 89%) separated from the solution. After reprecipitation from ethanol–water it melted at 213–214° dec, $[\alpha]^{23}_{D}$ −27.8° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent B) gave a single spot of R_F 0.47 (Pauly, iodine). *Anal.* Calcd for $C_{32}H_{34}N_6O_5$ (582.6): C, 66.0; H, 5.9; N, 14.4. Found: C, 66.3; H, 5.8; N, 14.5.

Chromatographic examination (solvent C) of a peptic hydrolysate of this compound (2 mM substrate, 0.04 mg of pepsin/ml, pH 4.0, 37°, 24 hr) showed the presence of a single Pauly-positive component of R_F 0.70 (identical with that for an authentic sample of Z-His-Phe) and a single ninhydrin-positive component of R_F 0.58 (identical with that for an authentic sample of Phe-NH_2). The ninhydrin-positive spot was well separated from those given by Phe-Phe (R_F 0.74) and Phe-Phe-NH_2 (R_F 0.73), but not from that given by L-phenylalanine (R_F 0.61). By paper chromatography (Whatman No. 1, solvent D), the ninhydrin-positive component (purple spot) had R_F 0.69 (identical with that of Phe-NH_2), and phenylalanine had R_F 0.57 (blue spot).

An attempt to prepare Z-His-Phe-Phe-NH_2 by ammonolysis of Z_2-His-Phe-Phe-OEt (Inouye *et al.*, 1966) gave a product (yield, 47%) whose mp 200–203° dec suggests that base-catalyzed racemization had occurred, as in the treatment of Z_2-His-Phe-Phe-OEt with sodium ethoxide.

Z-Gly-His-Phe-Phe-OEt. To a solution of His-Phe-Phe-OEt (0.48 g, 1 mmole, prepared by hydrogenolysis of the benzyloxycarbonyl compound) in dioxane–water (9:1, 5 ml) was added Z-Gly-N_3 (mp 62–63°

dec, 0.35 g, 1.5 mmoles). After 40 hr at 0°, the precipitate (0.45 g) was collected. From the filtrate, an additional crop (0.08 g) was obtained. After recrystallization from ethanol–water, the product melted at 210–211°, yield 66%, $[\alpha]^{23}_{D}$ −17.5° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent C) gave a single spot of R_F 0.73 (Pauly, iodine). *Anal.* Calcd for $C_{36}H_{40}N_6O_7$ (668.7): C, 64.6; H, 6.0; N, 12.6. Found: C, 64.5; H, 5.9; N, 12.4.

Chromatographic examination (solvent C) of a peptic hydrolysate of this compound (2 mM substrate, 0.04 mg of pepsin/ml, pH 4.0, 37°, 24 hr) showed the presence of a single Pauly-positive component of R_F 0.65, and a single ninhydrin-positive component of R_F 0.68. The latter was identical with that given by an authentic sample of Phe-OEt and well separated from those for L-phenylalanine (R_F 0.60), Phe-Phe (R_F 0.74), and Phe-Phe-OEt (R_F 0.77).

Treatment of Z-Gly-His-Phe-Phe-OEt (0.29, g 0.5 mmole) with a saturated solution of HBr in glacial acetic acid (2 ml) in the usual manner, followed by treatment of the product with Amberlite IR-4B, gave the free tetrapeptide ester (0.21 g, 91%).

Z-His-Gly-Phe-Phe-OEt. To an ethyl acetate solution of Z-His-N_3, derived from 0.46 g (1.5 mmoles) of the hydrazide, was added Gly-Phe-Phe-OEt prepared from 1.2 mmoles of the hydrobromide or the acetate (Inouye *et al.*, 1966). The mixture was kept at 0° for 40 hr to yield a gelatinous product which was reprecipitated from ethanol–water, yield 0.6 g (72%), mp 164–166°, $[\alpha]^{23}_{D}$ −15.0° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent B) gave a single spot of R_F 0.46 (Pauly, iodine). *Anal.* Calcd for $C_{36}H_{40}N_6O_7$ (668.7): C, 64.6; H, 6.0; N, 12.6. Found: C, 64.5; H, 5.9; N, 12.3.

Chromatographic examination (solvent C) of a peptic hydrolysate of this compound (2 mM substrate, 0.2 mg of pepsin/ml, pH 4.0, 37° 24 hr) showed the presence of one Pauly-positive component (R_F 0.65) and of one ninhydrin-positive component of R_F 0.68 (identical with that for an authentic sample of Phe-OEt.

Z_2-Tyr-Phe-OMe. Z_2-Tyr (mp 117°, 4.5 g, 10 mmoles) and Phe-OMe, derived from 2.6 g (12 mmoles) of the hydrochloride, were coupled in the usual manner in the presence of DCC (2.1 g, 10 mmoles), with CH_2Cl_2 (40 ml) as the solvent. The precipitate that formed during 40 hr at 0° contained both a portion of the coupling product and dicyclohexylurea; the latter was removed by means of dioxane. The filtrate obtained from the reaction mixture, upon evaporation *in vacuo*, yielded the remainder of the coupling product, total yield 5.0 g (82%). After recrystallization from dioxane–ether, the product melted at 186–188°. *Anal.* Calcd for $C_{35}H_{34}N_2O_8$ (610.6): N, 4.6. Found: N, 4.7.

Z-His-Tyr-Phe-OMe. Z_2-Tyr-Phe-OMe (2.0 g, 3.3 mmoles) was subjected to catalytic hydrogenolysis (palladium black) in the presence of 2 equiv of HCl, with methanol (45 ml) as the solvent. Evaporation of the filtrate *in vacuo* gave an oil that could not be crystallized; chromatography (solvent C) gave a single spot of R_F 0.52 (ninhydrin, phenol reagent). The free dipeptide ester (derived from the hydrochloride in the usual manner) was added to an ethyl acetate solution of Z-His-N_3, derived from 1.2 g (4 mmoles) of the hydrazide. The reaction mixture was kept at 0° for 2 days, and the product that separated was collected; yield 1.5 g (75%), mp 174–176°, $[\alpha]^{23}_{D}$ −22.4° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent A) gave a single spot of R_F 0.42 (phenol reagent, iodine). *Anal.* Calcd for $C_{33}H_{35}N_5O_7$ (613.7): C, 64.7; H, 5.8; N, 11.4. Found: C, 64.7; H, 5.9; N, 11.3.

Chromatographic examination (solvent C) of a peptic hydrolysate of this compound (2 mM substrate, 0.2 mg of pepsin/ml, pH 4.0, 37°, 24 hr) showed the presence of a single component of R_F 0.68 that was reactive toward both the Pauly and phenol reagents (R_F of authentic sample of Z-His-Tyr, 0.68), and a single ninhydrin-positive component of R_F 0.64 (identical with that for an authentic sample of Phe-OMe).

Z-His-Tyr. To a solution of $N^{\alpha},N^{\mathrm{Im}}$-$Z_2$-His-Tyr-OEt (Inouye *et al.*, 1966) (0.77 g, 1.25 mmoles) in ethanol (6 ml) was added 2.75 ml of 1 N NaOH. After 2 hr at 0°, the solution was neutralized wih 1 N HCl, and then concentrated *in vacuo* to yield 0.52 g (92%) of product. After recrystallization from ethanol, it melted at 230–231° dec. *Anal.* Calcd for $C_{23}H_{24}N_4O_6 \cdot H_2O$ (470.5): N, 11.9. Found: N, 11.7.

Z-Trp-Phe-OMe. Z-Trp (3.4 g, 10 mmoles) and Phe-OMe, prepared from 2.4 g (11 mmoles) of the hydrochloride, were coupled in the usual manner in the presence of DCC (2.1 g, 10 mmoles), with CH_2Cl_2 (35 ml) as the solvent. The filtrate obtained upon removal of dicyclohexylurea was concentrated to dryness *in vacuo* and the residue was taken up in ethyl acetate; the solution was washed successively with 1 N HCl, water, and 5% $NaHCO_3$, dried over $MgSO_4$, and concentrated to yield 4.8 g (96%) of the product. After recrystallization from ethyl acetate–ether, it melted at 132–133°. *Anal.* Calcd for $C_{29}H_{29}N_3O_5$ (499.5): N, 8.4. Found: N, 8.2.

