[CONTRIBUTION FROM THE CHEMOTHERAPY DIVISION, STAMFORD RESEARCH LABORATORIES, AMERICAN CYANAMID COMPANY]

Synthesis of Model, High Molecular Weight Peptides by the Mixed Carbonic–Carboxylic Acid Anhydride Procedure

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This method of synthesis has been extended to the preparation of two high molecular weight, optically active peptides, in each of which no amino acid unit is repeated. As model compounds, a neutral hexapeptide and a basic octapeptide are described.

In the course of another investigation currently in progress in this Laboratory, several model, high molecular weight, synthetic peptides were desired for a study of their chemical and physical properties and a comparison of these with those of naturally isolated peptides. Since stepwise degradation and partial hydrolysis studies were also contemplated, the most useful peptides were considered to be those in which all of the amino acids present in any one peptide were different. By an extension of the mixed carbonic–carboxylic acid anhydride procedure for the synthesis of optically-active peptides,[1,2] two such synthetic, optically-active peptides have now been prepared. These are the neutral hexapeptide, L-leucyl-L-alanyl-L-valyl-L-phenylalanylglycyl-L-proline (VI), and the basic octapeptide, L-tryptophyl-L-lysyl-L-leucyl-L-alanyl-L-valyl-L-phenylalanylglycyl-L-proline (VII).

First, a mixed anhydride between carbobenzoxy-L-leucyl-L-alanine and isobutylcarbonic acid was formed in tetrahydrofuran solution by the method previously reported.[1] This anhydride was then caused to react with ethyl L-valyl-L-phenylalanylglycinate, also in tetrahydrofuran, to give ethyl carbobenzoxy-L-leucyl-L-alanyl-L-valyl-L-phenylalanylglycinate (I) in good yield, as an amorphous solid from aqueous ethanol. When this product was heated in dioxane containing hydrochloric acid, the ester group was hydrolyzed to give the corresponding peptide acid derivative II. This material was converted to the mixed anhydride as before, but in dimethylformamide solution, and caused to react with benzyl L-prolinate to give benzyl carbobenzoxy-L-leucyl-L-alanyl-L-valyl-L-phenylalanylglycyl-L-prolinate (III) in 60% yield as colorless crystals from 50% acetic acid. The removal of the carbobenzoxy group from III was effected by the method of Anderson, *et al.*[3] A solution of the compound in *N* hydrogen bromide in glacial acetic acid was warmed on a steam-bath for 3–5 minutes or until carbon dioxide evolution ceased. Dilution with ether then precipitated the hydrobromide IV in high yield and of suitable purity for further use.

For reaction with IV, carbobenzoxy-L-tryptophyl-L-(ε-N-carbobenzoxy)-lysine was prepared from the mixed anhydride of carbobenzoxy-L-tryptophan in tetrahydrofuran solution and the sodium salt of L-(ε-N-carbobenzoxy)-lysine in aqueous solution. This dipeptide derivative was in turn converted to a mixed anhydride in tetrahydrofuran, in the presence of 2 equivalents of triethylamine, and caused to react with IV in aqueous solution, to give benzyl carbobenzoxy-L-tryptophyl-L-(ε-N-carbobenzoxy)-lysyl-L-leucyl-L-alanyl-L-valyl-L-phenylalanylglycyl-L-prolinate (V) in 31% yield after crystallization from 80% acetic acid. The scheme leading to the synthesis of III and V is[4]

Z·Leu-Ala·OH(2L) + H·Val-Phe-Gly·OEt(2L) ⟶ Z·Leu-Ala-Val-Phe-Gly·OEt(4L) (I)

I + aq. HCl ⟶ Z·Leu-Ala-Val-Phe-Gly·OH(4L) (II)

II + H·Pro·OBz(L) ⟶ Z·Leu-Ala-Val-Phe-Gly-Pro·OBz(5L) (III)

III + HBr·HOAc ⟶ H·Leu-Ala-Val-Phe-Gly-Pro·OBz·HBr(5L) (IV)

