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Added in Proof

We recently became aware of similar work on tritium labeling of the 5′-CH_2 by Nossen and Froholan (1967).

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Side Reactions in the Synthesis of Peptides Containing the Aspartylglycyl Sequence*

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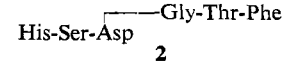
ABSTRACT: The hexapeptides L-histidyl-L-seryl-L-aspartylglycyl-L-threonyl-L-phenylalanine (**1**) and L-histidyl-L-seryl-β-L-aspartylglycyl-L-threonyl-L-phenylalanine (**2**) have been synthesized by the standard procedures of peptide synthesis. When the preparation of the same hexapeptides was attempted by the solid-phase approach, the only major product isolated was the succinimido derivative, namely, L-histidyl-L-seryl-L-aspartimidylglycyl-L-threonyl-L-phenylalanine (**13**). Formation of succinimido derivatives was also observed during the synthesis of **1** by the standard procedure, but only when the β-carboxyl group of aspartic acid was esterified. The lack of a side chain in the glycyl residue appears to be the most important contributing factor to the pronounced tendency of the aspartylglycine sequence to rearrange through the formation of cyclic imide intermediates.

In the course of our work on the synthesis of the intestinal hormone secretin (Bodanszky *et al.*, 1966, 1967; Ondetti *et al.*, 1968a,b) it became apparent that the aspartic acid in position 3 had a marked tendency to rearrange to the β-acyl form. The postulation of rearrangements of this type finds support in the experimental evidence already on record in the literature (Sondheimer and Holley, 1954; Battersby and Robinson, 1955; Bernhard *et al.*, 1962; Iselin and Schwyzer, 1962; Hanson and Rydon, 1964; Fölsch, 1966; Merrifield, 1967). However, the size and complexity of the secretin molecule made it very difficult to ascertain the nature and extent of this side reaction. In an effort to clarify this point we have synthesized two small peptides with the amino acid sequence surrounding the suspected aspartyl residue of secretin. One of these peptides represents the N-terminal portion of the secretin molecule, with the aspartic acid in position 3 linked to the glycine residue through its α-carboxyl (**1**); in the other the amide linkage is formed through the β-carboxyl (**2**).

His-Ser-Asp-Gly-Thr-Phe
1



2

Two alternative approaches were employed for the synthesis of the two hexapeptides: the standard procedure with isolation of the intermediates, and the solid-

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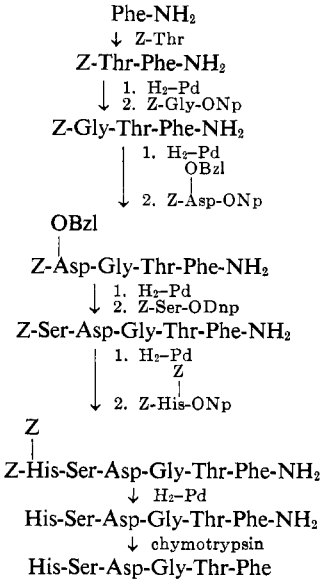


FIGURE 1: Synthesis of **1**.

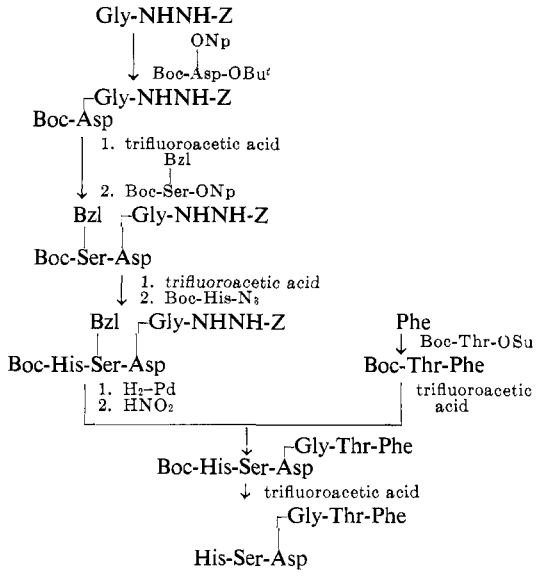


FIGURE 2: Synthesis of **2**.

phase method (Merrifield, 1963, 1964). The standard syntheses of **1** and **2** are presented schematically in Figures 1 and 2.[1]