Z-His-Trp-Phe-OMe. Z-Trp-Phe-OMe (1.25 g, 2.5 mmoles) was subjected to catalytic hydrogenolysis (palladium black) in the presence of methanol (20 ml) and glacial acetic acid (0.5 ml). The resulting dipeptide ester acetate was converted to the free base with cold 50% K_2CO_3, with CH_2Cl_2 as the organic solvent. Evaporation of the solvent gave an oily dipeptide ester, which was added to an ethyl acetate solution of Z-His-N_3, derived from 0.91 (3 mmoles) of the hydrazide. After 20 hr at 0°, the reaction mixture was concentrated *in vacuo*, and the residue was crystallized from ethanol–water; yield 1.3 g (80%), mp 156–158°, $[\alpha]^{23}_{D}$ −20.4° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent A) gave a single spot of R_F 0.53 (Pauly, Ehrlich). *Anal.* Calcd for $C_{35}H_{36}N_6O_6$ (636.7): C, 66.0; H, 5.7; N, 13.2. Found: C, 65.9; H, 5.7; N, 13.3.

Chromatographic examination (solvent C) of a peptic hydrolysate of this compound (2 mM substrate, 0.2 mg of pepsin/ml, pH 4.0, 37°, 24 hr) showed the presence of a single component of R_F 0.67 that was reactive toward both the Pauly and Ehrlich reagents, and of a single ninhydrin-positive component of R_F 0.64 (identical with that for an authentic sample of Phe-OMe).

Z-Phe-Leu-OMe. Z-Phe (3.0 g, 10 mmoles) and Leu-OMe, derived from 2.0 g (11 mmoles) of the hydrochloride, were coupled in the usual manner in the presence of DCC (2.1 g, 10 mmoles), with CH_2Cl_2 (40 ml) as the solvent, yield 4.05 g (95%), mp 108–110° (unaltered by recrystallization from ethyl acetate–ether–petroleum ether (bp 30–60°)). *Anal.* Calcd for $C_{24}H_{30}N_2O_5$ (426.5): N, 6.6. Found: N, 6.4.

The dipeptide ester hydrobromide was prepared from the above product (2.1 g, 5 mmoles) by treatment for 60 min with acetic acid (5 ml) saturated with HBr. After removal of the solvent *in vacuo*, the residue was dissolved in water (10 ml). The aqueous solution was washed with ether, treated at 0° with 50% K_2CO_3, and extracted with CH_2Cl_2; after having been dried over $MgSO_4$, the extract was concentrated *in vacuo* to yield the dipeptide ester as an oil.

Z-His-Phe-Leu-OMe. To an ethyl acetate solution of Z-His-N_3, derived from 1.8 g (6 mmoles) of the hydrazide, was added Phe-Leu-OMe prepared from 2.1 g (5 mmoles) of Z-Phe-Leu-OMe in the manner described above. The mixture was kept at 0° for 40 hr to yield a crystalline product, yield 2.3 g (80%), mp 178–180°. After recrystallization from ethanol–water it melted at 180–181°, $[\alpha]^{23}_{\mathrm{D}}$ −39.9° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent B) gave a single spot of R_F 0.48 (Pauly, iodine). *Anal.* Calcd for $C_{30}H_{37}N_5O_6$ (563.6): C, 63.9; H, 6.6; N, 12.4. Found: C, 64.0; H, 6.5; N, 12.2.

Chromatographic examination (solvent C) of a peptic hydrolysate (2 mM substrate, 0.4 mg of pepsin/ml, pH 4.0, 37°, 24 hr) showed the presence of a single Pauly-positive component of R_F 0.71 (identical with that for an authentic sample of Z-His-Phe) and a single ninhydrin-positive component of R_F 0.65 (identical with that for an authentic sample of Leu-OMe).

Z-Leu-Phe-OMe. Z-Leu, derived from the crystalline DCHA salt (mp 153–155°, 4.5 g, 10 mmoles) (Kliegler *et al.*, 1961) by treatment with Dowex 50W (H^+ form, 200–400 mesh), and Phe-OMe, prepared from 2.2 g (10 mmoles) of the hydrochloride, were coupled in the presence of DCC (2.1 g, 10 mmoles), with CH_2Cl_2 as solvent, yield 3.8 g (89%), mp 89–91°. After recrystallization from ethyl acetate–ether–petroleum ether, it melted at 90–91°. *Anal.* Calcd for $C_{24}H_{30}N_2O_5$ (426.5): N, 6.6. Found: N, 6.5. Catalytic hydrogenolysis (palladium black) of 2.1 g (5 mmoles) of this product gave the dipeptide ester hydrochloride (mp 200–201°) in quantitative yield. *Anal.* Calcd for $C_{16}H_{25}ClN_2O_3$ (328.8): N, 8.5. Found: N, 8.4.

Z-His-Leu-Phe-OMe. To an ethyl acetate solution (35 ml) of Z-His-N_3, derived from 0.9 g (3 mmoles) of the hydrazide, was added Leu-Phe-OMe prepared in the usual manner from 0.82 g (2.5 mmoles) of the hydrochloride. The mixture was kept at 0° for 40 hr to yield a gelatinous product which was crystallized from ethanol–water, yield 1.3 g (91%), mp 185–187°, $[\alpha]^{23}_{\mathrm{D}}$ −33.4° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent B) gave a single spot of R_F 0.48 (Pauly, iodine). *Anal.* Calcd for $C_{30}H_{37}N_5O_6$ (563.6): C, 63.9; H, 6.6; N, 12.4. Found: C, 63.7; H, 6.6; N, 12.3.

Chromatographic examination (solvent C) of a peptic hydrolysate (2 mM substrate, 0.2 mg of pepsin/ml, pH 4.0, 37°, 24 hr) showed the presence of a single Pauly-positive component of R_F 0.69, and a single ninhydrin-positive component of R_F 0.64 (identical with that for an authentic sample of Phe-OMe); the latter spot was clearly separated from that given by Leu-Phe-OMe (R_F 0.73).

Z-Phe-Pol. L-Phenylalaninol (Pol, L-2-amino-3-phenylpropan-1-ol) was prepared from Phe-OMe by reduction with lithium aluminum hydride in tetrahydrofuran,[4] mp 91–92°, $[\alpha]^{24}_{\mathrm{D}}$ −26.0° (*c* 1, ethanol). Toyoda *et al.* (1963) reported mp 95° and $[\alpha]^{24}_{\mathrm{D}}$ −22.0° (ethanol) for this compound. Paper chromatography (solvent D) showed the presence of a single ninhydrin-reactive component (R_F 0.82), clearly separated from phenylalanine (R_F 0.56) and Phe-OMe (R_F 0.75).

Z-Phe (1.2 g, 4 mmoles) and Pol (0.67 g, 4.4 mmoles) were coupled in the usual manner in the presence of ethyl chlorocarbonate (0.42 ml, 4.4 mmoles) and tri-*n*-butylamine (1.05 ml, 4.4 mmoles), with tetrahydrofuran (15 ml) as the solvent. After being stirred at 0° for 1 hr, and kept at 0° overnight, the reaction mixture was concentrated *in vacuo*, and the residue was dissolved in ethyl acetate. The solution was washed successively with 1 N HCl (twice), water, and 5% $NaHCO_3$ (twice), dried over $MgSO_4$, and evaporated *in vacuo* to yield 1.33 g (77%) of the product, mp 166–167°. *Anal.* Calcd for $C_{26}H_{28}N_2O_4$ (432.5): N, 6.5. Found: N, 6.3. Catalytic hydrogenolysis (palladium black) of 1.1 g (2.5 mmoles) of this product gave 0.54 g (73%) of Phe-Pol, mp 61–63°. Chromatography (solvent B) gave a single spot of R_F 0.47 (iodine).

Z-His-Phe-Pol. To an ethyl acetate solution of Z-His-N_3, prepared from 0.55 g (1.8 mmoles) of the hydrazide, was added 0.45 g (1.5 mmoles) of Phe-Pol. The mixture was kept at 0° for 20 hr and then concentrated to a small volume. The addition of ether gave 0.68 g (80%) of the product. After reprecipitation from ethanol–water, it melted at 177–179°, $[\alpha]^{23}_{\mathrm{D}}$ −41.0° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent B) gave a single spot of R_F 0.46 (Pauly, iodine). *Anal.* Calcd for $C_{32}H_{35}N_5O_5$ (569.6): N, 12.3. Found: N, 12.1.