IV + Z·Try-(ε·Z)Lys·OH(2L) ⟶ Z·Try-(ε·Z)Lys-Leu-Ala-Val-Phe-Gly-Pro·OBz(7L) (V)

Hydrogenation of compounds III and V in glacial acetic acid using a palladium catalyst led to the neutral hexapeptide VI and the basic octapeptide VII, respectively. Both compounds were obtained as hydrates, VI as a trihydrate and VII as a hexahydrate monoacetate. Some difficulty was experienced in the isolation of VII due to the apparent instability of synthetic peptides containing tryptophan in the presence of traces of impurities. Using glacial acetic acid and a 10% palladium on charcoal catalyst, however, satisfactory yields of the peptide were obtained.

The purity of compounds VI and VII was determined mainly by elemental analysis. An attempted demonstration of their purity by chromatography on paper was unsatisfactory in the solvent systems tried. The aminoacid compositions of compounds I-VII, however, was satisfactorily shown by acid hydrolysis in 6 *N* hydrochloric acid followed by chromatography on paper using a *n*-butanol:water:acetic acid (5:4:1) solvent system. The expected, well-defined aminoacid spots were observed in all cases except in those of compounds V and VII. These compounds gave seven well-defined spots corresponding to all of the aminoacids present except tryptophan, which is destroyed under the conditions of the acid hydrolysis. No attempt to demonstrate the optical purity of the individual aminoacids was made.

(1) J. R. Vaughan, Jr., THIS JOURNAL, **74**, 6137 (1952).

(2) J. R. Vaughan, Jr., and J. A. Eichler, *ibid.*, **75**, 5556 (1953).

(3) G. W. Anderson, J. Blodinger and A. D. Welcher, *ibid.*, **74**, 5309 (1952).

(4) The abbreviations used are those proposed by E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946)(*cf.* B. F. Erlanger and E. Brand, THIS JOURNAL, **73**, 3508 (1951)).

Experimental[5]

Carbobenzoxy-L-leucyl-L-alanine.[6]—A solution of 13.3 g. (0.05 mole) of carbobenzoxy-L-leucine,[7] oil, $[\alpha]^{24}D$ −16.8 ± 0.5° (*c* 2.1, ethanol) and 5.1 g. (0.05 mole) of triethylamine in 100 cc. of tetrahydrofuran was cooled to −5° and 6.9 g. (0.05 mole) of isobutyl chlorocarbonate added with stirring. After 5 minutes at this temperature, a solution of 4.46 g. (0.05 mole) of L-alanine, $[\alpha]^{22}D$ +14.4 ± 0.2° (*c* 6.5, 1 *N* HCl) in 50 cc. (1 equivalent) of *N* sodium hydroxide was added with stirring. The mixture was thus allowed to warm to room temperature during 30 minutes, acidified with 55 cc. of *N* hydrochloric acid and concentrated under a stream of air. The partially crystalline residue was extracted into three 50-cc. portions of ethyl acetate; these were combined, treated with decolorizing charcoal, and diluted to cloudiness with petroleum ether. On standing, the product separated slowly as colorless needles; wt. 11.4 g. (68%), m.p. 144–152°. Recrystallization from ethyl acetate–petroleum ether gave 10.3 g. (62%) of colorless crystals, m.p. 150–155°, $[\alpha]^{23}D$ −23.8 ± 0.4° (*c* 2.6, ethanol). The literature[6] records a m.p. of 152–153° and $[\alpha]^{27}D$ −25° (ethanol) for this product; however, further crystallization of the above material did not improve either property.

