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The Synthesis of Regular Copolymers of Glycine and Alanine

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The synthesis of polypeptides with a repeating sequence of glycines and L-alanines, *i. e.*, poly-(L-Ala-Gly-Gly), poly-(L-Ala-L-Ala-Gly), and poly-(L-Ala-L-Ala-L-Ala-Gly), are reported. Polymerization was achieved by a self-condensation of *p*-nitrophenyl esters of the corresponding tri- or tetrapeptides.

In 1963, Gratzer and Doty[1] reported the remarkable stability of the α-helical conformation of poly-L-alanine. On the other hand, the simplest polyamino acid, poly-glycine, has the so-called β-conformation in the solid state, as is well known.[2] It seemed to us to be very much meaningful to study the conformation of a series of copolymers made from glycine and alanine, since the interactions between their side chains were the simplest to interpret theoretically. To analyze the problem unambiguously, the glycine-alanine copolymers must be sequential. (It has been shown by Nylund and Miller[3] that different monomer reactivity ratios are observed between the beginning and the close of the random copolymerization of glutamic acid- and leucine-*N*-carboxy anhydride). In this paper, we wish to report the synthesis of sequential polypeptides consisting of glycine and L-alanine.

Efforts to obtain sequential copolymers of glycine and alanine have been made by many workers, such as the attempt to get poly-(L-alanyl-glycyl-glycine) from methyl L-alanyl-glycyl-glycinate by Fischer[4] and those to get poly-(D,L-alanyl-glycyl-glycine) from the analogous tripeptide methylester or thiophenylester by Wilson and Pascu[5] and Schramm *et al.*[6] Other references are cited in the review by Katchalsky and Sela.[7] These polymers, however, had rather low molecular weights, and the reproducibility was poor. It was necessary to carry out self-condensation reactions at elevated temperatures because of the relatively low reactivity of peptide alkylesters.

In the past few years, several papers related to the synthesis of sequential polymers of other amino acids have appeared. The methods which have been reported may be classified into two categories: (a) the treatment of a free peptide in solution with a suitable condensing agent (dicyclohexylcarbodiimide,[8a-c] tetraethylpyrophosphite,[8c,9] pyrophosphoric acid,[10] and ethyl metaphosphate[11] have been used as condensing agents.) and (b) the self-condensation of peptide, one of whose terminals has been activated by an adequate substituent towards the attack of the other terminal of another molecule. Noguchi[12] developed the *N*-carbothiophenyl amino acid method and synthesized poly-(L-alanyl-glycine), which has also been synthesized recently by Australian workers.[13a] An active ester method has also been applied; the results are

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1) W. B. Gratzer and P. Doty, *J. Am. Chem. Soc.*, **85**, 1193 (1963); P. Doty and W. B. Gratzer, in "Polyamino Acids, Polypeptides, and Proteins," ed. by M. A. Stahmann, The University of Wisconsin Press, Madison (1962), p. 111.

2) For instance, A. Elliott, E. M. Bradbury, A. R. Downie and W. E. Hanby, in "Polyamino Acids, Polypeptides, and Proteins," p. 255.

3) R. E. Nylund and W. G. Miller, *J. Am. Chem. Soc.*, **87**, 3537 (1965).

4) E. Fischer, *Ber.*, **39**, 2893 (1906).

5) E. J. Wilson and E. Pascu, *J. Org. Chem.*, **7**, 126 (1942).

6) G. Schramm and G. Thumm, *Z. Naturforsch.*, **3b**, 218 (1948); G. Schramm and H. Restle, *Makromol. Chem.*, **13**, 103 (1954); I. Brunn-Leube and G. Schramm, *Chem. Ber.*, **89**, 2045 (1956).

7) E. Katchalsky and M. Sela, *Adv. Protein Chem.*, **13**, 449 (1958).

8) a) V. Bruckner, M. Szekerke and J. Kovacs, *Naturwissenschaften*, **43**, 107 (1956). b) T. Vajda, *Chem. Ind.*, **1963**, 785. c) H. Kitaoka, S. Sakakibara and H. Tani, This Bulletin, **31**, 802 (1958).