Z-Phe(NO_2). To a solution of *p*-nitro-L-phenylalanine monohydrate (2.1 g, 9.2 mmoles, Cyclo Chemical Corp.) in 1 N NaOH (10 ml) was added Na_2CO_3 (1.2 g, 11 mmoles); benzyloxycarbonyl chloride (1.9 g, 11 mmoles) was then added dropwise with stirring at 0° for 1 hr. After being washed with ether, the solution was acidified with 6 N HCl (4 ml), and the resulting precipitate was dissolved in ethyl acetate. The ethyl acetate solution was washed with 1 N HCl and water, dried over $MgSO_4$, and evaporated *in vacuo* to yield a product

---

[4] We are indebted to Dr. G. R. Delpierre of this laboratory for valuable modifications in the procedure.

that was crystallized from ethyl acetate–petroleum ether, yield 3.0 g (96%), mp 135–136°. *Anal.* Calcd for $C_{17}H_{16}N_2O_6$ (344.3): C, 59.4; H, 4.7; N, 8.1. Found: C, 59.4; H, 5.0; N, 8.0.

Z-Phe(NO₂)-Phe-OMe. Z-Phe(NO_2) (1.7 g, 5 mmoles) and Phe-OMe, derived from 1.2 g (5.5 mmoles) of the hydrochloride, were coupled in the usual manner in the presence of DCC (1.0 g, 5 mmoles), with CH_2Cl_2 as the solvent. The filtrate obtained upon removal of dicyclohexylurea was concentrated to dryness *in vacuo* and the residue was taken up in ethyl acetate; the solution was washed successively with 1 N HCl, water, and 5% $NaHCO_3$, dried over $MgSO_4$, and concentrated to yield 2.4 g (96%) of the product. After recrystallization from ethyl acetate–ether–petroleum ether, it melted at 167–168°. *Anal.* Calcd for $C_{27}H_{27}N_3O_7$ (505.5): N, 8.3. Found: 8.4.

For the preparation of the dipeptide ester hydrobromide, the above product (1.5 g, 3.0 mmoles) was dissolved in glacial acetic acid (5 ml), a saturated solution of HBr in acetic acid (10 ml) was added, and the reaction mixture was kept at room temperature for 1 hr. Ether (100 ml) was added to yield 1.1 g (86%) of the product, mp 207–208° dec. *Anal.* Calcd for $C_{19}H_{22}BrN_3O_5$ (452.3); N, 9.3. Found: N, 9.1.

Z-His-Phe(NO₂)-Phe-OMe. An ethyl acetate solution of Z-His-N_3, derived from 0.73 g (2.4 mmoles) of the hydrazide, was added to Phe(NO_2)-Phe-OMe, derived from the hydrobromide (0.9 g, 2 mmoles) in the usual manner. The reaction mixture was kept at 0° for 24 hr, and concentrated *in vacuo* to yield 1.1 g (85%) of the product. After recrystallization from ethanol–water, it melted at 217–218° dec, $[\alpha]_D^{23}$ −33.0° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent B) gave a single spot of R_F 0.56 (Pauly, iodine). *Anal.* Calcd for $C_{33}H_{34}N_6O_8$ (642.7): C, 61.7; H, 5.3; N, 13.1. Found: C, 61.4; H, 5.5; N, 13.05.

Treatment of this compound (0.26 g, 0.4 mmole) with pepsin (5 mg) at pH 4 and 26° (total volume, 57 ml) for 16 hr resulted in the deposition of a crystalline precipitate. The mixture was chilled, and the crystals (0.17 g, 86%) were collected and recrystallized from ethanol–dioxane–water, mp 236° dec. The product is Z-His-Phe(NO_2). *Anal.* Calcd for $C_{23}H_{23}N_5O_7$ (481.5): C, 57.4; H, 4.8; N, 14.5. Found: C, 57.0; H, 4.8; N, 14.3. Samples of the filtrate were examined by thin layer chromatography (solvent C), which showed the presence of a single ninhydrin-positive component of R_F 0.60 (identical with that for an authentic sample of Phe-OMe).

*Z-Phe(NO₂)-*D*-Phe-OMe.* This compound was prepared in the manner described for the L-L isomer from Z-Phe(NO_2) (2.6 g, 7.5 mmoles) and D-Phe-OMe (derived from 1.8 g (8.25 mmoles) of the hydrochloride), with DCC (1.55 g, 7.5 mmoles) as the coupling agent, yield 3.55 g (94%), mp 160–161° (unchanged after recrystallization from ethyl acetate–ether). *Anal.* Calcd for $C_{27}H_{27}N_3O_7$ (505.5): N, 8.3. Found: N, 8.3. This product was converted to the dipeptide ester hydrobromide in the manner described for the L-L isomer.

*Z-His-Phe(NO₂)-*D*-Phe-OMe.* This compound was prepared in the same manner as the L-L-L isomer by the coupling of Z-His-N_3, derived from 0.73 g (2.4 mmoles) of the hydrazide, and Phe(NO_2)-D-Phe-OMe prepared from 0.94 g (2 mmoles) of the hydrobromide, yield 1.1 g (85%), mp 198–199°, $[\alpha]_D^{23}$ −17.5° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent B) gave a single spot of R_F 0.56 (Pauly, iodine). *Anal.* Calcd for $C_{33}H_{34}N_6O_8$ (642.7): C, 61.7; H, 5.3; N, 13.1. Found: C, 62.1; H, 5.3; N, 12.8.

Z-Phe-Pla-OMe. Z-Phe (6.0 g, 20 mmoles) was dissolved in dry pyridine (30 ml), and to the chilled solution was added dropwise benzenesulfonyl chloride (2.55 ml, 20 mmoles). After the solution had been stirred for 15 min at 0°, a solution of β-phenyl-L-lactic acid methyl ester (3.6 g, 20 mmoles, Pierce Chemical Co.) in pyridine (10 ml) was added. The mixture was stirred for 2 hr at 0° and for 2 hr at room temperature, poured into ice-cold water (200 ml), and the resulting oil was extracted with ether. The ethereal solution was washed successively with cold water, 1 N HCl, 5% $NaHCO_3$, dried over $MgSO_4$, and evaporated *in vacuo* to yield an oil that crystallized from ether–petroleum ether, yield 7.7 g (83%), mp 79–80°. Chromatography (solvent B) gave single spot of R_F 0.78 (iodine). *Anal.* Calcd for $C_{27}H_{27}NO_6$ (461.5): C, 70.2; H, 5.9; N, 3.0. Found: C, 70.1; H, 5.8; N, 3.05. The corresponding ethyl ester was prepared in a similar manner, to yield a product melting at 82–83°. *Anal.* Calcd for $C_{28}H_{29}NO_6$: (475.5): C, 70.8; H, 6.15; N, 2.95. Found: C, 70.65; H, 6.2; N, 3.2. Attempts to prepare this depsipeptide derivative by means of the acid chloride or mixed anhydride (ethyl chlorocarbonate) method did not yield a satisfactory product.

Z-Phe(NO₂)-Pla-OMe. Z-Phe(NO_2) (3.4 g, 10 mmoles) was coupled with β-phenyl-L-lactic acid methyl ester (1.8 g, 10 mmoles, Pierce Chemical Co.) by means of benzenesulfonyl chloride (1.3 ml, 10 mmoles), with dry pyridine as the solvent. The procedure was similar to that described above for the synthesis of Z-Phe-Pla-OMe, except that the *p*-nitro derivative was found to be less soluble in ether, yield 3.4 g (67%); after recrystallization from ethyl acetate–ether–petroleum ether the product melted at 135–136°. Chromatography (solvent B) gave a single spot of R_F 0.75 (iodine). *Anal.* Calcd for $C_{27}H_{26}N_2O_8$ (506.5): C, 64.0; H, 5.2; N, 5.5. Found: C, 64.05; H, 5.3; N, 5.6.

Upon treatment of the above compound (2.5 g, 5 mmoles) with HBr in glacial acetic acid (7 ml) in the usual manner, 2.2 g (96%) of the depsipeptide ester hydrobromide was obtained, mp 136–137°. Chromatography (solvent B) gave a single spot of R_F 0.67 (ninhydrin). *Anal.* Calcd for $C_{19}H_{21}BrN_2O_6$ (453.3): N, 6.2. Found: N, 6.0. The hydrobromide was converted to the free base by neutralization with cold saturated $NaHCO_3$, followed by extraction with CH_2Cl_2.