Ethyl Carbobenzoxy-L-leucyl-L-alanyl-L-valyl-L-phenylalanylglycinate (I).—A solution of 1.95 g. (0.0058 mole) of carbobenzoxy-L-leucyl-L-alanine and 0.60 g. (0.0058 mole) of triethylamine in 50 cc. of tetrahydrofuran was cooled and treated with 0.80 g. (0.0058 mole) of isobutyl chlorocarbonate as above. After 5 minutes, a slurry of 2.50 g. (0.0058 mole) of ethyl L-valyl-L-phenylalanylglycinate hydrobromide[2] in 50 cc. of tetrahydrofuran containing 0.60 g. (0.0058 mole) of triethylamine was added and the mixture was stirred, heated rapidly to the point of reflux and then immediately cooled and diluted with 400 cc. of 2% sodium bicarbonate solution. The product separated on standing as an amorphous, gel-like solid; wt. 3.79 g. (98%), m.p. 190–192°. This was purified by first reprecipitating it from glacial acetic acid by dilution with water followed by solution of the material in 300 cc. of hot 80% ethanol. On cooling, the product again separated as an amorphous, gel-like solid; wt. 2.70 g. (70%). The material melts at 223–225°, resolidifies at about 230° and remelts at 241–242°, $[\alpha]^{24}D$ −43.8 ± 0.3° (*c* 2.2, glacial acetic acid).

Anal. Calcd. for $C_{35}H_{49}N_5O_8$: C, 63.0; H, 7.40; N, 10.5. Found: C, 62.8; H, 7.33; N, 10.6.

Carbobenzoxy-L-leucyl-L-alanyl-L-valyl-L-phenylalanylglycine (II).—A 6.7-g. (0.01 mole) sample of I was dissolved with warming in 500 cc. of dioxane and 50 cc. (5 equivalents) of *N* hydrochloric acid added. The solution was then heated on a steam-bath for 1.25 hours and finally concentrated under an air stream to a colorless, gelatinous solid. This residue was dissolved in 200 cc. of hot 95% ethanol and the solution filtered and diluted with 350 cc. of water. On cooling, the clear solution set to a white, waxy solid. This was broken up and filtered off under pressure to express most of the solvent. The colorless solid remaining was redissolved in 400 cc. of hot 90% ethanol and the solution cooled to again give a solid, waxy mass. This was pressed almost dry, as before, and finally dried under high vacuum to give 5.3 g. (82%) of product as a colorless, non-crystalline solid, m.p. 237–242° dec., $[\alpha]^{22}D$ −44.4 ± 1.0° (*c* 2.3, glacial acetic acid). A mixed m.p. with the ester starting material was depressed to 210°. The material analyzes for a hemihydrate.

Anal. Calcd. for $C_{33}H_{45}N_5O_8 \cdot {}^1/_2H_2O$: C, 61.1; H, 7.15; N, 10.8. Found: C, 61.1; H, 7.33; N, 10.8.

Benzyl Carbobenzoxy-L-leucyl-L-alanyl-L-valyl-L-phenylalanylglycyl-L-prolinate (III).—A solution of 1.30 g. (0.002 mole) of II and 0.40 g. (0.004 mole) of triethylamine in 25 cc. of dimethylformamide was cooled to −5° and 0.31 g. (10% excess) of isobutyl chlorocarbonate added with stirring. After 10 minutes at this temperature a second solution of 0.48 g. (0.002 mole) of benzyl L-prolinate hydrochloride,[8] m.p. 143–144°, $[\alpha]^{24}D$ −47.6 ± 0.2° (*c* 2, water), in 20 cc. of dimethylformamide was added and the mixture heated rapidly to about 70°, then immediately recooled. On addition of an excess of water, the product precipitated as a colorless solid. Two recrystallizations of this from 40-cc. portions of 50% acetic acid gave 1.00 g. (60%) of product as colorless, micro-crystalline prisms, m.p. 210–212°, $[\alpha]^{22}D$ −53.5 ± 0.3° (*c* 2.1, glacial acetic acid).

Anal. Calcd. for $C_{45}H_{58}N_6O_9$: C, 65.4; H, 7.07; N, 10.2. Found: C, 65.2; H, 7.07; N, 10.2.