Experimental Section

Melting points were taken in capillary tubes and are uncorrected. The copolymer used for the solid-phase syntheses (1.8 mequiv/g) was obtained from Bio-Rad Laboratories (Richmond, Calif.). The *t*-butyloxycarbonylamino acids were purchased from Schwarz Bio-Research Inc. (Orangeburg, N. Y.). The shaker was of the wrist-action type (Palo-Meyers Inc., New York, N. Y.). Paper chromatography was carried out on Whatman No. 1 by the descending technique with the solvent system *n*-butyl alcohol–pyridine–acetic acid–water (30:20:6:24). Paper electrophoresis was carried out on Whatman No. 4 with pyridinium–formate buffer (pH 33) (Werum *et al.*, 1960). Electrophoretic mobilities are expressed as fractions, E_{His} of the distance traveled by histidine in the same system. α-Chymotrypsin was purchased from Worthington Biochemical Corp. (Freehold, N. J.), and leucine aminopeptidase from Boehringer Mannheim Corp. (New York, N. Y.). The leucine aminopeptidase digestion was carried out as described by Hofmann *et al.* (1962). Quantitative amino acidanalyses were performed in a modified Technicon amino acid Autoanalyzer. A Craig countercurrent distribution apparatus (H. O. Scientific Instrument Co., New York, N. Y.) with 500 tubes (10 ml of each phase) was utilized for the countercurrent distributions.

Solid-Phase Syntheses. A. ATTEMPTED SYNTHESIS OF HISTIDYLSERYLASPARTYLGLYCYLTHREONYLPHENYLALANINE. Chloromethylpolystyrene–2% divinylbenzene copolymer (10 g) was added to a solution of *t*-butyloxycarbonyl-L-phenylalanine (5.16 g) and triethylamine (1.96 g) in absolute ethanol (75 ml). The suspension was refluxed for 72 hr, filtered, and the resin was washed three times each with ethanol, water, methanol, and dichloromethane. A 3-g batch of this *t*-butyloxycarbonyl-L-phenylalanine-resin (0.72 mequiv of phenylalanine/g of resin) was placed in a reaction vessel essentially identical with that described by Merrifield (1963) and the following cycle of reactions was carried out: (1) deprotection with 1 N HCl in dioxane–dichloromethane (1:1) (30 ml) for 30 min, (2) washing with dichloromethane (three 50-ml portions), (3) washing with ethanol (three 50-ml portions), (4) washing with methanol (three 50-ml portions), (5) washing with dichloromethane (three 50-ml portions), (6) neutralization with a mixture of triethylamine (3 ml) and dichloromethane (30 ml) and shaking for 10 min, (7) washing with dichloromethane (four 50-ml portions), (8) addition of *t*-butyloxycarbonylthreonine (7.56 mmol) dissolved in dichloromethane (20 ml) and shaking for 10 min, (9) addition of *N,N'*-dicyclohexylcarbodiimide (7.56 mmol) dissolved in dichloromethane (10 ml) and shaking for 2 hr, (10) washing with dichloromethane (three 50-ml portions), (11) washing with warm (*ca.* 45°) dimethylformamide (three 50-ml portions), (12) washing with warm ethanol (three 50-ml portions), (13) washing with acetic acid (three 50-ml portions), (14) washing with methanol (three 50-ml portions), and (15) washing with dichloromethane (three 50-ml portions). The same cycle of reactions was repeated for the introduction of the following *t*-butyloxycarbonylamino acid residues (7.56 mmol each): glycine, β-benzylaspartate, *O*-benzylserine, and N^{im}-benzylhistidine (dimethylformamide was used instead of dichloromethane in the coupling step). After the final coupling step the resin was thoroughly washed and dried (yield 4.8 g). For the removal of the hexapeptide, the peptide-resin was suspended in trifluoroacetic acid (30 ml) and hydrogen bromide was bubbled through for 7 min. The resin was filtered and washed with trifluoroacetic acid. The filtrate was concentrated to dryness *in vacuo*

[1] All the abbreviations employed are in accordance with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (*Biochemistry 5*, 2485 (1966)). All amino acids are of the L configuration.

at room temperature and the residue was dissolved in trifluoroacetic acid (10 ml). This solution was diluted with ether (100 ml), and the precipitate was filtered and washed thoroughly with ether (yield 900 mg). This material (880 mg) was dissolved in a mixture of methanol–water–acetic acid (10:1:1) (400 ml) and hydrogenated at atmospheric pressure with 10% palladium on charcoal (900 mg) for 88 hr.[2] The catalyst was filtered and the filtrate was concentrated to dryness. The residue (650 mg) was distributed for 300 transfers in the system *n*-butyl alcohol–pyridine–acetic acid–water (4:2:1:7). The material obtained from the main peak[3] (K, 0.22; 162 mg) showed on paper chromatography one main spot (R_F 0.47). It was further purified by partition chromatography on a column of Sephadex G-25 (1.8-cm diameter, 100 cm long) equilibrated with the solvent system *n*-butyl alcohol–pyridine–acetic acid–water (30:20:6:24): yield 94 mg, R_F 0.47, E_{His} 0.78, $[\alpha]_D^{23}$ −33.8° (*c* 1, 1 N AcOH). The infrared spectrum (KBr) showed absorption bands at 5.62 and 5.88 μ. Quantitative amino acid analysis after acid hydrolysis gave the following molar ratio of amino acids: Asp (1.04), Thr (0.85), Ser (0.95), Gly (1.08), Phe (1.07), and His (1.00). Leucine aminopeptidase treatment followed by quantitative amino acid analysis of the hydrolysate gave the following molar ratio of amino acids: Asp (0.14), Thr (0.78), Ser (0.78), Gly (0.19), Phe (0.82), and His (1.00).