Z-His-Phe(NO₂)-Pla-OMe. To an ethyl acetate solution of Z-His-N_3, derived from 0.49 g (1.6 mmoles) of the hydrazide, was added Phe(*p*-NO_2)-Pla-OMe, derived from 0.60 g (1.32 mmoles) of the hydrobromide. After 42 hr at 0°, the solution was evaporated *in vacuo* and the residue was crystallized by the addition of ether, yield 0.54 g (64%); after recrystallization from ethyl acetate, the product melted at 134–136°, $[\alpha]^{23}_{\mathrm{D}}$ −32.5° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent B) gave a single spot of R_F 0.54 (Pauly, iodine). *Anal.* Calcd for $C_{33}H_{33}N_5O_9$ (643.6): C, 61.6; H, 5.2; N, 10.9. Found: C, 61.7; H, 5.3; N, 10.8.

Treatment of this compound (20 mg, 0.031 mmole) with pepsin (0.5 mg) at pH 4 and 26° (total volume, 11 ml) for 16 hr resulted in the deposition of Z-His-Phe(NO_2) (11.8 mg, 78%), mp 234–235° dec. Samples of the filtrate were examined by thin layer chromatography (solvent B), which showed the presence of a single mobile iodine-positive component of R_F 0.65 (identical with that for an authentic sample of Pla-OMe).

D-*Pla-OMe.* To a solution of D-phenylalanine (5 g, 30 mmoles) in 1 N HCl (33 ml) and water (50 ml) was added dropwise an aqueous solution of $NaNO_2$ (2.3 g, 33 mmoles) at 0°. The mixture was stirred for 60 min at room temperature, allowed to stand overnight, and NaCl was added to saturation. Extraction with ether gave a product that was crystallized from ether–petroleum ether, yield 2.5 g (51%), mp 120–123°, $[\alpha]^{24}_{\mathrm{D}}$ +20.8 (*c* 1, water). Suwa (1911) reported mp 121° and $[\alpha]^{20}_{\mathrm{D}}$ +19.7° (*c* 1, water) for β-phenyl-D-lactic acid. The corresponding L compound, prepared in the manner described above, melted at 119–122°, $[\alpha]^{24}_{\mathrm{D}}$ −21.6° (*c* 1, water). Suwa (1911) reported mp 121° and $[\alpha]^{20}_{\mathrm{D}}$ −19.7° (*c* 1, water) for the L isomer. Winitz *et al.* (1956) reported $[\alpha]^{25}_{\mathrm{D}}$ +20.0 and −20.0° (*c* 1, water) for the D and L isomers of β-phenyllactic acid, respectively.

Esterification of β-phenyl-D-lactic acid (2.2 g, 13 mmoles) with thionyl chloride in methanol gave the desired methyl ester. After recrystallization from ether–petroleum ether, the yield was 1.9 g (81%), mp 48–50°, $[\alpha]^{25}_{\mathrm{D}}$ +12.8° (*c* 1, benzene). A commercial preparation (Pierce Chemical Co.) of L-Pla-OMe melted at 47–49.5°, $[\alpha]^{25}_{\mathrm{D}}$ −12.9° (*c* 1, benzene). L-Pla-OEt was prepared by esterification of β-phenyl-L-lactic acid with thionyl chloride in ethanol, $[\alpha]^{24}_{\mathrm{D}}$ −21.4° (*c* 1, benzene).

*Z-Phe(NO_2)-*D*-Pla-OMe.* This compound was prepared in the manner described above for the L-L isomer from Z-Phe(NO_2) (2.6 g, 7.5 mmoles) and D-Pla-OMe (1.35 g, 7.5 mmoles), yield 2.4 g (63%), mp 81–83°. After recrystallization from ethyl acetate–ether, the product melted at 82.5–84°. *Anal.* Calcd for $C_{27}H_{26}N_2O_8$ (506.5): C, 64.0; H, 5.2; N, 5.5. Found: C, 64.1; H, 5.0; N, 5.4. Treatment of the above product (1.5 g, 3 mmoles) with glacial acetic acid (3 ml) saturated with HBr gave the depsipeptide ester hydrobromide as an amorphous solid; it was converted to the free base by neutralization with cold saturated $NaHCO_3$, followed by extraction with CH_2Cl_2.

*Z-His-Phe(NO_2)-*D*-Pla-OMe.* To an ethyl acetate solution (35 ml) of Z-His-N_3, derived from 0.9 g (3 mmoles) of the hydrazide, was added Phe(NO_2)-D-Pla-OMe prepared as described above. The mixture was kept at 0° for 40 hr to yield a crystalline product (1 g, 50%), mp 174–177°. After recrystallization from ethanol–water, it melted at 176–177°, $[\alpha]^{23}_{\mathrm{D}}$ −13.8° (*c* 1, 50% aqueous acetic acid). Chromatography (solvent B) gave a single spot of R_F 0.54 (Pauly, iodine). *Anal.* Calcd for $C_{33}H_{33}N_5O_9$ (643.6): C, 61.6; H, 5.2; N, 10.9. Found: C, 61.6; H, 5.1; N, 11.1.

Results

Effect of Structural Changes in Pepsin Substrates. Previous studies (Baker, 1951; Inouye *et al.*, 1966; Jackson *et al.*, 1966) have indicated that the action of pepsin at a sensitive peptide bond is favored by the presence, on both sides of the bond, of the L forms of aromatic amino acid residues (Phe, Tyr, and Trp). Until recently, however, the number of synthetic substrates for pepsin has been relatively limited, and the specificity of pepsin requires closer examination through kinetic studies with a larger variety of substrates whose structure has been systematically altered. To facilitate the further discussion, we denote a typical synthetic substrate of pepsin as AX-YB, where X and Y are the residues joined by the sensitive bond; A is the group linked to the α-amino group of X, and B is the group attached to the α-carbonyl group of Y. In the present work, the kinetic parameters $K_{M\,\mathrm{app}}$ and k_{cat} were determined routinely at pH 4.0 because previous work (Inouye *et al.*, 1966) had shown that for substrates with A = Z-His and B = OEt this pH value is near the optimum for the cleavage of the X-Y bond. It is recognized that comparison of the kinetic data at a single pH value can only give evidence of large specificity effects, and that relatively small differences in K_M or k_{cat} values may be a consequence of differences in the pH dependence curves of closely related substrates. We believe, however, that initial studies at a single pH value can give useful data to guide the selection of substrates for more detailed study of the pH dependence of the kinetic parameters. The substrates tested were found to be cleaved by pepsin only at the X-Y bond, as judged by the identification of the products through isolation or chromatography (see Experimental Section) and by the ninhydrin assay.

Examination of the kinetic data in Table I indicates that, for the various substrates listed, the values of $K_{M\,\mathrm{app}}$ do not differ greatly from one another, and that the differences in the rates of hydrolysis are largely reflected in widely varying values of k_{cat}. Thus, whereas the K_M values range between 0.2 and 0.8 mM, the k_{cat} values vary over a 1000-fold range (0.0025–2.4 sec^{-1}).

