Reactivity of Cyclic Peptides. III. Reaction of Isomeric Histidine, Tyrosine Peptides with *p*-Nitrophenyl Acetate*

KENNETH D. KOPPLE,† REBECCA R. JARABAK, AND PARSHOTAM LAL BHATIA

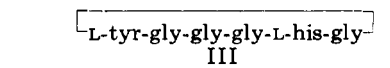
From the Department of Chemistry, University of Chicago

Received February 19, 1963

cyclo-Glycyl-L-tyrosyldiglycyl-L-histidylglycyl (Ia) and its D-tyrosyl isomer (Ib) were synthesized. They were compared with the diastereomeric *cyclo*-histidyltyrosyls in behavior toward *p*-nitrophenyl acetate. Imidazole-dependent acetylation of tyrosine hydroxyl occurs in the cyclic peptides bearing the side chains in a *cis* relationship. Hydrolysis of the resulting phenyl esters is not appreciably facilitated by proximity of the side-chain imidazole.

In a previous paper (Kopple and Nitecki, 1962) we reported the synthesis and some properties of *cyclo*-L-tyrosyltriglycyl-L-histidylglycyl (III) and *cyclo*-L-tyrosyl-L-histidyl (IIa); upon reaction with *p*-nitrophenyl acetate, these peptides were acetylated at the tyrosine hydroxyl. This paper deals with the synthesis of isomeric peptides and with studies relating to their *O*-acetylation by the same reactive ester.

cyclo-Glycyl-L-tyrosyldigylcyl-L-histidylglycyl (Ia) was obtained by a synthesis similar to that used for the earlier preparation of its position isomer, III. The



scheme is shown in Figure 1. The same sequence was used for preparing a diastereomeric mixture, incorporating racemic tyrosine and L-histidine, from which the epimeric cyclic peptide Ib, *cyclo*-glycyl-D-tyrosyldiglycyl-L-histidylglycyl, was isolated.

Synthesis of these peptides was more convenient

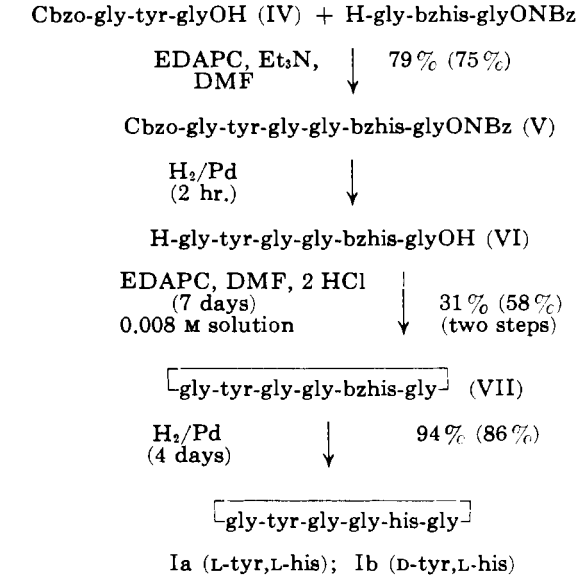


FIG. 1.—Synthesis of the "1,4" cyclic hexapeptides. Yields given in parentheses are for the series incorporating DL-tyrosine. L-Histidine was used throughout. Abbreviations: bzhis, N^{im}-benzylhistidine; Cbzo, carbobenzoxy; NBz, *p*-nitrobenzyl; DMF, dimethylformamide; EDAPC, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide.

* This work was supported by a National Science Foundation grant (G 14324), and by a grant from the U. S. Public Health Service, Division of General Medical Sciences (RG 7573). (Paper II, Kopple, Nitecki, 1962).

† Present address, General Electric Company Research Laboratory, Box 1088, Schenectady, New York.

than that of isomer III. Debenzylation of imidazole-blocked histidine, a side reaction in removing the terminal blocking groups from the linear peptide intermediate (V → VI, Figure 1), was sufficiently unimportant in the present case that no purification of unblocked hexapeptide (VI) prior to cyclization was necessary, although in the corresponding step of the earlier synthesis it was extensive. This good fortune was the result of a more facile hydrogenolysis at N-terminal carbobenzoxyglycyl than occurred at N-terminal carbobenzoxytyrosyl (by a factor of about 3). More gratifying, however, was the fact that in the final step of the present synthesis (Figure 1) the imidazole-protecting benzyl group could be removed by catalytic hydrogenolysis. The corresponding final step in the sequence leading to position isomer III could be accomplished, in poor yield, only by means of sodium in liquid ammonia. It seems reasonable to ascribe this difference to steric factors that prevent proper orientation of the benzyl derivative of the 1,3-cyclic peptide (III) on the catalytic surface, although this is the only clear-cut difference so far observed between the 1,3 and 1,4 isomeric peptides.

The method adopted for the preparation of the *trans* hexapeptide (Ib) was the result of the availability of carbobenzoxyglycyl-DL-tyrosylglycine ester and by hydrolysis, the corresponding acid, DL-IV. Coupling of carbobenzoxyglycyl-L-tyrosine with glycine ester was carried out using dicyclohexylcarbodiimide. This reaction, in ethyl acetate–methanol at 25°, gave 38% of crystalline optically inactive tripeptide ester, plus a noncrystallizable mixture of peptides that was hydrolyzed to partially active crystalline tripeptide acid. When coupling occurred in tetrahydrofuran at 0°, 77% of crystalline L-isomer and 6% of DL-isomer were obtained. The DL-isomer regularly crystallized first from ethyl acetate and could be removed. Synthesis of carbobenzoxyglycyltyrosylglycine ester is therefore analogous to that of carbobenzoxyglycylphenylalanylglycine ester in possessing utility as a test for racemization in peptide coupling (Anderson and Callahan, 1958).

When carbobenzoxyglycyl-DL-tyrosylglycine was used for synthesis of the hexapeptide, V, it was not possible, even on paper chromatograms, to separate the resulting diastereomeric linear hexapeptides, nor could separation be achieved at subsequent intermediate stages (VI or VII). Only when the final product (Ia + Ib) was obtained did partition chromatography on cellulose powder result in isolation of separate, pure isomers of cyclic hexapeptides Ia and Ib.