B. ATTEMPTED SYNTHESIS OF HISTIDYLSERYL-β-ASPARTYLGLYCYLTHREONYLPHENYLALANINE. A 2-g batch of *t*-butyloxycarbonylphenylalanine-resin (0.65 mequiv of phenylalanine/g of resin) was placed in the reaction vessel and the same cycle of reactions described above was used for the introduction of the following *t*-butyloxycarbonylamino acids: threonine, glycine, α-benzylaspartate,[4] *O*-benzylserine, and N^{im}-benzylhistidine. The hexapeptide was removed from the resin (2.9 g) by a 7-min treatment with hydrogen bromide in trifluoroacetic acid, yield 580 mg. This material (530 mg) was dissolved in 10% aqueous acetic acid (250 ml) and hydrogenated with 5% palladium on barium sulfate (500 mg) for 78 hr.[2] The catalyst was filtered and the filtrate was concentrated to dryness. The residue was distributed for 500 transfers in the solvent system described above. The material from the main peak[3] (K, 0.21) was recovered by removal of the solvents *in vacuo* and freeze drying from dilute acetic acid: yield 66 mg, R_F 0.47, E_{His} 0.78, $[\alpha]_D^{23}$ −33.2° (*c* 1, 1 N AcOH). The infrared spectrum was superimposable on that of the material described in section A. Quantitative amino acid analysis after acid hydrolysis gave the following molar ratios: Asp (1.00), Thr (0.85), Ser (0.85), Gly (1.09), Phe (1.05), and His (0.95); after leucine aminopeptidase digestion: Asp (0.10), Thr (0.87), Ser (0.49), Gly (0.20), Phe (0.87), and His (1.00).

The material (4 mg) obtained according to A or B was dissolved in aqueous 1% ammonium bicarbonate and the solution was stored at 40° for 24 hr. The material isolated after freeze drying showed on paper chromatography and on paper electrophoresis two spots (R_F 0.36 and 0.29; E_{His} 0.70 and 0.64). The spot with R_F 0.29 and E_{His} 0.64 represented approximately 80% of the mixture. A time study showed that this reaction was practically completed in the first hour.

Synthesis of **1**

Benzyloxycarbonylthreonylphenylalaninamide (**3**). A solution of benzyloxycarbonylthreonine (5.06 g) and triethylamine (2.9 ml) in acetonitrile (40 ml) was added to a suspension of *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward *et al.*, 1961) (5.06 g) in acetonitrile (40 ml), and the suspension was stirred in an ice bath until a clear solution was obtained (0.5 hr). This solution was added to one of phenylalaninamide hydrochloride (3.9 g) in acetonitrile (50 ml) containing triethylamine (2.9 ml). The reaction mixture was allowed to stand at room temperature for 72 hr. The gelatinous precipitate formed was filtered, washed, and crystallized from ethyl acetate: yield 3 g, mp 197–198°, $[\alpha]_D^{24}$ +1.2° (*c* 1, dimethylformamide). *Anal.* Calcd for $C_{21}H_{25}N_3O_5$: C, 63.1; H, 6.3; N, 10.5. Found: C, 63.1; H, 6.4; N, 10.4.

Benzyloxycarbonylglycylthreonylphenylalaninamide (**4**). The protecting group of **3** (1.48 g) was removed by catalytic hydrogenolysis, and the dipeptide amide was allowed to react with benzyloxycarbonylglycine *p*-nitrophenyl ester (1.3 g) in the usual way (Bodanszky and Williams, 1967). The product (1.6 g) was crystallized from ethyl acetate: yield 1.37 g, mp (sintering at 155°) 163–164°, $[\alpha]_D^{23}$ −1.7° (*c* 1, dimethylformamide). *Anal.* Calcd for $C_{23}H_{28}N_4O_6$: C, 60.5; H, 6.2; N, 12.3. Found: C, 60.6; H, 6.5; N, 12.3.