For the group of substrates with A = Z-His and X = Y = Phe, the change in the B group from OEt or OBu^t to OMe or NH_2 produces a relatively small (but significant) decrease in the susceptibility of the Phe-Phe bond to peptic hydrolysis. For the group of substrates with A = Z-His, Y = Phe, and B = OMe, replacement of X = Phe by Phe(NO_2) causes little change in the rate of cleavage, but the introduction of X = Tyr, Trp, or Leu leads to a considerable decrease (about tenfold) in susceptibility, all three substrates being cleaved at similar rates. On the other

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

Substrate	K_M (mM)	k_{cat} (sec^{-1})	k_{cat}/K_M (mM^{-1} sec^{-1})	Rel Reactivity
Z-His-Phe-Phe-OMe	0.37	0.15	0.40	1
Z-His-Phe-Phe-OEt[b]	0.18	0.31	1.72	4.3
Z-His-Phe-Phe-OBu^t	0.43	0.45	1.05	2.6
Z-His-Phe-Phe NH$_2$	0.49	0.21	0.43	1.1
Z-His-Phe(NO$_2$)-Phe-OMe[c]	0.46	0.29	0.63	1.6
Z-His-Tyr-Phe-OMe	0.68	0.013	0.019	0.048
Z-His-Trp-Phe-OMe	0.25	0.013	0.052	0.13
Z-His-Leu-Phe-OMe	0.54	0.017	0.031	0.078
Z-His-Phe-Tyr-OEt[b]	0.23	0.16	0.70	1.8
Z-His-Phe-Trp-OEt[b]	0.23	0.51	2.22	5.5
Z-His-Phe-Leu-OMe	0.56	0.0025	0.0044	0.011
Z-His-Tyr-Tyr-OEt[b]	0.24	0.0094	0.039	0.098
Z-Gly-His-Phe-Phe-OEt	0.80	2.43	3.04	7.6
Z-His-Gly-Phe-Phe-OEt	0.28	0.032	0.11	0.28
Gly-Gly-Phe-Phe-OEt[b]	0.28	0.18	0.64	1.6
Z-His-Phe(NO$_2$)-Pla-OMe[d]	0.40	0.77	1.93	4.8

[a] pH 4.0 (0.04 M citrate), 37° (for details, see Experimental Section). [b] Previously reported data (Inouye *et al.*, 1966) included for comparison with new results. [c] At pH 2.0 (0.04 M citrate) and 37°, the values for K_M and k_{cat} were 0.52 mM and 0.07 sec^{-1}, respectively. [d] At pH 2.0 (0.4 M citrate) and 37°, the values for K_M and k_{cat} were 0.40 mM and 0.13 sec^{-1}, respectively.

hand, for the substrates A = Z-His, X = Phe, and B = OMe or OEt (the appropriate ester of Z-His-Phe-Phe used for comparison), the replacement of Y = Phe by Tyr, Trp, or Leu produces substrates of widely different susceptibility, the compound with Y = Trp being a somewhat better substrate than the comparison compound, the one with Y = Tyr a poorer substrate, and the one with Y = Leu a much poorer substrate. The data in Table I suggest therefore that, for the series of substrates discussed above, the relative specificity of pepsin for the X group is Phe > Tyr $\simeq$ Trp $\simeq$ Leu, and for the Y group is Trp > Phe > Tyr $\gg$ Leu (when the other residue forming the sensitive bond is Phe).

It was of considerable interest to find a sizeable increase in susceptibility when the A group (Z-His) of Z-His-Phe-Phe-OEt was replaced by Z-Gly-His. That this effect is not solely a consequence of the position of the benzyloxycarbonyl group in relation to the sensitive bond, or of the number of CONH groups in the substrate, is indicated by the slow hydrolysis of the isomeric Z-His-Gly-Phe-Phe-OEt. The possibility exists that the presence of a suitably located cationic group (as in A = Z-His or Gly-Gly) promotes the enzymic action; it should be noted, however, that Gly-Phe-Phe-OEt, in which the positively charged group is in closer proximity to the sensitive bond, is hydrolyzed by pepsin much more slowly than Gly-Gly-Phe-Phe-OEt (Inouye *et al.*, 1966). Further evidence of the exacting specificity of pepsin is provided by the observation that Z-His-Phe-L-phenylalaninol is resistant to peptic cleavage (0.5 mM peptide, 0.2 mg of pepsin/ml, pH 2.0 and 4.0, 37°, 20 hr) as judged by the ninhydrin assay (L-phenylalaninol used as the standard).

Spectrophotometric Method for Pepsin Kinetics. The ninhydrin method for following the kinetics of pepsin action gives reproducible results, and the recent automatic recording procedures described by Lenard *et al.* (1965) and Cornish-Bowden and Knowles (1965) facilitate the accumulation of kinetic data by this method. Unpublished experiments by Dr. T. R. Hollands in this laboratory have shown the applicability of the automatic ninhydrin method to the study of the kinetics of pepsin action on substrates of the general type Z-His-X-Y-OR. It seemed desirable, however, to have in addition to the ninhydrin method reliable spectrophotometric procedures for following pepsin kinetics, especially for the examination of the esterase activity of pepsin and for the determination if K_I values for competitive inhibitors. Silver *et al.* (1965) and Clement and Snyder (1966) used a spectrophotometric method patterned after that of Schwert and Takenaka (1955), involving measurement of the change in absorbance at 237 mμ.

In the spectrophotometric procedure used in the present work, advantage was taken of the change in absorbance when a bond linking the carbonyl group of a *p*-nitrophenylalanyl residue is cleaved (Figure 1). As indicated in the Experimental Section, the value of $\Delta\epsilon$ is appreciable, and automatic recording of the change in absorbance permits satisfactory estimation of initial rates of hydrolysis. The results obtained by the spectrophotometric method for the peptic cleavage of Z-His-Phe(NO$_2$)-Phe-OMe were identical with those determined by the ninhydrin method, with samples of the same incubation mixture used for the two assay procedures. For the measurement of the rate of cleavage of the ester linkage between the Phe(NO$_2$) and Pla residues in Z-His-Phe(NO$_2$)-Pla-OMe, the

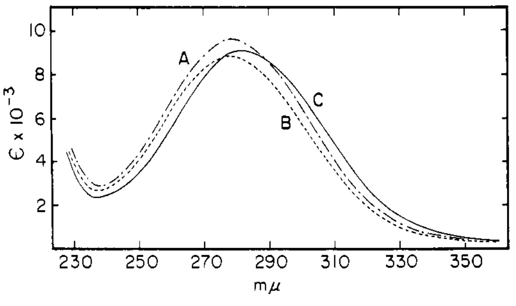

FIGURE 1: Absorption spectra of *p*-nitro-L-phenylalanine derivatives at pH 4.0. Curve A: Z-His-Phe(NO_2)-Phe-OMe; curve B: Z-His-Phe(NO)$_2$-Pla-OMe; and curve C: Z-His-Phe(NO_2).

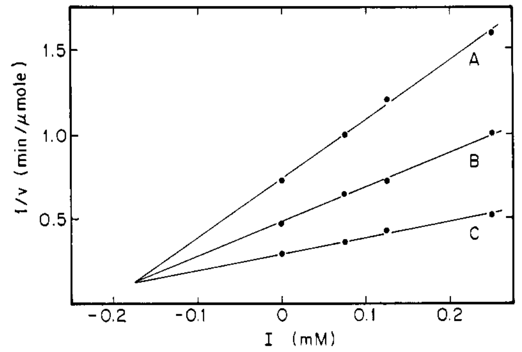

FIGURE 2: Competitive inhibition by Z-His-D-Phe-Phe-OEt of peptic hydrolysis of Z-His-Phe(NO_2)-Phe-OMe. Enzyme concentration, 0.02 mg/ml; pH 4.0; 37°. Substrate concentration: curve A, 0.075 mM; curve B, 0.125 mM; curve C, 0.25 mM. Plot according to Dixon (1953).

ninhydrin procedure is not applicable, and spectrophotometry is the method of choice.

That the enzymic cleavage of Z-His-Phe(NO_2)-Phe-OMe is restricted to the cleavage of the Phe(NO_2)-Phe bond was shown by the finding that the increase in ninhydrin color stopped after 100% hydrolysis of one peptide bond, the isolation from the peptic hydrolysate of Z-His-Phe(NO_2) in 86% yield, and the demonstration by thin layer chromatography that phenylalanine methyl ester was the only ninhydrin-reactive component of the hydrolysate. It will be noted from Table I that the kinetic parameters for Z-His-Phe(NO_2)-Phe-OMe and Z-His-Phe-Phe-OMe are very similar, indicating that the introduction of the *p*-nitro group does not cause a significant change in the susceptibility of the sensitive bond.

Since the spectrophotometric method measures the formation of the AX product of hydrolysis, while the ninhydrin method measures the YB product, it was of special interest to determine whether the two products are formed at different initial rates. Under the conditions of these experiments, no significant differences could be observed, and no "burst" formation of Z-His-Phe(NO_2) could be detected. Further studies on this question are in progress.