Racemic *trans*-3-(4-hydroxybenzyl)-6-(4-imidazolylmethyl)-piperazine-2,5-dione (IIb) was also obtained through chromatographic resolution of a mixture of diastereomers. Investigation of the low yield (31%) of *cyclo*-L-histidyl-L-tyrosyl obtained via path A,

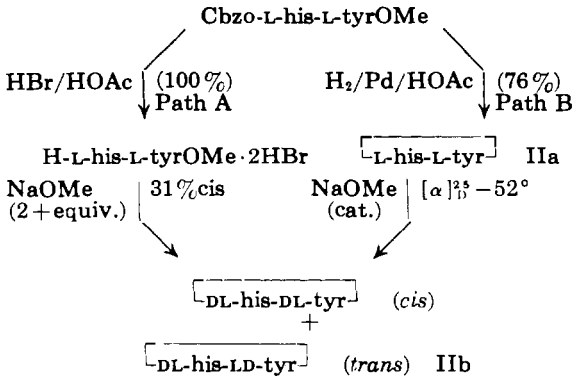


FIG. 2.—Preparations of *cyclo*-histidyltyrosyl. Path A, which gave rise to a mixture of optically inactive diastereomers, is that described by Kopple and Nitecki (1962).

Figure 2 (Kopple and Nitecki, 1962) revealed that catalytic amounts of sodium methoxide, probably present in the second step of that path, caused epimerization of the diketopiperazine. When carbobenzoxy-L-histidyl-L-tyrosine methyl ester in acetic acid solution was unblocked by hydrogenolysis over palladium (path B, Figure 2), a 76% yield of cyclic peptide was isolated directly. This product was homogeneous and chromatographically identical to that already described as *cyclo*-L-histidyl-L-tyrosyl, except that in acetic acid it had $[\alpha]_D^{25}$ −52° while the products of preparations by path A exhibited specific rotations between −9° and 0°. Treatment of the path B preparation with a catalytic quantity of sodium methoxide (under reflux in ethanol, 18 hours) converted it to a mixture of two components: starting material and a second ninhydrin-inactive material of higher R_F. Usable amounts of this second component were isolated chromatographically from path A residues after crystallization of the less soluble *cis* component. The isolated material analyzed for *cyclo*-histidyltyrosyl and therefore was assumed to be the *trans* substituted diketopiperazine, IIb.[1]

With the synthesis of the above peptides, there were at hand five cyclic peptides; described by side-chain positions, these were the 1,3-*cis* (III), 1,4-*cis* (Ia), and 1,4-*trans* (Ib) cyclohexapeptides, plus the *cis* (IIa) and *trans* (IIb) cyclic dipeptides. Chemical differences could be expected between the *cis* and *trans* dipeptides to the extent that interaction of side-chain functional groups occurred in the *cis* form; the necessary rigidity of the diketopiperazine ring insures their independence in the *trans* isomer. Similar differences could be expected to appear in a comparison of the stereoisomeric 1,4-cyclohexapeptides, but these might be less marked because of the greater distance across the larger ring and its probable greater flexibility. Differences between the 1,3 and 1,4 *cis* hexapeptides are not readily predictable; in any event, examination of reactivity toward protons and *p*-nitrophenyl acetate revealed no significant differences between the members of this last-mentioned pair. (See Tables I and II.)

Table I gives the pK_A of the cyclic peptides and some of their derivatives. It is seen that there is no appreciable difference in pK between *cis* and *trans* isomers in either the dipeptide or hexapeptide series, and thus strong histidyl-tyrosyl hydrogen bonding is absent in even the favorable *cis* cases. It can also be noted that the imidazole pK_A in each of the three *cis* *O*-acetyl derivatives is not different from that of the corresponding unacylated compound, an argument against transannular interaction of imidazole and ester groups. It therefore appears that the two side-chain functional groups in these peptides are independent of each other in the five peptides examined. This independence is indicated also by the similarity in rate of phenol liberation from *p*-nitrophenyl acetate by the *cis*-substituted cycles and their *O*-acetyl derivatives (Table II).

Despite the lack of evidence for side-chain interaction just cited, Table II reveals that *O*-acetylation of tyrosine in these peptides by *p*-nitrophenyl acetate does depend on the proximity of the imidazole function, being faster for the *cis*-isomers than for the *trans*, and faster for the *cis* dipeptide than for the *cis* hexapeptides. Sufficient data has been obtained for the acetylation reactions of Ia and IIa to establish their certain dependence on the presence, in the peptide molecule, of free (unprotonated) imidazole. Although *O*-acetylation of the *trans* peptides, Ib and IIb, by nitrophenyl acetate does occur, it occurs at a lower rate that can be accounted for without invoking assistance by imidazole. Published data (Jencks and Gilchrist, 1962) give a rate constant of 65 liters mole^{-1} min^{-1} for the reaction of

TABLE I
pK_A OF CYCLIC PEPTIDES

Peptide	Histidine H_2O[a]	Histidine 90% CH_3CN[b]	Tyrosine H_2O[a]
cyclo-			
His-tyr (*cis*) (IIa)	6.4_2	6.1_0	9.6_4
His-tyr (*trans*) (IIb)	6.4_4	6.0_3	9.6_0
His-*O*-Ac-tyr (*cis*) (*O*-AcIIa)	6.4_2	—	—
Gly-L-tyr-gly$_2$-L-his-gly (Ia)	6.7_3	6.3_4	9.8_5
Gly-D-tyr-gly$_2$-L-his-gly (Ib)	6.6_0	6.2_3	9.7_9
L-Tyr-gly$_3$-L-his-gly (III)	6.6_5[c]	6.3_1	9.9_6[c]
Gly-*O*-Ac-L-tyr-gly$_2$-L-his-gly (*O*-Ac-Ia)	6.5_5	—	—
O-Ac-L-Tyr-gly$_3$-L-his-gly (*O*-Ac-III)	6.5_5[c]	—	—
Gly-L-tyr-gly$_2$-bz-L-his-gly (VII)	5.9_7	5.7_8	9.7_9
L-Tyr-gly$_3$-bz-L-his-gly	5.8_2	5.7_3	9.9_2

[a] 25°, 0.1 M KCl, NaOH titrant. [b] 25°, 0.07 M $(C_4H_9)_4N^+I^-$, $(C_2H_5)_4N^+OH^-$ titrant. [c] Kopple and Nitecki, 1962.