Benzyloxycarbonyl-β-benzylaspartylglycylthreonylphenylalaninamide (**5**). The tripeptide amide obtained by catalytic hydrogenolysis of **4** (6.8 g) was allowed to react with benzyloxycarbonyl-α-*p*-nitrophenyl β-benzylaspartate (9.0 g) under the usual conditions. The crude product was triturated with ethyl acetate, filtered, and dried: yield 9.0 g, mp 165–167°;[5] $[\alpha]_D^{22}$ −14.3° (*c* 1, dimethylformamide); nuclear magnetic resonance (CD_3COOD) spectra at τ 2.66 (s, 10), 2.76 (s, 5), and 4.88 (s, 4). *Anal.* Calcd for $C_{34}H_{39}N_5O_9$: C, 61.7; H, 5.9; N, 10.6. Found: C, 61.3; H, 5.8; N, 10.5.

Crystallization of this material from boiling 95% ethanol gave a product of mp 218–221°; nuclear magnetic resonance (CD_3COOD) spectra of τ 2.65 (s, 6), 2.75 (s, 5), and 4.86 (s, 2); infrared spectra (KBr) 5.60 and 5.82 μ.

Benzyloxycarbonylserylaspartylglycylthreonylphenylalaninamide (**6**). Catalytic hydrogenolysis of **5** (7.9 g) gave the tetrapeptide amide, which was coupled with benzyloxycarbonylserine 2,4-dinitrophenyl ester (Bodanszky and Ondetti, 1966) (4.8 g). The crude product

[2] Removal of the N^{im}-benzyl-protecting group was still incomplete, but the hydrogenation was interrupted to avoid the reduction of the aromatic ring of phenylalanine.

[3] Several other minor peaks were observed but were not investigated.

[4] Prepared from Boc-N_3 and Asp-OBzl: mp 97–99°, $[\alpha]_D^{25}$ −30.3° (*c* 0.6, dimethylformamide).

[5] Capillary introduced in the bath when the temperature was 150°. If the capillary is introduced in a cold bath and heated slowly, sintering at 189° and mp 194–195° is observed.

was triturated with ether and ethyl acetate, filtered, and dried: yield 3.8 g, mp (sintering 148°) 152–155°, $[\alpha]_D^{29}$ −14.5° (*c* 1, dimethylformamide). *Anal.* Calcd for $C_{30}H_{38}N_6O_{11}$: C, 54.7; H, 5.8; N, 12.8. Found: C, 54.1; H, 6.3; N, 12.0.

Histidylserylaspartylglycylthreonylphenylalaninamide (**7**). The free pentapeptide amide (1.6 g) obtained from **6** by catalytic hydrogenolysis was allowed to react with N^{α}-benzyloxycarbonyl-N^{im}-benzyloxycarbonylhistidine *p*-nitrophenyl ester (1.9 g) (Meienhofer, 1962). After standing overnight at room temperature the solvent was removed *in vacuo*. The residue was triturated with ether, filtered, resuspended in ethyl acetate, filtered, and dried, yield 2.1 g. The protected hexapeptide (1.6 g) was hydrogenated in the usual manner and the free hexapeptide was crystallized from ethanol–water (10:1): yield 575 mg, $[\alpha]_D^{26}$ −26° (*c* 1, H_2O), R_F 0.41. Quantitative amino acid analysis after acid hydrolysis gave the following molar ratios: Asp (1.01), Thr (0.96), Ser (0.96), Gly (0.96), Phe (0.90), NH_3 (1.03), and His (1.10). *Anal.* Calcd for $C_{28}H_{39}N_9O_{10}\cdot 2H_2O$: C, 48.2; H, 6.2; N, 18.1; TV, 5.1. Found: C, 48.3; H, 6.7; N, 17.6; TV 4.9.

Histidylserylaspartylglycylthreonylphenylalanine (**1**). A solution of the hexapeptide amide **7** (100 mg) in 1% aqueous ammonium bicarbonate (10 ml) was incubated at 40° with 0.8 ml of a 0.25% α-chymotrypsin solution in 1% ammonium bicarbonate. After 5 hr another 0.8-ml portion of chymotrypsin solution was added and the incubation was continued for 16 hr. The incubation mixture was freeze dried and the residue was applied to a column of Sephadex G-25 (1.8-cm diameter, 100 cm long) equilibrated with *n*-butyl alcohol–pyridine–acetic acid–water (30:20:6:24). The elution was carried out with the same solvent mixture and the eluate was collected in 5-ml fractions. The elution was followed with Pauly and Ninhydrin reagents, and the fractions containing the desired material were pooled, concentrated to dryness, and the residue was freeze dried from water: yield 75.9 mg, $[\alpha]_D^{23}$ −17.9° (*c* 1, 1 N AcOH), R_F 0.36, E_{His} 0.70. Quantitative amino acid analysis after leucine aminopeptidase hydrolysis gave the following molar ratios: Asp (0.99), Thr (1.10), Ser (0.95), Gly (0.95), Phe (1.06), and His (0.98).