Pepsin as an Esterase. The spectrophotometric method described above was used to examine the activity of pepsin as an esterase toward the depsipeptide Z-His-Phe(NO_2)-Pla-OMe, whose synthesis is described in the Experimental Section. The change in absorbance (*cf.* Figure 1) was found to be in accord with that to be expected for the cleavage of the Phe(NO_2)-Pla bond. Furthermore, there was isolated from the peptic hydrolysate Z-His-Phe(NO_2), and Pla-OMe was shown by thin layer chromatography to be the only other product of hydrolysis. The depsipeptide is cleaved more rapidly per unit weight of pepsin than is the corresponding peptide Z-His-Phe(NO_2)-Phe-OMe. It was found that the cleavage of the ester bond follows satisfactory Michaelis–Menten kinetics; the values of $K_{M\,\mathrm{app}}$ and of k_{cat} at pH 2 and 4 are given in Table I. The $K_{M\,\mathrm{app}}$ values are essentially the same as those for the peptide, k_{cat} at pH 4 for the cleavage of the ester linkage being nearly three times that for scission of the peptide bond.

In an earlier report on the esterase activity of pepsin, Lokshina *et al.* (1964) indicated that, in the presence of 0.027 mM pepsin, acetyl-Phe-Pla (2.7 mM) is cleaved to 30% (as measured by the disappearance of material reacting with NH_2OH to form a hydroxamate) in 48 hr at 37° and pH 2 (10–15% ethanol was present); no hydrolysis was observed at pH 4. When expressed as moles of substrate cleaved per minute per mole of enzyme, the rate measured by Lokshina *et al.* at pH 2 is approximately 0.01 min^{-1}; this may be compared to the hydrolysis of Z-His-Phe(NO_2)-Pla-OMe (0.25 mM) by 0.00014 mM pepsin at a rate of 20 min^{-1} at 37° and pH 4. It is clear that the latter compound is a more sensitive substrate for pepsin than was the preparation of acetyl-Phe-Pla used by Lokshina *et al.* (1964). Furthermore, it should be noted that the data of Baker (1951) on the hydrolysis of acetyl-Phe-Phe (0.5 mM, present in a mixture of the four possible diastereoisomers) by pepsin (*ca.* 0.0085 mM) at pH 2 and 37° indicate a rate of approximately 0.2 min^{-1}, a value considerably higher than that found for the corresponding ester by Lokshina *et al.* (1964).

Competitive Inhibition by Diastereoisomers of Peptide Substrates. The stereochemical specificity of pepsin with respect to both amino acid residues involved in the sensitive bond is evident from the resistance of Z-His-Phe-D-Phe-OEt and of Z-His-D-Phe-Phe-OEt to enzymic cleavage. Under conditions (0.5 mM peptide, 0.1 mg of pepsin/ml, pH 4.0, 37°) that gave rapid and complete hydrolysis of the L-L-L isomer at the bond between the two phenylalanyl residues (100% cleavage in 0.5 hr), no measurable liberation of Phe-OEt (ninhydrin reaction) in 18 hr could be demonstrated with either of the other two diastereoisomers.

The spectrophotometric method for following pepsin kinetics, with Z-His-Phe(NO_2)-Phe-OMe as the substrate, provides a convenient procedure for the study of the inhibitory effect of test compounds. As will be seen from Figure 2, Z-His-D-Phe-Phe-OEt behaves as competitive inhibitor of pepsin in this assay, and a value of K_I = 0.175 mM (pH 4.0, 37°) has been determined. Similar linear plots have been obtained with Z-His-Phe-D-Phe-OEt (K_I = 0.275 mM) as an inhibitor of the hydrolysis of the peptide substrate. Furthermore, with the depsipeptide Z-His-Phe(NO_2)-Pla-OMe as the substrate, the K_I values for the above L-D-L and L-L-D peptides were found to be 0.20 and 0.275 mM, respectively. The compounds Z-His-Phe(NO_2)-D-Phe-OMe and Z-His-Phe(NO_2)-D-Pla-OMe were resistant to peptic cleavage (0.125 mM peptide, 0.01 mg of pepsin/ml, pH 4.0, 37°, 18 hr) as judged by the spectrophotometric assay.

Enzymic Test for Racemization. The resistance of the L-L-D and L-D-L isomers of Z-His-Phe-Phe-OEt to the action of pepsin permitted closer examination of an apparent racemization previously encountered in the treatment of N^α,N^{Im}-Z_2-His-Phe-Phe-OEt with 0.1 M sodium ethoxide in ethanol (Inouye *et al.*, 1966). Because the resulting product was cleaved by pepsin to only about 50% of that expected for the hydrolysis of a single peptide bond, it was suggested that this product represented a mixture of diastereoisomeric forms of the desired peptide Z-His-Phe-Phe-OEt, and that a base-catalyzed racemization had occurred during the removal of the benzyloxycarbonyl group from the imidazolyl side chain of the histidine residue.

To establish the nature of the amino acid residue (or residues) racemized upon treatment of Z_2-His-Phe-Phe-OEt with sodium ethoxide, the product of the reaction was subjected to the prolonged action of pepsin (see Experimental Section), and the material insoluble in aqueous bicarbonate was isolated. It proved to be identical with an authentic sample of Z-His-Phe-D-Phe-OEt, and different from an authentic sample of Z-His-D-Phe-Phe-OEt. The fact that the L-L-D compound was isolated from the peptic hydrolysate in good yield (74%) indicates that the main site of racemization in the treatment of Z_2-His-Phe-Phe-OEt with sodium ethoxide is the L-phenylalanyl residue bearing the OEt group. The fact that Z-His-Phe was isolated in 80% yield from the bicarbonate-soluble fraction, after peptic hydrolysis, supports the conclusion that about 50% of the diastereoisomeric mixture was the L-L-L compound.

That the racemizing action of sodium ethoxide on the sensitive phenylalanyl residue does not depend on the presence of the benzyloxycarbonyl group at the imidazolyl group of Z_2-His-Phe-Phe-OEt is shown by the fact that Z-His-Phe-Phe-OEt is rapidly racemized by sodium ethoxide. To estimate the rate at which the racemization occurs, Z-His-Phe-Phe-OEt was subjected to the action of 0.1 M sodium ethoxide in ethanol, and samples withdrawn at various times were incubated with pepsin at pH 4 for 20 hr. The proportion of pepsin-resistant material increased rapidly, and within 1 hr the racemization to form the L-L-D compound was nearly complete (92%). The occurrence of a much slower racemization of the L-L-L isomer to the L-D-L form is suggested by the finding that after 2 hr, the proportion of pepsin-resistant product had increased about 60% of the starting material.

Several compounds structurally related to Z-His-Phe-Phe-OEt were examined for their susceptibility to the racemizing action of sodium ethoxide. The replacement of the ethoxy group by the *t*-butoxy group caused a significant decrease in the rate of racemization (49% in 1 hr), and the corresponding *N*-acylated tripeptide (Z-His-Phe-Phe) was much more resistant to racemization (10% in 1 hr) than either of the esters. The racemization of Z-His-Phe-Tyr-OEt was found to be negligible under the conditions of this study, a result consistent with the successful preparation of this compound from Z_2-His-Phe-Tyr-OEt by treatment with sodium ethoxide (Inouye *et al.*, 1966). These findings are consistent with a mechanism involving direct proton abstraction (Neuberger, 1948), favored in the racemization of amino acid residues that have an electron-withdrawing substituent at the β carbon (Liberek, 1963; Photaki, 1963; Bohak and Katchalski, 1963).

Because of the considerable attention given to the problem of racemization in the synthesis of peptides, it was of interest to determine whether Z-His-Phe-Phe-OEt is racemized in ethanol by triethylamine (up to tenfold molar proportion), the base usually employed in coupling reactions. As judged by the susceptibility of the reisolated product to the action of pepsin, no evidence of such racemization was found. Anderson *et al.* (1966) have shown that bases such as *N*-methylmorpholine are less likely than triethylamine to cause racemization in coupling reactions. Although methods are available for reducing the extent of base-catalyzed racemization in peptide synthesis through proper choice of coupling methods, bases, and solvents, the type of racemization encountered in the present study reemphasizes the need for careful check of the stereochemical purity of protected intermediates in the synthesis of long-chain peptides.