TABLE II
REACTION OF CYCLIC PEPTIDES WITH *p*-NITROPHENYL ACETATE[a]

Peptide	Phenol Liberation k	Phenol Liberation pK_A	*O*-Acetylation k	*O*-Acetylation pK_A
IIa	3.9_5	6.2_4	3.7_5	6.3_8
IIa (D_2O)	3.0	6.8		
IIb	5.3_1	6.2_0	0.63 at *p*H 7.5	
IIb (D_2O)	4.7	6.8		
O-Ac IIa	4.3_5	6.2_4		
Ia	4.5	6.4_8	1.3_8	6.4_5
Ib	2.8	6.2_8	0.43 at *p*H 7.5	
III[c]	6.0	6.6_5	1.0	(6.5)
O-Ac Ia	5.4[b]	6.3_1		

[a] 0.09 M KCl, 0.01 M phosphate buffer, 9% (v/v) ethanol, 25°. Rate constants in liters mole^{-1} min^{-1}. See under Experimental for discussion of measurements. [b] From second-order analysis, neglecting blank hydrolysis of ester; therefore 5–10% high. [c] From Kopple and Nitecki (1962).

[1] Infrared examination of diketopiperazine itself, before and after treatment with sodium carbonate in hot deuterium oxide, indicated that its α-protons are exchangeable.

TABLE III
HYDROLYSIS OF O-ACETYL CYCLIC HISTIDINE, TYROSINE PEPTIDES

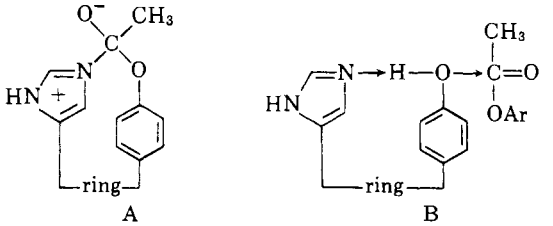
Peptide	pH	$10^4\ k_{obs}$[a]	$10^4\ k_{calc}$[b]
O-Ac Ia	6.5	0.23	
	7.4	0.75	
O-Ac IIa	6.41	2.3	2.4
	6.89	3.2	3.2
	7.18	3.8	3.8
	7.28	3.8	4.0
	7.43	4.4	4.5
	7.64	5.6	5.5

[a] In 0.09 M potassium chloride, 0.01 phosphate buffer, 25°, peptide concentration 10^{-3} M. Constants in min^{-1}. [b] Calculated on the basis of the rate law and constants given in the text.

phenolate ion (pK_A 10.0) with *p*-nitrophenyl acetate, corresponding to an observed rate constant of 0.2 liter $mole^{-1}$ min^{-1} at *p*H 7.5. The observed rate constants for the *trans* peptides (Table II) are not far from this value. That they are larger may result from the lower pK_A of the peptide tyrosine and/or reaction of un-ionized phenol.

The hydrolysis of the *O*-acetyl *cis* peptides, for which data are given in Table III, is a slow reaction. For the hydrolysis of *cyclo*-L-histidyl-*O*-acetyl-L-tyrosyl (IIa) the observations are consistent with a rate law of the form $k = k_w + k_{OH}[OH^-] + k_i\alpha$, where α is the fraction of peptide imidazole in the neutral form. The data do not permit precise evaluation of the three constants, but they are about 0.00013 min^{-1}, 520 liters $mole^{-1}$ min^{-1}, and 0.0002 min^{-1}. The value for k_i is in marked contrast to the corresponding term reported for the hydrolysis of phenyl 4-(4-imidazolyl)-butyrate: 2.6 min^{-1} (Bruice and Sturtevant, 1959). (It can readily be computed that for the hexapeptide Ia the hydrolysis rate is almost of the same order of magnitude as that to be expected if the side-chain imidazole and acetoxyphenyl groups were not in the same molecule. Bruice and Schmir (1957) report a specific rate for reaction of imidazole and phenyl acetate of 0.108 liter $mole^{-1}$ min^{-1} (30°, 28.5 [v/v] ethanol-water). Assuming that the peptide imidazole is about one-fourth as reactive as imidazole itself, as is the case for reaction with nitrophenyl acetate, at the concentration of peptide imidazole used to obtain the data of Table III one calculates a pseudo first-order rate constant of about 0.3×10^{-4} min^{-1}.)

Any mechanism of imidazole-dependent acetylation of tyrosine oxygen, upon reaction with *p*-nitrophenyl acetate, requires proximity of histidyl and tyrosyl side chains and should, as is found, be most effective in the *cis* diketopiperazine (IIa) and least in the *trans* hexapeptide (Ib). Intramolecular acetyl transfer to tyrosine from acetylimidazole formed in a prior step is the obvious possibility, and seems most likely for reaction of IIa, where acetylation of phenolic oxygen is effectively quantitative. Consistent with this mechanism is the fact that the phenol liberation rates for the *cis* and *trans* diketopiperazines are comparable, suggesting similarity of site attacking the ester, whether the acetyl group winds up as acetate or phenyl acetate. In addition, for reaction of IIa, where *O*-acetylation accounts initially for almost all phenol liberated, k_H/k_D for phenol liberation is 3.95/3.0 = 1.3, while for hydrolysis of *p*-nitrophenyl acetate by IIb (presumably by nucleophilic imidazole catalysis) k_H/k_D = 5.3/4.7 = 1.1. A general-base-catalyzed reaction in which the acetyl group is transferred directly from ester to phenol as a proton moves from hydroxyl to imidazole (B) should be associated with a solvent isotope effect greater than 2 (Bruice *et al.*, 1962; Jencks and Carriuolo, 1961), but a negligible isotope effect is to be expected if imidazole is involved as an attacking nucleophile.



A B

Facile transfer of the acetyl group from side-chain imidazole to side-chain hydroxyphenyl, as must occur in the acetylation of the *cis* diketopiperazine, presumably involves the intermediate (A), though (A) need not be present in detectable concentrations. If (A) exists, some acetylated imidazole is also present, but a sufficiently favorable equilibrium acetylimidazole $\rightleftharpoons$ phenyl acetate can account for the slow observed hydrolysis of the *cis* acetylated diketopiperazine. If it is assumed that the observed hydrolysis of the *O*-acetyl *cis* diketopiperazine proceeds via the N^{im}-acetyl peptide, and that the hydrolysis rate of the latter is that for acetylimidazole at *p*H 7.5 in the buffer used, ~0.015 min^{-1} (Jencks and Carriuolo, 1959), the further assumption of a 50:1 equilibrium ratio of *O*-acetyl to N^{im}-acetyl species leads to a predicted apparent first-order rate constant for hydrolysis of acetyl IIa of about 0.0003 min^{-1}. The observed value of k_i is about 0.0002.