Compound **1** (2 mg) was dissolved in aqueous 1% ammonium bicarbonate (1 ml) and the solution was kept at 40° for 24 hr. The material recovered after freeze drying the solution showed on paper chromatography and paper electrophoresis the same mobility of the starting material **1**.

Compound **1** (10 mg) was dissolved in trifluoroacetic acid (1 ml) and hydrogen bromide was bubbled through for 30 min. Ether was added (15 ml) and the precipitate was isolated by centrifugation, washed several times with ether, and dried, yield 9.6 mg. This material showed on paper chromatography and paper electrophoresis the same mobility of the the starting material **1**.

Synthesis of **2**

β-Aspartylglycine Benzyloxycarbonylhydrazide (**8**). *t*-Butyloxycarbonyl-α-*t*-butyl-β-*p*-nitrophenylaspartate[6] (11.5 g) was allowed to react with glycinebenzyloxycarbonylhydrazine trifluoroacetate (Ondetti *et al.*, 1968a,b) (11.6 g). The product was dissolved in trifluoroacetic acid (55 ml) and the solution was kept at room temperature for 1 hr. The trifluoroacetic acid was removed *in vacuo* and the residue was disintegrated with ether: yield 6 g, mp 180–185°, $[\alpha]_D^{25}$ +3.1° (*c* 0.8, methanol containing 2.5% of 6 N HCl). *Anal.* Calcd for $C_{14}H_{18}N_4O_6$: C, 49.7; H, 5.4; N, 16.6. Found: C, 49.8; H, 5.4; N, 16.4.

t-Butyloxycarbonyl-O-benzylseryl-β-aspartylglycine Benzyloxycarbonylhydrazide (**9**). *t*-Butyloxycarbonyl-*O*-benzylserine *p*-nitrophenyl ester (prepared from 9 g of the corresponding protected amino acid derivative (Bodanszky and Williams, 1967)) was allowed to react with **8** (5.7 g) in the usual manner. The product was crystallized from methanol–water: yield 6.1 g, mp 153–155°, $[\alpha]_D^{25}$ −2.7° (*c* 0.6, dimethylformamide). *Anal.* Calcd for $C_{29}H_{37}N_5O_{10}$: C, 56.6; H, 6.1; N, 11.4. Found: C, 56.6; H, 6.4; N, 11.4.

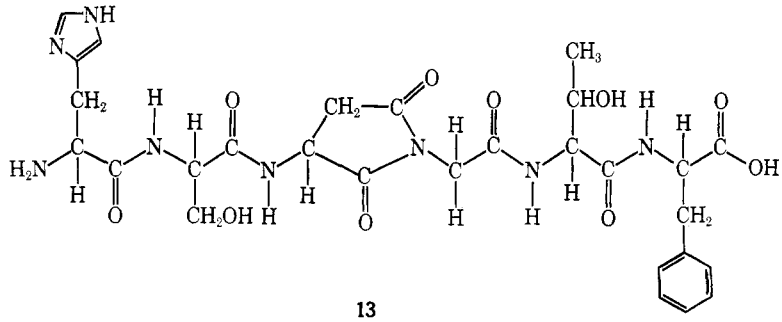
t-Butyloxycarbonylhistidyl-O-benzylseryl-β-aspartylglycine Benzyloxycarbonylhydrazide (**10**). *t*-Butyloxycarbonylhistidine azide (prepared from 3.85 g of the corresponding hydrazide) in ethyl acetate was allowed to react with *O*-benzylseryl-β-aspartylglycine benzyloxycarbonylhydrazide trifluoroacetate (5.5 g, prepared from 6 g of **9**) (Ondetti *et al.*, 1968a,b). This crude material (6.6 g) was purified by countercurrent distribution in the solvent system: chloroform–methanol–0.1 M pyridinium acetate (50:45:20). The major component (K, 0.5) was the protected tetrapeptide hydrazide: yield 2.5 g, mp 132–135°, $[\alpha]_D^{25}$ −10.5° (*c* 1.6, dimethylformamide). *Anal.* Calcd for $C_{35}H_{44}N_8O_{11}\cdot H_2O$: C, 54.5; H, 6.0; N, 14.5. Found: C, 54.2; H, 6.0; N, 14.6.