Discussion

The results presented above confirm and extend the conclusion that the action of pepsin on synthetic substrates is favored by the presence of the side-chain groups of certain amino acids (L-phenylalanine, L-tyrosine, L-tryptophan, and L-leucine) on both sides of the sensitive bond. This preference is also clearly evident in the condensation reactions catalyzed by pepsin (Determann *et al.*, 1965); it was also found that carboxyl-terminal L-isoleucine or L-valine did not participate effectively in such condensation reactions. Since, in the action of pepsin, both the AX and YB components of a hydrolytic substrate AX-YB appear to be specifically bound near the catalytic site, the relative magnitude of the binding constants for these components may be expected to influence the sequence of the release of products, and the kinetic parameters in the cleavage of closely related substrates.

Also, if the binding constants are differentially affected by changes in pH, this may be reflected in the pH dependence of the kinetic parameters for different substrates. Factors of this kind may be involved in the complex kinetic behavior of pepsin substrates such as acetyl-L-phenylalanyl-L-tyrosine (Baker, 1954; Jackson *et al.*, 1966). The binding to pepsin of split products (*e.g.*, Z-His-Phe(NO_2) and Phe-OEt) and of substrate analogs is currently under study by means of the gel filtration method (Fairclough and Fruton, 1966), and a kinetic analysis of the type of product inhibition operative in the cleavage of the new substrates is in progress, with the aid of the spectrophotometric method described above.

The most striking feature of the data summarized in Table I is the lack of large variation in the values of K_M among substrates of widely different susceptibility. This result and the similarity of the K_M values to the K_I values for diastereoisomeric inhibitors suggest that $K_{M\,\mathrm{app}}$ for these substrates approximates K_S, the dissociation constant of the initial enzyme–substrate complex, and that the rate-limiting process in the hydrolysis is the first step in the conversion of the enzyme–substrate complex to products. The interpretation of the large differences in the values of k_{cat} will require further kinetic studies. As indicated above, the possible contribution of product inhibition to k_{cat} merits attention, especially in view of the report (Kozlov *et al.*, 1966) that the free-energy change for the pepsin-catalyzed hydrolysis of Ac-Phe-Tyr-OEt at pH 4 is near zero.

The unequivocal demonstration that pepsin is an esterase whose action is favored by aromatic side chains on both sides of the sensitive bond provides an additional property that must be accommodated by theories of the mechanism of pepsin catalysis. A further consequence of this finding is suggested by the results of Vallee and his associates (Vallee, 1964) that, upon chemical modification of its tyrosine residues, carboxypeptidase A loses peptidase activity and acquires enhanced esterase activity; this enzyme exhibits preference for the presence, in its substrates, of an aromatic side chain on the imino or alkoxy side of the sensitive bond. The inhibitory effect of the substitution of the tyrosine side chains of pepsin has long been known (Herriott, 1937). In view of the current interest in the development of specific chemical reagents for pepsin (Erlanger *et al.*, 1965; Delpierre and Fruton, 1965, 1966; Rajagopalan *et al.*, 1966; Hamilton *et al.*, 1967), it will be desirable to determine the effect of chemical modification of pepsin not only with proteins and synthetic peptides as substrates, but also with suitable synthetic esters. In this connection, it may be noted that Dr. T. R. Hollands of this laboratory has found that the acetylation of pepsin by means of acetylimidazole at pH 5.8 (Lokshina and Orekhovich, 1966; Perlmann, 1966) yields an enzyme preparation with enhanced peptidase activity toward Z-His-Phe(NO_2)-Phe-OMe and enhanced esterase activity toward Z-His-Phe(NO_2)-Pla-OMe. On the other hand, complete inhibition of the hemoglobin-cleaving ability of pepsin by L-1-diazo-4-phenyl-3-tosylamidobutanone (Delpierre and Fruton, 1966) also inhibits completely peptidase activity and esterase activity toward the above substrates.

It was of interest to find that replacement of the terminal $COOCH_3$ group of Z-His-Phe-Phe-OMe by a CH_2OH group renders the resulting derivative of L-phenylalaninol resistant to peptic cleavage. The explanation of this resistance, despite the presence of hydrophobic side chains of L components on both sides of a CONH bond, must await further study. Such derivatives of L-phenylalaninol may be of value in the study of the competitive inhibition of pepsin action and in the determination of the binding constants of substrate analogs.

Some years ago it was suggested (Bergmann and Fruton, 1942; Fruton and Bergmann, 1942) that the relative specificity in the action of a proteolytic enzyme may be denoted as the ratio of "proteolytic coefficients" for pairs of substrates that differ only in a single feature of their structure. The proteolytic coefficient, as originally defined, is approximately equal to $k_{\mathrm{cat}}/2.3\ K_{M\,\mathrm{app}}$ (Neurath and Schwert, 1950). The use of k_{cat}/K_M ratios for the description of the relative specificity of proteolytic enzymes has recently been discussed by Knowles (1965) and by Bender and Kézdy (1965). The values of k_{cat}/K_M for the pepsin substrates listed in Table I suggest that the rate of cleavage of the X-Y bond depends both on the nature of X and of Y, and that the effect of varying X or Y is not independent of the nature of the other partner in the bond. Furthermore, a relatively small change in the nature of the A group (Z-His-Gly in place of Z-Gly-His) can produce a large effect on the cleavage of the X-Y bond; in cases such as this one, the possibility of "wrong fit" may be considered. It would seem that the topography of the catalytic region of pepsin is such as to respond to small changes in substrate structure through significant changes in catalytic activity. Although a satisfactory comparison cannot be made of the limited data on the peptic hydrolysis of synthetic substrates with the extensive results obtained on the peptic cleavage of proteins and natural polypeptides, it would appear that the results reported in the present communication are in agreement with the conclusions of Tang (1963) on the preferential sites of pepsin action on complex substrates.

In Table II are collected some quotients of k_{cat}/K_M values for pairs of substrates that differ in an amino acid residues in either the X or Y position of the type substrate AX-YB. It may be seen that when X = Phe or Tyr, the change of Y from Phe to Tyr produces relatively little change in susceptibility, whereas when Y = Phe or Tyr, the change of X from Phe to Tyr leads a marked (*ca.* 20-fold) decrease in the rate of cleavage of the X-Y bond. It was of interest to compare these calculations with those made from the recent data of Jackson *et al.* (1966) on the peptic cleavage (pH 2, 37°) of the *N*-acetyl derivatives of Phe-Phe, Phe-Tyr, Tyr-Phe, and Tyr-Tyr (A = Ac; B = OH). The qualitative similarity of the two sets of data for the effect of replacement of a Phe residue by a Tyr residue

TABLE II: Quotients of $k_{\mathrm{cat}}/K_{M\,\mathrm{app}}$ Values for Pairs of Pepsin Substrates (AX-YB).

Variable X or Y	A	B	Invariant X		Invariant Y	
			Phe	Tyr	Phe	Tyr
Phe/Tyr	Z-His	OAlk	2.5^a	2.1^b	21^c	18^a
Phe/Tyr	Ac	OH	2.3^d	1.0^d	36^d	16^d
Phe/Trp	Z-His	OAlk	0.8^a		8^c	
Phe/Leu	Z-His	OAlk	91^c		13^c	

[a] Quotient of values for ethyl esters. [b] Quotient of value for methyl ester to value for ethyl ester multiplied by 4.3 (quotient of value for Z-His-Phe-Phe-OEt to value for Z-His-Phe-Phe-OMe). [c] Quotient of values for methyl esters. [d] Calculated from the data of Jackson *et al.* (1966).

in the X or Y position is noteworthy. The data in Table II indicate that the change of X from Phe to Trp also causes a decrease in the susceptibility of the X-Y bond, whereas the change in Y from Phe to Trp leads to a small increase. The result for the replacement of Phe by Leu in the Y position, however, does not follow the general pattern seen with the Phe-Tyr and Phe-Trp pairs.