In the reactions of the *cis* cyclic hexapeptides with *p*-nitrophenyl acetate, *O*-acetylation accounts for only a fraction of the ester consumed. If the initial attack on ester is by imidazole, the intramolecular reaction of phenolic hydroxyl and acetylimidazole must be somewhat slower than hydrolysis of the acetylimidazole derivative, a conclusion consistent with other evidence indicating lack of serious side-chain interaction in these peptides and with the slow hydrolysis observed for their acetyl derivatives. If the hydrolysis of N^{im}-acetyl Ia is described by a rate constant of 0.015 min^{-1} then the rate constant for transfer of acetyl to hydroxyphenyl is 1.4/(4.5 − 1.4) as large, or about 0.007 min^{-1}. Assuming as before that the *O*-acetyl derivative is 50-fold favored over the N^{im}-derivative at equilibrium, the predicted constant for hydrolysis of the *O*-acetylated peptide would be that for $O \rightarrow N^{im}$ transfer, about 0.00014, which is at least of the observed order of magnitude.

EXPERIMENTAL

Measurements.—The determinations of *pK* and measurements of reaction rate were carried out as described earlier (Kopple and Nitecki, 1962). Rate of hydrolysis of the *O*-acetyl peptides was determined by following the increase in absorption of a solution of the acetyl peptide at the 275 mμ tyrosine maximum, using a Cary Model 14 spectrophotometer with thermostatted cell compartment.

The second-order rate constants and kinetic *pK*'s given in Table II were determined from the best straight line drawn through points for individual runs on plots of $k_{obs}[H^+]$ vs. k_{obs}; rate constants were taken as the intercept on the k_{obs} axis and the apparent acidity constants were obtained from the slope. At least eight runs at *p*H between 6.0 and 7.7 were made for each plot. The values of k_{obs} in individual runs differed from those calculated using the values of

Table II by no more than 7%. In following phenol liberation, observed second-order rate constants were determined from a pseudo first-order analysis (zero order in peptide) of the first 15% of reaction, by correction for blank ester hydrolysis, and division by initial peptide concentration; peptide concentration was 1–2 $\times 10^{-3}$ M and ester 2–4 $\times 10^{-3}$ M. The observed rates for O-acetylation were derived from runs using 0.5–1.0 $\times 10^{-3}$ M peptide and 4–5 $\times 10^{-3}$ M ester, assuming constant ester concentration over the first 25–30% of peptide reaction.

For the determinations in deuterium oxide solvent, a stock solution of 0.01 M dipotassium deuterium phosphate and 0.09 M potassium chloride was made up and used to prepare stock solutions of peptides (2.04 $\times 10^{-3}$ M) and a solution of *p*-nitrophenol (0.325 $\times 10^{-3}$ M) for determination of an $\epsilon 400 m\mu$ vs. *p*D curve. Acidity of these solutions was adjusted by addition of 1.48 N deuterium chloride. The *p*D was measured at 25° using a Beckman Model G *p*H meter with No. 40498 (formerly No. 1190–80) glass electrode. The meter was standardized against *p*H 4.00 potassium acid phthalate buffer in water and the correction of +0.40 determined by Glasoe and Long (1960) was used to convert readings in deuterium oxide to *p*D. A pK_A in D_2O of 7.54 was so obtained for *p*-nitrophenol. Kinetic runs in deuterium oxide were made by addition of 0.030 ml of 0.0163 M *p*-nitrophenyl acetate in ethanol to 0.30 ml of peptide stock. The resulting protium concentration (from ethanol and peptide) was estimated at 2.1 atom %.

*N-Carbobenzoxyglycyl-*L*-tyrosine.*—Glycyl-L-tyrosine (Mann Research Laboratories) (19.0 g, 0.08 mole) and sodium bicarbonate (16.8 g, 0.20 mole) were dissolved in 320 ml of water. The solution was cooled in an ice bath and stirred vigorously while carbobenzoxychloride (13.7 g, 0.08 mole) was added dropwise over 30 minutes. Stirring at 0° was continued for 3 hours. The resulting gelatinous mixture was extracted with ether and then acidified to Congo red by addition of 2 N hydrochloric acid. Precipitated product was taken up in ethyl acetate and combined with ethyl acetate washings of the aqueous phase. The ethyl acetate solution was washed with water and dried over anhydrous magnesium sulfate, then evaporated under reduced pressure to an oil that crystallized on trituration with petroleum ether. Crystallization from ethyl acetate gave *N*-carbobenzoxyglycyl-L-tyrosine of mp 104°, reported 107° (Bergmann and Fruton, 1937), and $[\alpha]_D^{25}$ +39.0 (*c* 1, acetic acid). Repeated recrystallization did not raise its melting point.

*N-Carbobenzoxyglycyl-*L(DL)*-tyrosylglycine Ethyl Ester.*—Glycine ethyl ester (3.2 g, 0.031 mole) and *N*-carbobenzoxyglycyl-L-tyrosine (11.5 g, 0.031 mole) were combined in 25 ml of freshly distilled tetrahydrofuran. At 0° was added 6.4 g (0.031 mole) of dicyclohexylcarbodiimide. The reaction mixture was stirred at 0° for 6 hours and at room temperature overnight; then unreacted carbodiimide was destroyed by the addition of one ml of 50% acetic acid. After 30 minutes dicyclohexylurea was removed by filtration and washed with ethyl acetate. The tetrahydrofuran solution, combined with the ethyl acetate washings, was washed at 0° with water, 0.5 N hydrochloric acid, 1 M sodium bicarbonate, and water, then it was dried over magnesium sulfate and concentrated at reduced pressure to a small volume. This concentrated solution of product was seeded with *N*-carbobenzoxy-DL-tyrosylglycine ethyl ester and stored at 5° overnight. Racemic product was collected by filtration (830 mg, mp 120°); the mother liquors were returned to the refrigerator until optically active product crystallized. In three crops was collected 10.9 g (77% crude yield) of L-isomer, recrystallized from methylene chloride–petroleum ether or from small volumes of ethyl acetate, mp 94°.