t-Butyloxycarbonylhistidylseryl-β-aspartylglycine Hydrazide (**11**). A solution of **10** (2.3 g) in a mixture of methanol–acetic acid–water (2:1:1) was hydrogenated over 10% palladium on charcoal for 6 hr. The catalyst was filtered and the filtrate was evaporated to dryness *in vacuo*. The residue was triturated with ethyl acetate, filtered, and dried: yield 1.3 g, mp 152–155°, $[\alpha]_D^{25}$ −1.9° (*c* 1.2, dimethylformamide). *Anal.* Calcd for $C_{20}H_{32}N_8O_9$: hydrazide N, 5.3 (Medzihradszky-Schweiger, 1962). Found: hydrazide N, 5.5.

t-Butyloxycarbonylthreonylphenylalaninedicyclohexylammonium Salt (**12**). Phenylalanine (330 mg) was dissolved in a mixture of water (3.5 ml) and pyridine (3.5 ml) and the pH was adjusted to 8.6 with 2 N NaOH. Butyloxycarbonylthreonine *N*-hydroxysuccinimide ester[7] (632 mg) was added in portions with stirring, maintaining the pH between 8.5 and 8.7 by addition of 2 N NaOH. After the consumption of alkali stopped (*ca.* 3 hr) the reaction mixture was cooled in an ice bath, acidified to pH 3 with 1 N HCl saturated with NaCl, and extracted

[6] Prepared by the general procedure described in *Biochem. Prepn.* 9, 110: mp 127–129°, $[\alpha]_D^{25}$ 18.5° (*c* 2, chloroform). Boc-Asp-OBu^t was prepared by the same procedure described by Schröder and Klieger (1964) for the β analog: mp 105–107°, $[\alpha]_D^{25}$ −34.4° (*c* 0.74, dimethylformamide).

[7] Prepared from Boc-Thr and *N*-hydroxysuccinimide (Anderson *et al.*, 1963): mp 136–137°, $[\alpha]_D^{25}$ −29.1° (*c* 2, dioxane).



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four times with ethyl acetate. The ethyl acetate extract was washed once with saturated NaCl, dried ($MgSO_4$), and the solvent was removed *in vacuo*. The protected dipeptide acid was isolated as a dicyclohexylammonium salt: yield 713 mg, mp 203–204°, $[\alpha]_D^{26}$ +21.6° (*c* 1.1, dimethylformamide). *Anal.* Calcd for $C_{30}H_{49}N_3O_6$: C, 65.8; N, 9.0; N, 7.7. Found: C, 66.3; H, 9.2; N, 7.4.

The dipeptide (218 mg) was deprotected with trifluoroacetic acid in the usual manner, yield 140 mg.

Histidylseryl-β-aspartylglycylthreonylphenylalanine (**2**). Concentrated HCl (0.1 ml) was added to a solution of **11** (106 mg) in dimethylformamide (1.65 ml) cooled in a Dry-Ice–acetone bath at −20°. The temperature of the bath was allowed to rise to −15° and an aqueous 14% solution of sodium nitrite (0.15 ml) was added. After 5 min the temperature of the bath was lowered to −25° and *N*-ethylpiperidine (0.23 ml) was added, followed by a solution of threonylphenylalanine (140 mg) in dimethylformamide (1.65 ml). The reaction mixture was stored at 5° for 48 hr and then evaporated to dryness *in vacuo*. The residue was dissolved in trifluoroacetic acid (5 ml) and the solution was kept at room temperature for 20 min. The trifluoroacetic acid was removed *in vacuo* and the residue was triturated with ether, centrifuged, washed with ether, and dried. The crude residue (193 mg) was purified by partition chromatography on a Sephadex G-25 column (1.8 × 100 cm) with the solvent system *n*-butyl alcohol–pyridine–acetic acid–water (30:20:6:24) as described for **1**: yield 51 mg, $[\alpha]_D^{25}$ −4.7° (*c* 1.2, 1 N acetic acid), R_F 0.29, E_{His} 0.64. Quantitative amino acid analysis showed Asp (1.01), Thr (0.90), Ser (0.95), Gly (1.00), Phe (1.04), and His (0.97).

Discussion

The two hexapeptides obtained from the solid-phase syntheses had identical properties and they could be easily distinguished from the hexapeptide synthesized by the standard procedure described in Figure 2. The fact that the latter could be completely digested by leucine aminopeptidase confirmed that it had the expected structure **1**, in other words, that the aspartic acid residue was joined to the peptide chain through the α-carboxyl group. The higher cathodic mobility of the hexapeptide prepared by the solid-phase procedure, when compared with that of the hexapeptide **1**, indicated a higher basicity of the former. These data, in conjunction with its comparatively large optical rotation, and the presence in the infrared spectrum of carbonyl bands typical of succinimido derivatives, led us to conclude that this compound was neither the α- nor the β-hexapeptide expected (**1** or **2**), but the cyclic imide **13**. This succinimido derivative was not the only product formed in the solid-phase synthesis, but it was the major component.