Benzyl L-Leucyl-L-alanyl-L-valyl-L-phenylalanylglycyl-L-prolinate Hydrobromide Trihydrate (IV).—A 500-mg. (0.0006 mole) sample of III was placed in 2 cc. (50% excess) of *N* hydrogen bromide in glacial acetic acid and the mixture warmed for 3 minutes on a steam-bath. During this time, the sample dissolved and carbon dioxide was evolved. The hot solution was then diluted with 50 cc. of ether to precipitate the product as a colorless solid, which was filtered off, washed with ether and dried; wt. 450 mg. (91%). This was of suitable purity for further reaction. For analysis, a sample was dissolved in 95% ethanol, filtered and reprecipitated with ether to give 33% of material which analyzed for a trihydrate and on melting decomposed above 250°; $[\alpha]^{23}D$ −65.3 ± 1.5° (*c* 0.7, water).

Anal. Calcd. for $C_{37}H_{52}N_6O_7 \cdot HBr \cdot 3H_2O$: C, 53.7; H, 7.18; N, 10.2. Found: C, 54.1; H, 6.97; N, 10.2.

Carbobenzoxy-L-tryptophyl-L-(ε-N-carbobenzoxy)-lysine.—A solution of 5.1 g. (0.015 mole) of carbobenzoxy-L-tryptophan,[9] m.p. 123–125°, $[\alpha]^{23}D$ +3.2 ± 0.1° (*c* 3.3, glacial acetic acid), and 1.5 g. (0.015 mole) of triethylamine in 50 cc. of tetrahydrofuran was cooled to −5°, and 2.06 g. (0.015 mole) of isobutyl chlorocarbonate added with stirring. After 10 minutes at this temperature, a second solution of 4.2 g. (0.015 mole) of L-(ε-N-carbobenzoxy)-lysine,[10] m.p. about 240°, $[\alpha]^{22}D$ +14.6 ± 0.4° (*c* 1.5, water plus 2 equiv. of HCl), in 30 cc. of 0.5 *N* sodium hydroxide was added with stirring. A white precipitate separated immediately but redissolved after several minutes. After 30 minutes the solution was acidified with hydrochloric acid, diluted with 100 cc. of water, and the precipitated oil extracted into two 50-cc. portions of ethyl acetate. The combined extract was dried over sodium sulfate, filtered and diluted with petroleum ether to reprecipitate the product as a light brown oil. This was separated and placed under high vacuum to give a light tan solid; wt. 6.35 g. (71%). This material turned pink on overnight standing.

For purification, the crude product was dissolved in 25 cc. of chloroform and the solution diluted hot with petroleum ether until it just failed to turn cloudy. On cooling, a colorless product crystallized after several hours. This was filtered off and washed with 10 cc. of chloroform–petroleum ether (3:1) solution to give colorless material melting at 131.5–133°. Recrystallization from chloroform–petroleum ether as above gave 1.76 g. (20%) of product melting at 130.5–131.5° and having zero rotation in glacial acetic acid.

Anal. Calcd. for $C_{33}H_{36}N_4O_7$: C, 66.0; H, 6.04; N, 9.3. Found: C, 65.8; H, 5.91; N, 9.4.

Reworking of the crystallization mother liquors gave an additional 1.50 g. (17%) of product as colorless crystals also melting at 130.5–131.5°.

Benzyl Carbobenzoxy-L-tryptophyl-L-(ε-N-carbobenzoxy)-lysyl-L-leucyl-L-alanyl-L-valyl-L-phenylalanylglycyl-L-prolinate (V).—A solution of 1.20 g. (0.002 mole) of carbobenzoxy-L-tryptophyl-L-(ε-N-carbobenzoxy)-lysine and 0.41 g. (0.004 mole) of triethylamine in 20 cc. of tetrahydrofuran was cooled to −5° and 0.28 g. (0.002 mole) of isobutyl chlorocarbonate added with stirring. After 5 minutes at this temperature, a solution of 1.55 g. (0.002 mole) of IV in 100 cc. of water was added with stirring. The reaction mixture was allowed to warm to room temperature during 15 minutes. Saturated sodium bicarbonate solution (25 cc.) and 50 cc. of chloroform were then added and the mixture stirred rapidly for 30 minutes to cause separation of a colorless, solid product. This was separated, dissolved in glacial acetic acid, reprecipitated by addition of an excess of water and damp dried. The material was then dissolved in 35 cc. of hot glacial acetic acid and the solution was filtered and diluted with water until it just failed to turn cloudy. On cooling, the product separated slowly as colorless, micro-crystalline blades; wt. 0.78 g. (31%), m.p.