Storage at 5° of an ethyl acetate solution of the 94° product for about a month resulted in precipitation of a mp 134° modification that was obtained in all subsequent work with this peptide. An analytical sample, mp 134°, was prepared by recrystallization from ethyl acetate. Optical rotation: 134° form, $[\alpha]_D^{25}$ 4.1 ± 0.2° (*c* 10, acetic acid); 94° form, $[\alpha]_D^{25}$ 4.4 ± 0.8° (*c* 1.25, acetic acid).

Anal.[2] Calcd. for $C_{23}H_{27}N_3O_7$: C, 60.38; H, 5.95; N, 9.19. Found: C, 60.59; H, 6.02; N, 9.15.

Carrying out the above coupling in a mixture of ethyl acetate and methanol at room temperature, using glycine ester hydrochloride and an equivalent of triethylamine in place of the ester free base, led to a 38% yield of optically inactive tripeptide, mp 118–120°. An analytical sample was obtained by recrystallization from ethyl acetate.

Anal. Calcd. for $C_{23}H_{27}N_3O_7$: C, 60.38; H, 5.95. Found: C, 60.50; H, 6.03.

Racemic and optically active products ran identically on paper chromatography.

N-Carbobenzoxyglycyltyrosylglycine (IV). L-*Form.*—To a solution of the 134° tripeptide ester (5.45 g, 0.0119 mole) in 30 ml of pyridine was added 17.9 ml of 1 N sodium hydroxide. The reaction mixture was stored 1 hour at room temperature and 2 hours at 5°, then brought to *p*H 7 by means of 1 N hydrochloric acid. After most of the solvent had been removed at reduced pressure, 50 ml of water was added and the solution was acidified to Congo red. Precipitated oil was taken up in ethyl acetate and the aqueous layer was extracted with ethyl acetate. The combined organic solutions were washed with cold water and dried over magnesium sulfate. Removal of solvent under reduced pressure left an oil that crystallized on trituration with petroleum ether, 4.4 g (86%). In other preparations the product crystallized upon seeding the concentrated ethyl acetate solution; material of analytical quality was so obtained, mp 180°.[3] Optical rotation $[\alpha]_D^{25}$ 5.6 ± 0.4° (*c* 5, acetic acid).

Anal. Calcd. for $C_{21}H_{23}N_3O_7$: C, 58.73; H, 5.40; N, 9.79. Found: C, 58.76; H, 5.48; N, 9.83.

Racemic Form.—The isomeric DL-tripeptide acid was obtained in 84% yield by similar hydrolysis, in pyridine-water, of the corresponding ester. This product was obtained from ethyl acetate with mp 172°.

Anal. Calcd. for $C_{21}H_{23}N_3O_7$: C, 58.73; H, 5.40. Found: C, 58.66; H, 5.53. Optically active and racemic products behaved identically on paper chromatograms.

*N-Carbobenzoxyglycyl-*L-(DL)*-tyrosyldiglycyl-*N^{im}*-benzyl-*L*-histidylglycine p-Nitrobenzyl Ester (V).* L,L-*Form.*—Glycyl-N^{im}-benzyl-L-histidylglycine *p*-nitrobenzyl ester dihydrobromide (Kopple and Nitecki, 1962) (7.1 g, 0.0109 mole) and *N*-carbobenzoxyglycyl-L-tyrosylglycine (3.7 g, 0.0086 mole) were dissolved in 65 ml of freshly distilled dimethylformamide. The solution was cooled and treated with 1.5 ml (0.0109 mole) of triethylamine and 1.7 ml of *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide. The reaction mixture was held in the dark at room temperature. Progress of the coupling was followed by paper chromatography. After 6 days the product was precipitated by addition of 200 ml of water and was triturated with two 100-ml

[2] We are indebted to Mr. William Saschek for the microanalyses reported in this paper.

[3] A previous report (Abderhalden *et al.*, 1938) gives 134° as mp for this peptide.

portions of water; then it was dissolved in boiling 95% ethanol. The gelatinous precipitate which appeared on cooling was collected by filtration and dried in vacuum. A second crop was obtained from the ethanolic mother liquors. The total yield was 6.2 g, 79%.

The hexapeptide product was ninhydrin negative and gave a single spot on chromatography in 2-butanol-water (4:1), R_F 0.85, and 2-butanol–acetic acid–water (4:1:1), R_F 0.81. An analytical sample was prepared by recrystallization from 95% ethanol and dried in vacuum at 100° for 48 hours. Optical rotation $[\alpha]_D^{30}$ −4.8 ± 0.6° (*c* 3.1, acetic acid).

Anal. Calcd. for $C_{45}H_{47}N_9O_{12}$: C, 59.68; H, 5.22; N, 13.92. Found: C, 59.53; H, 5.42; N, 13.83.

DL,L-*Form.*—When the above procedure was carried out using DL-tyrosine tripeptide, the mixture of diastereomeric hexapeptides was obtained in 75% yield. The mixture was not resolvable on paper chromatography in any of a variety of solvent systems.

Anal. Calcd. for $C_{45}H_{47}N_9O_{12}$: C, 59.68; H, 5.22; N, 13.92. Found: C, 59.27; H, 5.30; N, 14.09.

*cyclo-Glycyl-*L*-tyrosyldiglycyl-N^{im}-benzyl-*L*-histidylglycyl* (L,L-*V*).—Hydrogen was bubbled for 2 hours through a solution of the blocked linear L,L-hexapeptide, V, (3.50 g, 0.00386 mole) in 200 ml of glacial acetic acid; 1.75 g of 10% palladium on charcoal was used as catalyst. The catalyst was removed by centrifugation and washed with acetic acid. Filtration of combined acetic acid solutions followed by lyophilization gave amorphous unblocked product, which was dissolved in water and extracted several times with ethyl acetate. Two equivalents of hydrochloric acid (38.6 ml of 0.2 N) was added to the aqueous solution, which was lyophilized to give a hygroscopic powder used directly in the next step.