The undesirable formation of succinimido derivatives was also observed during the synthesis of **1** by the standard procedure. Attempts to recrystallize the protected tetrapeptide benzyloxycarbonyl-β-benzylaspartylglycylthreonylphenylalanin amide from boiling alcohol led to products grossly contaminated with the succinimido derivative, as evidenced by their nuclear magnetic resonance and infrared spectra. Deprotection of the same tetrapeptide with hydrogen bromide in trifluoroacetic acid led to the same type of side reactions. In contrast, those intermediates that had the β-carboxyl group of the aspartyl residue in its free unesterified form showed no tendency to undergo the aforementioned cyclization either under acid or alkaline conditions. This behavior is probably due to the fact that alkoxy groups are better leaving groups than hydroxyls, and are, therefore, more easily displaced in the nucleophilic attack of the glycine nitrogen (Figure 3). The same argument applies to the recent finding of Haley *et al.* (1966) that the conversion of asparaginylglycine into β-aspartylglycine proceeds approximately ten times faster than the conversion of aspartylglycine into the same isomer. The succinimido hexapeptide **13**, on the other hand, opens readily under alkaline conditions to give a mixture of α-and β-hexapeptides, with a large predominance of the latter. These results can be attributed to the greater electrophilicity of the α-carbonyl carbon of the aspartimidyl residue (Battersby and Robinson, 1955).

The formation of succinimido intermediates has been invoked by several investigators to explain the facile alkaline hydrolysis of aspartyl β esters in aspartyl peptides with the formation of mixtures of α- and β-aspartyl peptides (Battersby and Robinson, 1955; Sondheimer and Holley, 1954; Bernhard *et al.*, 1962; Iselin and Schwyzer, 1962; Hanson and Rydon, 1964; Fölsch, 1966). In some instances the cyclic intermediates were isolated and characterized (Battersby and Robinson, 1955; Iselin and Schwyzer, 1962). The evidence presented in this paper indicates that this rearrangement is a real danger during the synthesis of aspartylglycine sequences, even when alkaline hydrolysis is avoided. The formation of succinimido derivatives during treatment with hydrogen bromide in acetic acid is a case in point. Swallow and Abraham (1958) reported that treatment of ε-(α-L-aspartyl)-L-lysine or ε-(β-L-aspartyl)-L-lysine with 11 N HCl at 80°

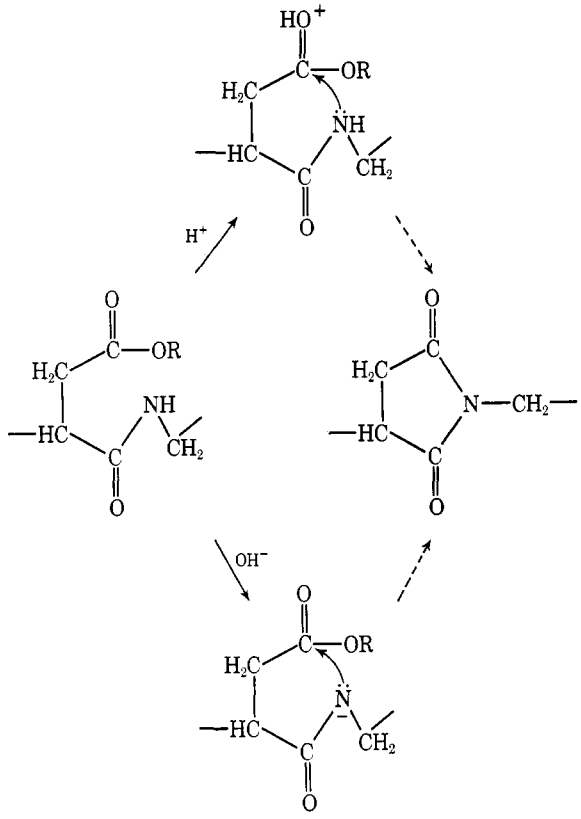

FIGURE 3: Mechanism of formation of aspartimidyl derivatives.

leads to the formation of succinimido derivatives. It is interesting to note that these compounds have some structural resemblance to aspartylglycine, in that the amino group involved in the peptide linkage is next to a primary carbon.