(5) All melting points were taken on a Fisher–Johns block and are corrected. The analyses reported were determined in these laboratories under the direction of Dr. J. A. Kuck.

(6) W. F. Polglase and E. L. Smith, THIS JOURNAL, **71**, 3081 (1949).

(7) M. Bergmann, L. Zervas and J. S. Fruton, *J. Biol. Chem.*, **115**, 593 (1936).

(8) R. E. Neuman and E. L. Smith, *ibid.*, **193**, 97 (1951).

(9) E. L. Smith, *ibid.*, **175**, 39 (1948), gives m.p. 126°.

(10) A. Neuberger and F. Sanger, *Biochem. J.*, **37**, 515 (1943).

246–248°. Recrystallization from a mixture of 10 cc. of dimethylformamide and 4 cc. of water gave 0.65 g. (84% recovery) of crystalline product also melting at 246–248°, $[\alpha]^{22}D$ −43.6 ± 0.3° (*c* 1.4, glacial acetic acid).

Anal. Calcd. for $C_{70}H_{88}N_{10}O_{13}$: C, 65.9; H, 6.80; N 11.0. Found: C, 65.5; H, 6.55; N, 11.4.

L-Leucyl-L-alanyl-L-valyl-L-phenylalanylglycyl-L-proline Trihydrate (VI).—To a solution of 165 mg. (0.0002 mole) of III in 15 cc. of glacial acetic acid was added approximately 100 mg. of palladium black catalyst[11] and the mixture was shaken under 50 lb./in.^{2} of hydrogen pressure at room temperature for 18 hours. The catalyst was then filtered off and washed with 5–10 cc. of distilled water. The clear, colorless filtrate was freeze-dried under high vacuum to give the product as a colorless, fluffy powder; wt. 119 mg. (91%), m.p. 228–233° dec. This was redissolved in 20 cc. of distilled water and the solution filtered to remove a small amount of insoluble material. The filtrate was then again freeze-dried under high vacuum to give 87 mg. (66%) of product as a colorless powder; m.p. 228–230° dec., $[\alpha]^{23}D$ −56.3 ± 0.5° (*c* 0.7, 3 *N* HCl).

Anal. Calcd. for $C_{36}H_{46}N_6O_7 \cdot 3H_2O$: C, 54.9; H, 7.98; N, 12.8. Found: C, 54.8; H, 7.89; N, 12.7.

Paper chromatography using a *n*-butyl alcohol:water:acetic acid (5:3:2) solvent system gave a single, fast running spot (R_f 0.89); however, basic solvent systems, such as 80% aqueous lutidine or *n*-butyl alcohol saturated with 3% ammonium hydroxide, were unsatisfactory in that two fast running spots invariably were obtained (lutidine, R_f 0.58 and 0.83; butanol, R_f 0.61 and 0.92), even after continued hydrogenation of the product to remove contaminating traces of the benzyl ester.

L-Tryptophyl-L-lysyl-L-leucyl-L-alanyl-L-valyl-L-phenylalanylglycyl-L-proline Monoacetate Hexahydrate (VII).—A 50-mg. (0.00004 mole) sample of V was dissolved in 10 cc. of glacial acetic acid and 50 mg. of 10% palladium-on-charcoal catalyst[12] added. The mixture was then hydrogenated and the product was isolated as in the above case. The peptide acetate was obtained as a colorless powder; wt. 33 mg. (77%), m.p. 240–245° dec. (dependent upon rate of heating), $[\alpha]^{24}D$ +15.3 ± 2.5° (*c* 0.8, water).

Anal. Calcd. for $C_{47}H_{68}N_{10}O_9 \cdot CH_3COOH \cdot 6H_2O$: C, 54.2; H, 7.80; N, 12.9. Found: C, 54.2; H, 7.99; N, 12.6.