The hydrogenolysis product was separated into two components on paper chromatography using 2-butanol–acetic acid–water (4:1:1). The major component, R_F 0.23, presumably glycyl-L-tyrosyl-diglycyl-N^{im}-benzyl-L-histidylglycine (VI), was ninhydrin-positive and gave an orange spot with diazotized *p*-bromoaniline; the second peptide, present in relatively small amount, R_F 0.05, was ninhydrin-positive and gave a bright red spot with diazotized *p*-bromoaniline, indicating debenzylation of the histidine imidazole. No *N*-carbobenzoxy hexapeptide acid was detected.

The mixture was dissolved in 460 ml of freshly distilled dimethylformamide. Two equivalents (1.2 ml) of *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide was added and the clear solution was kept in the dark, at room temperature, for 7 days. Progress of the cyclization was followed on paper chromatograms. Removal of solvent at 1 mm pressure left an orange oil which solidified when treated with dry ether. The solid was washed with several fresh portions of ether and then dried in vacuum. The crude cyclized mixture was purified by chromatography on a 1-kg cellulose powder column using 1-butanol-water (6:1). Those fractions comprising the center of the eluted band of desired product deposited crystalline material on standing; this product was collected by filtration and dried *in vauco*, 436 mg. The filtrate was combined with those fractions at the edges of the band that were shown by paper chromatograms also to contain pure VII. Removal of solvent at 1 mm pressure gave an amorphous solid, 319 mg. The over-all yield of pure cyclic product for the two steps, unblocking and cyclization, was 31%. An additional 229 mg of material was isolated from later fractions off the column, but this contained traces of impurities. The first 31% was ninhydrin-negative and chromatographically homogeneous in several solvent systems, including 2-butanol-water (4:1), R_F 0.67, and 1-butanol–formic acid–water (Wiggins and Williams, 1952), R_F 0.42.

A small portion of the crystalline product was dried *in vacuo* 24 hours at 100° for use as an analytical sample. Optical rotation $[\alpha]_D^{30}$ −26.5 ± 1.3° (*c* 1.5, acetic acid).

Anal. Calcd. for $C_{30}H_{34}N_8O_7$: C, 58.25; H, 5.54; N, 18.11. Found: C, 58.18; H, 5.73; N, 18.40.

*cyclo-Glycyl-*DL*-tyrosyldiglycyl-N^{im}-benzyl-*L*-histidylglycyl.* (DL,L-*VII*).—Beginning with 3.50 g of the diastereomeric mixture of blocked linear hexapeptides (DL,L-V), the unblocking and cyclization were repeated exactly as described for the L,L series. Purification of the crude cyclic product as above gave 865 mg of crystalline material and 518 mg of amorphous material; the two fractions gave identical single spots on paper chromatography in all solvent systems used; no resolution of the diastereomeric mixture was noted. The over-all yield for the two steps in this case was 58%. An analytical sample was dried *in vacuo* 48 hours at 100°.

Anal. Calcd. for $C_{30}H_{34}N_8O_7$: C, 58.25; H, 5.54; N, 18.11. Found: C, 58.17; H, 5.96; N, 17.83.

*cyclo-Glycyl-*L*-tyrosyldiglycyl-*L*-histidylglycyl (Ia).*—Crystalline N^{im}-benzyl cyclic peptide, L,L-VIII (226 mg), was dissolved in 25 ml of glacial acetic acid. Ten per cent palladium on charcoal (113 mg) was added, and hydrogen was bubbled through the solution. After 2 days an additional 50 mg of catalyst was added. After a total reaction time of 4 days no starting material could be detected on paper chromatography of the reaction mixture.

The catalyst was removed by centrifugation and washed several times with acetic acid. The combined acetic acid solutions were filtered and lyophilized. The product was taken up in water, and the solution was extracted first with chloroform, then with ethyl acetate. Lyophilization of the aqueous solution gave a fluffy white solid, 181 mg, 94%. The product was ninhydrin-negative and chromatographically homogeneous in several solvent systems, including 2-butanol–water–acetic acid (4:1:1), R_F 0.45, and one-butanol-water (6:1), R_F 0.15. Its ultraviolet spectrum in aqueous solution exhibted a maximum at 275 mμ (ε 1210) at neutrality and at 293 mμ (ε 2080) at pH 12.

Anal. Calcd. for $C_{23}H_{28}N_8O_7$: C, 52.26; H, 5.34; N, 21.20. Found. C, 51.87; H, 5.46; N, 20.60.

*cyclo-Glycyl-*D*-tyrosyldiglycyl-*L*-histidylglycyl (Ib).*—Hydrogenolysis of the imidazole-protected mixture DL,L-VII as described for the L,L isomer gave debenzylated product in 83–89% yield. The product was chromatographically homogeneous in all solvent systems tried but one: it appeared as two spots, R_F 0.25 and 0.29, when 2-butanol-water (4:1) was used, these spots being cleanly separated when the solvent front was allowed to run off the paper. The slower-running diastereomer was identical on paper chromatography with the isomer synthesized from carbobenzoxyglycyl-L-tyrosylglycine. The D,L-cyclic peptide (Ib) was obtained by chromatography of 730 mg of the diastereomeric mixture of debenzylated cyclic peptides on a 1500-g cellulose column, using 2-butanol-water (4:1). Fractions containing pure D,L-isomer and pure L,L-isomer (as determined by paper strip chromatograms) were collected, the former being eluted first. Each set of fractions was stripped to dryness at 1 mm pressure. The D,L material could not be dissolved in water; instead it was taken up in 200 ml of water to which 3.4 ml of 0.2 N hydrochloric acid had been added. The aqueous solution was washed with ethyl acetate, then with chloroform, and was lyophilized to give 121 mg of chromato-

graphically homogeneous D,L material. About half the initial charge to the column was recovered as diastereomeric mixture, the separation of the two bands not being complete on one pass.

Anal.[4] Calcd. for $C_{23}H_{28}N_8O_7 \cdot HCl \cdot 3H_2O$: C, 44.62; H, 5.70; N, 18.11; Cl, 5.90. Found: C, 45.07; H, 6.05; N, 16.70; Cl, 6.29.