In its preference for hydrophobic side chains on both sides of the sensitive bond, pepsin exhibits a property that it shares in part with other proteolytic enzymes. Extensive studies on chymotrypsin have provided $k_{\mathrm{cat}}/K_{M\,\mathrm{app}}$ data for acetyl-L-amino acid esters and amides; a preference for the acyl component of the sensitive bond in the decreasing order Trp > Tyr > Phe > Met > Leu has been observed (Knowles, 1965). Unfortunately, insufficient data are available on the effect of changes in the nature of the L-amino acid that contributes the imino component of the sensitive bond in synthetic substrates for chymotrypsin. With papain, however, which also appear to act by an acyl-enzyme mechanism (Bender and Brubacher, 1966), there is clear evidence of specific interaction with a hydrophobic side chains in the imino component of the sensitive bond (Bergmann *et al.*, 1935; Mycek and Fruton, 1957). For carboxypeptidase A, the preferred substrates have carboxyl-terminal amino acids with hydrophobic side chains (Stahmann *et al.*, 1946; Smith, 1948), and the nature of the amino acid contributing the carbonyl group to the sensitive bond is believed to have relatively minor importance (Neurath, 1960); it should be noted, however, that the best of the known synthetic substrates of carboxypeptidase a have an aromatic acyl group (hippuryl or benzyloxycarbonylglycyl). The contribution of suitably located nonamino acid hydrophobic groups of this type to the susceptibility of substrates for a proteolytic enzyme has been made evident recently by Gerwin *et al.* (1966) for streptococcal proteinase.

References

Anderson, G. W., Zimmermann, E., and Callahan, F. M. (1966), *J. Am. Chem. Soc. 88*, 1338.

Baker, L. E. (1951), *J. Biol. Chem. 193*, 809.

Baker, L. E. (1954), *J. Biol. Chem. 211*, 701.

Bender, M. L., and Brubacher, L. J. (1966), *J. Am. Chem. Soc. 88*, 5880.

Bender, M. L., and Kézdy, F. J. (1965), *Ann. Rev. Biochem. 34*, 49.

Bergmann, M., and Fruton, J. S. (1942), *J. Biol. Chem. 145*, 247.

Bergmann, M., Zervas, L., and Fruton, J. S. (1935), *J. Biol. Chem. 111*, 225.

Bohak, Z., and Katchalski, E. (1963), *Biochemistry 2*, 228.

Clement, G. E., and Snyder, S. L. (1966), *J. Am. Chem. Soc. 88*, 5338.

Cornish-Bowden, A. J., and Knowles, J. R. (1965), *Biochem. J. 95*, 71P.

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

Delpierre, G. R., and Fruton, J. S. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1817.

Determann, H., Heuer, J., and Jaworek, D. (1965), *Ann. Chem. 690*, 189.

Dixon, M. (1953), *Biochem. J. 55*, 170.

Erlanger, B. F., Vratsanos, S. M., Wassermann, N., and Cooper, A. G. (1965), *J. Biol. Chem. 240*, PC3447.

Fairclough, G. F., and Fruton, J. S. (1966), *Biochemistry 5*, 673.

Folin, O., and Ciocalteu, V. (1927), *J. Biol. Chem. 73*, 627.

Fruton, J. S., and Bergmann, M. (1942), *J. Biol. Chem. 145*, 253.

Gerwin, B. I., Stein, W. H., and Moore, S. (1966), *J. Biol. Chem. 241*, 3331.

Hamilton, G. A., Spona, J., and Crowell, L. D. (1967), *Biochem. Biophys. Res. Commun. 26*, 193.

Herriott, R. M. (1937), *J. Gen. Physiol. 20*, 335.

Inouye, K., and Fruton, J. S. (1967), *J. Am. Chem. Soc. 89*, 187.

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.

Kliegler, E., Schröder, E., and Gibian, H. (1961), *Ann. Chem. 640*, 157.

Knowles, J. R. (1965), *J. Theoret. Biol. 9*, 213.

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

Lenard, J., Johnson, S. L., Hyman, R. W., and Hess, G. P. (1965), *Anal. Biochem. 11*, 30.

Liberek, B. (1963), *Tetrahedron Letters*, 925.

Lokshina, L. A., and Orekhovich, V. N. (1966), *Biokhymia 31*, 143.

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

Mycek, M. J., and Fruton, J. S. (1957), *J. Biol. Chem. 226*, 165.

Neuberger, A. (1948), *Advan. Protein Chem. 4*, 297.
Neurath, H. (1960), *Enzymes 4*, 11.
Neurath, H., and Schwert, G. W. (1950), *Chem. Rev. 46*, 69.
Perlmann, G. E. (1966), *J. Biol. Chem. 241*, 153.
Photaki, I. (1963), *J. Am. Chem. Soc. 85*, 1123.
Rajagopalan, T. G., Stein, W. H., and Moore, S. (1966), *J. Biol. Chem. 241*, 4295.
Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 16*, 570.
Silver, M. S., Denburg, J. L., and Steffens, J. J. (1965), *J. Am. Chem. Soc. 87*, 886.
Smith, E. L. (1948), *J. Biol. Chem. 175*, 39.
Stahmann, M. A., Fruton, J. S., and Bergmann, M. (1946), *J. Biol. Chem. 164*, 753.
Suwa, A. (1911), *Z. Physiol. Chem. 72*, 113.
Tang, J. (1963), *Nature 199*, 1094.
Toyoda, T., Suyama, T., and Kanao, S. (1963), *Yakugaku Zasshi 83*, 856.
Vallee, B. L. (1964), *Federation Proc. 23*, 8.
Winitz, M., Bloch-Frankenthal, L., Izumiya, N., Birnbaum, S. M., Baker, C. G., and Greenstein, J. P. (1956), *J. Am. Chem. Soc. 78*, 2423.

A Genetic Defect in Retention of Potassium by *Streptococcus faecalis*[*]

F. M. Harold, Ruth L. Harold, J. R. Baarda, and A. Abrams

ABSTRACT: Cells of *Streptococcus faecalis* 9790 selectively concentrate K^+ and Rb^+ even when growing in a medium in which Na^+ is the predominant cation. Mutants were isolated which require high concentrations of K^+ or Rb^+ for growth at pH 6. The requirement is seen even if the growth medium contains Tris or triethanolamine in place of Na^+. The mutant phenotype results from a defect in the concentrative uptake of K^+ and Rb^+ and is defined by three experimental procedures. (i) Measurements of ^{86}Rb uptake reveal no clear difference between wild type and mutant with respect to the kinetic parameters of Rb^+ entry into the cells. (ii) Wild-type cells retain K^+ and Rb^+ while the mutants rapidly lose these cations to the medium by an energy-dependent exchange with external Na^+. (iii) Wild-type cells preloaded with Na^+ and H^+ can carry out energy-dependent net uptake of K^+ with concurrent extrusion of Na^+ and H^+; the mutants are severely deficient in this process. It is proposed that the uptake of cations by the wild type involves an energy-dependent transport system which mediates cation exchanges across the membrane. In the inward direction K^+ and Rb^+ are strongly selected over Na^+, while in the outward direction the relative affinities are reversed. In the mutants, selective transport of K^+ and Rb^+ *into* the cells is normal; the genetic defect involves primarily the relative affinities for cation transport *out* of the cells.

Current concepts of membrane transport in microorganisms derive to a large extent from studies on mutants defective in the uptake of specific metabolites against a concentration gradient. According to the permease model (Kepes and Cohen, 1962; Koch, 1964) the initial step in transport involves a stereospecific protein, the permease; this catalyzes association of the substrate with a carrier molecule, which then crosses the membrane. In the majority of transport mutants that have been analyzed kinetically the genetic lesion affects primarily the *entry* of the metabolite into the cells, and many of these mutants are thought to have a defective permease. Examples include mutants deficient in the uptake of monosaccharides (Rickenberg *et al.*, 1956; Wiesmeyer and Cohn, 1960; Hagihara *et al.*, 1963; Egan and Morse, 1966), amino acids (Kessel and Lubin, 1962, 1963; Ames, 1964), and inorganic anions (Pardee *et al.*, 1966; Harold *et al.*, 1965; Harold and Baarda, 1966).

A transport mutant of very different phenotype was isolated by Lubin and Ennis (1963). A mutant of *Escherichia coli* was found to have lost the ability to *retain* K^+ when suspended in potassium-free medium, while exchange of cellular K^+ with external K^+ was accelerated (Lubin and Ennis, 1963; Lubochinsky *et al.*, 1964, 1966). Very recently, Günther and Dorn (1966) demonstrated that the primary defect is an increased efflux of K^+ from the cells, and proposed that the mutation has altered the specificity of a carrier involved in the transport of cations out of the cells.

---

[*] From the Division of Research, National Jewish Hospital, and the Department of Microbiology and Biochemistry, University of Colorado School of Medicine, Denver, Colorado. *Received January 16, 1967.* Supported by Grants AI-03658 and GM-5810 from the U. S. Public Health Service.