Chromatography on paper gave a single spot using either *n*-butyl alcohol:water:acetic acid (5:3:2) or 80% lutidine as the solvent system. The spot obtained in the basic system was slower running (R_f 0.69) and more diffuse than that obtained in the acidic system (R_f 0.94). In the case of the *n*-butyl alcohol saturated with 3% ammonium hydroxide system, the spot obtained was too diffuse for characterization.

(11) R. Willstätter and E. Waldschmidt-Leitz, *Ber.*, **54**, 128 (1921).

(12) Baker and Co., Inc., Newark, N. J.

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[CONTRIBUTION FROM THE RESEARCH DIVISION, ARMOUR AND COMPANY]

The Degradation of Collagen. A Method for the Characterization of Native Collagen

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The dye–protein precipitation method for the estimation of polar functional groups on the protein has been applied to an investigation of a highly purified bovine hide collagen and several mildly degraded collagens. There is no difference in the maximum number of basic dye binding sites on each sample per unit weight, but the concentration dependence of the binding is clearly related to the extent of degradation. Less than half of the theoretically available anionic groups are available as dye binding sites in the undegraded material. These become available in stages as the extent of degradation is increased. The thermodynamic functions $\Delta F°$ and $\Delta S°$ have been evaluated for the combination of the undegraded protein with Orange-G and Safranin-O.

The detailed nature of the path by which the connective-tissue proteins, the collagens, are converted into soluble gelatin has been an open question for many years. We have undertaken a re-examination of this problem with particular emphasis on bovine hide collagen. One logical starting point for such an investigation is the characterization of a starting material which represents collagen in its most nearly native state physically and chemically. The present discussion is a description of one method of analysis which gives information on both aspects of such a characterization and which has already led to some interesting information on the initial steps of the degradation.

Cassel, McKenna and Glime[1] have shown that there are no significant differences in the amino acid analyses of collagen, gelatin or hide powder. In particular, no changes could be found in the composition of the polar amino acid residues other than the loss of some amide nitrogen in the gelatin. Titration curves of insoluble collagen have been used to follow changes in the number of functional groups per unit weight, but drastic treatments are required to demonstrate these changes.[2,3]

Furthermore, as Ames[3] has pointed out, one can barely differentiate between the total nitrogen content of untreated collagen and exhaustively acid treated gelatin and all changes detected can be ascribed to the loss of amide nitrogen. Thus, the methods which have been most commonly used to study the collagen–gelatin transformation lack the sensitivity which is apparently required.

Dye–protein precipitation reactions have been used[4] to determine the number of protein functional groups. Such reactions are sufficiently sensitive to detailed structural features of the globular proteins to allow one to detect minor changes which occur on denaturation.[5,6] These methods have been applied, in modified form, to the present problem.

Experimental Part

A. **Preparation of the Collagen.**—Hide collagen was prepared by a combination of the methods of Bowes and Kenton[2] and of Loofbourow, Gould and Sizer.[7] The hide of a freshly slaughtered steer was immediately chilled in ice-water. The hide was then cut into small strips. These were washed thoroughly with cold water, then extracted with cold 10% sodium chloride. The supernatant was dis-

(1) J. Cassel, E. McKenna and A. Glime, *J. Am. Leather Chem. Assoc.*, **48**, 277 (1953).

(2) J. H. Bowes and R. H. Kenton, *Biochem. J.*, **43**, 358, 365 (1948); **44**, 142 (1949).

(3) W. M. Ames, *J. Sci. Food Agric.*, **3**, 454, 579 (1952).

(4) H. Fraenkel-Conrat and M. Cooper, *J. Biol. Chem.*, **511**, 239 (1944).

(5) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 1597 (1949).

(6) J. R. Calvin, *Can. J. Chem.*, **30**, 320, 973 (1952).

(7) J. R. Loofbourow, B. S. Gould and I. W. Sizer, *Arch. Biochem.*, **22**, 406 (1949).