*cyclo-Glycyl-O-acetyl-*L(D)*-tyrosyldiglycyl-*L*-histidylglycyl.*—Ten mg of the *cis*-1,4 hexapeptide (Ia) was dissolved in 5–6 drops of glacial acetic acid. One to 1.5 ml of acetic anhydride was added, and the solution was heated at 80° for 1 hour. Acetic acid and acetic anhydride were removed *in vacuo*. To the residue was added 0.4 ml of 0.05 N hydrochloric acid; after 40 minutes the solution was lyophilized. The product thus obtained was lyophilized again from water. Further treatment of this material with acetic anhydride did not alter its ultraviolet spectrum, and it was therefore assumed to be completely acetylated. A few mg of this acetylated peptide was treated with aqueous ammonia and then subjected to paper chromatography. A single spot, which ran with an authentic sample of unacetylated cyclopeptide (Ia), was observed, indicating the absence of peptide degradation upon acetylation. Paper chromatography of the acetylated material itself did reveal a detectable trace of starting material, but it is possible that some deacetylation had occurred in the chromatographic process.

The ultraviolet spectrum of an aqueous solution of the acetyl peptide at *p*H 4–5 was measured and maxima were observed at 262 and 268 mμ (ε 370) with a shoulder at 280 mμ. (This solution was made basic by the addition of a minute amount of 5 N sodium hydroxide. After 1 hour the absorption of the basic solution was measured and the intensity of the absorption maximum at 293 mμ [phenolate] was used to determine the concentration of the solution and thus the extinction coefficients for the acetylated substance. This method was chosen to obviate the necessity of weighing small samples of the hydroscopic acetyl peptide hydrochloride.)

The infrared spectrum of the acetyl peptide (potassium bromide pellet) had absorption maxima in the carbonyl region at 1735, 1655, and 1550 cm^{-1}.

The *trans*-1,4 hexapeptide (Ib) was more difficult to acetylate than the *cis* isomer. Treatment with acetic acid–acetic anhydride for 3.5 hours at 80° was necessary to complete the reaction according to the criterion used above.

*cyclo-*L*-Histidyl-*L*-tyrosyl (IIa).*—Palladium on charcoal (10%, 0.9 g) was suspended in a solution of carbobenzoxy-L-histidyl-L-tyrosine methyl ester (1.84 g) in 100 ml of glacial acetic acid. Hydrogen was bubbled through the mixture for 44 hours. Catalyst was removed by centrifugation and washed with more acetic acid. Acetic acid solutions were combined, filtered, and concentrated to dryness under reduced pressure. The residue was freed of acetic acid by suspension in ethanol and evaporation of the ethanol, followed by repetition of this process twice using toluene. The residue was then crystallized from water (*p*H adjusted to 7.5) and again from water. Nine-tenths gram, mp 272–276°d (76%) was obtained. Optical rotation $[\alpha]_D^{25}$ −52.3 ±0.3° (c 12.9, acetic acid). This product ran as a single component, R_F 0.45, in 2-butanol-water (4:1), and was identical in several other solvents with the diketopiperazine prepared by the procedure given in the previous paper (Kopple and Nitecki, 1962).

*Racemization of cyclo-*L*-Histidyl-*L*-tyrosyl.*—A sample of optically active *cis* diketopiperazine as prepared above (50 mg) was suspended in 20 ml of methanol containing 0.04 ml of 0.4 M sodium methoxide in methanol (0.1 molar equivalent), and the mixture was heated under reflux for 18 hours, then neutralized with excess acetic acid, and evaporated to dryness under vacuum. The residue was subjected to paper chromatography using 2-butanol-water (4:1) and contained two components; one, R_F 0.43, ran indistinguishably from starting material; the other, R_F 0.52 was distinct from starting material. Neither spot was ninhydrin active.

*cyclo-*LD*-Histidyl-*DL*-tyrosyl (IIb).*—Lowering melting, later crops from the recrystallization of presumed L,L-*cyclo*-histidyltyrosyl prepared as previously reported (path A) contained the same two ninhydrin-inactive components as resulted from the base-catalyzed racemization just described. A collection of such crops was combined and chromatographed on a 1-kg cellulose column using 1-butanol-water (6:1). Fractions containing each isomer free of the other (as determined on paper strips) were obtained, although complete separation of the bands did not occur in a single pass. Those fractions containing the faster running isomer (R_F 0.52) were combined and concentrated to a residue that was triturated with ethyl acetate and dried *in vacuo* at 100°, mp 269–271°d. This product was considerably more soluble in water than the *cis* isomer

Anal. Calcd. for $C_{15}H_{16}N_4O_3$: C, 59.99; H, 5.37; N, 18.66. Found: 59.81, 59.78; H, 5.49, 5.62; N, 17.57, 17.90.

*O,N-Diacetyl-cyclo-*L*-histidyl-*L*-tyrosyl.*—A sample of *cis-cyclo*-histidyltyrosyl (170 mg) was mixed with 4 ml of pyridine and 1 ml of acetic anhydride and heated at 80–90° until solution occurred. The solution was stored at room temperature for 3 hours and then concentrated to dryness *in vacuo* at 65–70° to a residue that was washed with five 20-ml portions of ice water and then with 5 ml of absolute ethanol. The crystalline product was dried over phosphorus pentoxide *in vacuo* at room temperature. An analytical sample was further dried at 100° *in vacuo* for 24 hours.

Anal. Calcd. for $C_{19}H_{20}N_4O_5$: C, 59.37; H, 5.24; N, 14.58. Found: C, 59.09, 59.15; H, 5.67, 5.57; N, 14.47, 14.03.

A 10-mg sample of this acetylated dipeptide dissolved in 0.1 ml of concentrated aqueous ammonia in about 3 minutes. After 10 minutes the resulting solution was evaporated at room temperature and the residue was subjected to paper chromatography. It proved to contain only one, ninhydrin-inactive component, and that identical to *cis*-diketopiperazine. The *O,N*-diacetyl compound dissolved only slowly in water at *p*H 3. Titration of this solution indicated acids of *pK* 4.8 (acetic acid) and 6.4 (imidazole). To obtain the mono-*O*-acetyl diketopiperazine, the remainder of the *O,N*-diacetyl compound was dissolved in 5 ml of water by addition of enough hydrochloric acid solution to give a final solution *p*H of 3, and this solution was lyophilized to yield the *O*-acetyl-*cis-cyclo*-histidyltyrosyl. The lyophilized solid was stored over phosphorus pentoxide.

The ultraviolet spectrum of this product (in water) possessed maxima at 261 mμ (ε 290), 268 mμ (ε 235), and a shoulder at 280 mμ (ε 70). Its infrared spectrum showed absorption at 1750 and 1660 cm^{-1}. (No distinct third carbonyl band was observed in the spectrum of the *O,N*-diacetyl compound.)