The absence of an alkyl side chain on the α-carbon of glycine facilitates the formation of diacyl intermediates (Wieland and Mohr, 1956; Kopple and Renick, 1958), not only for reasons of electron distribution but also for steric reasons. If an electronegative side chain were present, as in the case of the hydroxymethyl group of serine, the tendency toward the formation of cyclic intermediates would certainly be enhanced. Several investigators (Bernhard *et al.*, 1962; Shalitin and Bernhard, 1966; Fölsch, 1966; Schwyzer *et al.*, 1963) have demonstrated that this is indeed the case, and it has been argued (Bernhard *et al.*, 1962; Shalitin and Bernhard, 1966) that these cyclic intermediates might play a role in the mechanism of action of enzymes containing the sequence aspartylserine in their active site. Merrifield (1967; Marshall and Merrifield, 1965) observed the formation of neutral byproducts during removal of peptides containing the aspartylseryl sequence from a resin support using a 1-hr treatment with HBr–trifluoroacetic acid. The formation of this side product was essentially eliminated by reducing the duration of the cleavage treatment to 5 min. It is interesting to point out in this connection that even though the sequence aspartylserine occurs also in secretin, there is no indication of any rearrangement in this case. However, the hydroxyl group of serine was protected during the synthesis with a benzyl group and the steric hindrance exerted by this bulky substituent was probably the overriding factor in preventing the formation of cyclic intermediates. Similar observations (A. Deer, 1968, unpublished data) were made during the synthesis of the hexapeptide histidylserylaspartylthreonylglycylphenylalanine by the solid-phase procedure using *t*-butyloxycarbonyl-*O*-benzyl-L-threonine. The product cleaved from the resin with hydrogen bromide in trifluoroacetic acid showed no contamination with either the cyclic or the β-aspartyl form. Undoubtedly, the nature of the amino acid that follows the aspartyl residue in the peptide chain plays a very important role in the rate of formation of succinimido derivatives. This particular point is under investigation (M. Bodanszky, 1968, personal communication).

The possibility that rearrangements of the type described in this paper can take place with some aspartyl sequences is extremely important in planning the strategy and tactics to be followed in the synthesis of aspartyl peptides.

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An Examination of the Polymerization Behavior of *Jasus lalandii* Haemocyanin and Its Relation to the Allosteric Binding of Oxygen*

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ABSTRACT: *Jasus lalandii* haemocyanin in solution is shown by ultracentrifuge studies to undergo a pH-dependent polymerization. In acetate buffer of pH 5.5 and ionic strength of 0.1, the protein exists essentially as a form of mol wt 455,000, characterized by a weight-average sedimentation coefficient of 15 S; but as the pH is increased this unit slowly dissociates to a series of lower polymers in equilibrium. Plots of the amount of oxygen bound to holohaemocyanin *vs.* the partial pressure of unbound oxygen (binding curves) are sigmoidal, the extent of sigmoidality varying with pH in the range 5.5–8.9. It is shown that results obtained on the polymerization behavior of both holo- and apohaemocyanins are consistent with the postulate that polymerization of the protein is the basis of these observed allosteric binding effects. The regeneration of holohaemocyanin by the combination of apoprotein with added cuprous chloride is shown to be inhibited by silver ions. It, therefore, appears that these ions bind at the oxygen binding site. While cupric and magnesium ions do not inhibit the regeneration of haemocyanin, their addition to haemocyanin solutions at alkaline pH values results in a shift of the polymerization equilibria in favor of the formation of the 15S species. Inhibition studies performed with mixtures of these divalent metal ions and silver ions indicate that haemocyanin in the presence of divalent metal ions is capable of existing in various forms, all of the same size, which exhibit a differential capacity toward the binding of silver ions. These observations are employed to interpret oxygen binding curves obtained with the haemocyanin in the presence of cupric and magnesium ions in terms of the coexistence of various isomeric forms of the protein. Results obtained with *Jasus* serum further suggest that the allosteric binding of oxygen to haemocyanin, operating *in vivo*, is affected by the metal ion content of the serum.

Pantin and Hogben (1925) showed that oxygen binding curves obtained with *Palinurus* haemocyanin were sigmoidal and similar curves have been obtained with haemocyanins from other sources (Redfield, 1934; Wolvekamp, 1949; Redmond, 1955). Recently, considerable attention has been given to the physical basis of sigmoidal binding curves, because they manifest an allosteric effect important in metabolic control (Wyman, 1964; Atkinson, 1966; Stadtman, 1966; Changeux *et al.*, 1968; Gerhart and Schachman, 1968; Changeux and Rubin, 1968). It has been shown that multiple binding of ligand to equivalent sites on isomeric and/or polymeric forms of protein acceptor molecules leads to sigmoidal binding curves, provided the phenomena of binding and self-interaction of acceptor are competitive (Nichol *et al.*, 1967). Koshland *et al.* (1966) interpreted the sigmoi-

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