Anal.[4] Calcd. for $C_{17}H_{18}N_4O_4 \cdot HCl \cdot 3H_2O$: C, 47.17; H, 5.82; N, 12.95. Found: C, 47.16; H, 6.28; N, 12.37.

[4] Sample could not be dried without partial loss of hydrogen chloride.

References

Abderhalden, R., Weidle, H., Baertich, E., and Morneweg, W. (1938), *Fermentforschung 16*, 98.

Anderson, G. W., and Callahan, F. M. (1958), *J. Am. Chem. Soc. 80*, 2902.
Bergmann, M., and Fruton, J. S. (1937), *J. Biol. Chem. 118*, 405.
Bruice, T. C., Fife, T. H., Bruno, J. J., and Benkovic, P. (1962), *J. Am. Chem. Soc. 84*, 3012.
Bruice, T. C., and Schmir, G. L. (1957), *J. Am. Chem. Soc. 79*, 1663.
Bruice, T. C., and Sturtevant, J. M. (1959), *J. Am. Chem. Soc. 81*, 2860.
Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.
Jencks, W. P., and Carriuolo, J. (1959), *J. Biol. Chem. 234*, 1272.
Jencks, W. P., and Carriuolo, J. (1961), *J. Am. Chem. Soc. 83*, 1743.
Jencks, W. P., and Gilchrist, M. (1962), *J. Am. Chem. Soc. 84*, 2910.
Kopple, K. D., and Nitecki, D. E. (1962), *J. Am. Chem. Soc. 84*, 4457.
Wiggins, L. F., and Williams, J. (1952), *Nature 170*, 279.

Heat- and Alkali-Induced Changes in the Conformation of Pepsinogen and Pepsin

JOSEPH F. GERSTEIN, HELEN VAN VUNAKIS, AND LAWRENCE LEVINE

From the Graduate Department of Biochemistry, Brandeis University, Waltham 54, Massachusetts*

Received April 4, 1963

The conformational changes which occur when pepsinogen and pepsin are subjected to treatment with heat and alkali have been studied using (1) an immunologic method with antisera directed towards pepsinogen and pepsin, (2) potential enzyme activity of pepsinogen, and (3) tryptophan fluorescence of the proteins. The specificity of the antisera varied for the different conformational states of the proteins. Antipepsinogen was capable of distinguishing reversible and irreversible changes in the homologous antigen while antipepsin, directed toward pepsin denatured at *p*H 7, was capable of detecting an irreversibly denatured state of the zymogen in which the pepsin moiety was exposed. Antipepsin also could detect a more disorganized molecular state in pepsinogen and pepsin which was present when these proteins were subjected to elevated temperatures and alkali concentrations. The major structural transitions during alkali treatment, as measured by the three techniques, occurred at *p*H's between 9.4 and 10.2, confirming the results of other investigators that the ϵ-amino group of lysine or hydroxyl group of tyrosine may be involved in maintaining the structure of the zymogen.

The ability of antibodies to complex with globular proteins, since it is to some degree dependent upon the tertiary structure of the antigen as well as the integrity of the peptide sequence, provides a sensitive tool for the detection of structural changes produced in the antigen by the application of various chemical and physical treatments (Levine, 1962). The rabbit antisera directed against porcine pepsinogen and pepsin (denatured at *p*H 7.0) are capable of detecting structural alterations in their homologous antigens; in addition antipepsin is capable of detecting the "unmasking" of the pepsin moiety of pepsinogen (Van Vunakis *et al.*, 1963; Van Vunakis and Levine, 1963).

Herriott (1962) has reviewed the effects of some denaturing agents on pepsinogen and pepsin. This communication presents the results of investigations on the structural status of these proteins during treatment with heat or alkali. The techniques of quantitative complement fixation, estimation of potential peptic activity, and, as a physical parameter, the tryptophan fluorescence of the proteins have been utilized in an attempt to delineate the structural changes which occur during these modifications as well as to evaluate the discrimination of the various methods. Protein fluorescence was selected as the physical parameter for these studies because it has been shown to reflect major structural transitions in some proteins (Teale, 1960; Brand *et al.*, 1962; Gally and Edelman, 1962) and to vary in a nonlinear fashion when pepsinogen is subjected to increments of temperature and *p*H (Steiner and Edelhoch, 1962).[1] Considerable data on the structural modifications of pepsinogen and pepsin, utilizing the optical rotatory properties of the proteins as a physical constant, are available (Perlmann, 1959, 1963; Perlmann and Harrington, 1961). With these studies as a point of departure, the present investigation was undertaken.

* Contribution No. 230. We are grateful to the National Science Foundation (grant 20,029) and the National Institutes of Health (grants E-1940 and E-2792).

[1] J. A. Gally, G. E. Perlmann, and G. M. Edelman have also investigated the effect of denaturing agents on the fluorescence of pepsinogen. A preliminary report of some of their findings was made by Dr. Perlmann at the Symposium on Quantum Aspects of Polypeptides and Polynucleotides, and will appear in *Biopolymers 1* (1963, in press). A preprint of this manuscript was kindly made available to us by Dr. Perlmann.

[2] A strict comparison of our results with the optical rotation studies of Perlmann and Harrington (1961) and Perlmann (1963) is difficult since factors which influence this reaction, such as the concentration of pepsinogen and the duration of equilibration at each temperature, have varied between the two laboratories.

[3] The expression pH_f was introduced by White (1959) for the *p*H at which the fluorescence of a substance is quenched 50% by either acid or base addition.

Materials and Methods

Proteins.—Swine pepsinogen was prepared as previously described (Van Vunakis *et al.*, 1963); pepsin (Lot 661 twice crystallized from ethanol) was obtained from Worthington Biochemical Corporation.

Antisera.—The preparation, characterization, and immunologic properties of the antisera have been reported (Van Vunakis *et al.*, 1963; Van Vunakis and Levine, 1963).

Complement (C′) Fixation.—Reagents and procedures for the quantitative *C′* fixation technique are described by Wasserman and Levine (1961).

Fluorescence Measurements.—The Aminco-Bowman spectrofluorometer equipped with a 150-watt Osram high pressure xenon lamp and thermoregulated at 25° was used for most of the fluorescence measurements. Activation was at 286 mμ, and emission was monitored