9) S. G. Waley, *J. Chem. Soc.*, **1955**, 517.

10) G. Schramm and H. Wissmann, *Chem. Ber.*, **91**, 1073 (1958).

11) G. Spach, *Angew. Chem.*, **76**, 381 (1964).

12) J. Noguchi and T. Hayakawa, *J. Am. Chem. Soc.*, **76**, 2846 (1954); J. Noguchi *et al.*, *Nippon Kagaku Zasshi* (*J. Chem. Soc. Japan, Pure Chem. Sect.*), **74**, 961 (1953); *ibid.*, **75**, 639 (1954).

13) a) R. D. B. Fraser, T. P. MacRae, F. H. C. Stewart and E. Suzuki, *J. Mol. Biol.*, **11**, 706 (1965). b) F. H. C. Stewart, *Australian J. Chem.*, **18**, 887 (1965).

satisfactory with peptide *p*-nitrophenyl esters[13b,14] or pentachlorophenyl esters.[15]

We selected the *p*-nitrophenyl ester method for the synthesis of sequential polymers of glycine and L-alanine. The principles of the synthesis of such regular polypeptides with high molecular weights are the following: (1) the number of amino acids which build up a polymerization unit must be three or more in order to exclude a primary intramolecular cyclization, since dipeptides may form six-membered ring compounds (diketopiperazine derivatives); (2) in order to avoid an intramolecular cyclization, a satisfactory concentrated solution of reactants must be used.

Results

Synthesis of Polymerizing Units. In order to avoid racemization yielding peptide bonds during the course of the condensation reaction, we always used the so-called azide method if L-alanine was present in a position where the reaction occurred. The azide method is one of the two methods which have been recommended for the coupling of amino acids without racemization[16] (the other one[17] is coupling through 1-hydroxy-piperidine esters). Figure 1 outlines the synthetic steps for the required derivatives of tri- and tetra-peptides.

L-Alanine was ethylated in ethanol saturated with hydrogen chloride, and then converted into the hydrazide of carbobenzoxyalanine according to the method described by Erlanger and Brand.[18] The azide coupling of II with ethyl glycyl-glycinate hydrochloride, which has been derived from the ethanolysis of diketopiperazine (IV) in the presence of hydrogen chloride,[19] gave the ethyl ester of carbobenzoxy-L-alanyl-glycyl-glycine (III). The compound III was also obtained from step-by-step synthesis through the compound VI[18,20] and compound VII. The compound III was hydrolyzed with a slight excess of sodium hydroxide in aqueous methanol; this afforded a free acid (VIII) which was then converted into *p*-nitrophenyl ester (IX) by the use of dicyclohexylcarbodiimide as the condensing agent. Recently, Kovacs[15] recommended the use of a pentachlorophenyl ester instead of a *p*-nitrophenyl ester as the active ester for polymerization, since a pentachlorophenyloxy group is probably a more electron-attracting group than a *p*-nitrophenyloxy group; these catalytic hydrogenation will be used in contrast to the case of a *p*-nitrophenyl ester. We also tried to synthesize a pentachlorophenyl ester of VIII from VIII and pentachlorophenol using dicyclohexylcarbodiimide, but the surprisingly low solubilities of the ester towards general solvents made it very difficult to purify the crude ester. The carbobenzoxy group of the compound IX was smoothly eliminated in the usual manner in acetic acid half-saturated with hydrogen bromide, and the resulting *p*-nitrophenyl-L-alanyl-glycyl-glycinate was recrystallized enough times to obtain a high-molecular-weight polymer.

p-Nitrophenyl L-alanyl-L-alanyl-glycinate hydrobromide and *p*-nitrophenyl (L-alanyl)$_3$-glycinate hydrobromide were synthesized in the same manner. Of the derivatives appearing in Fig. 1, mention should be made of the remarkable crystallizability of the compound XVI. It gave large needles very easily in the presence of a small amount of methanol, even if we started from crude, half-crystalline XV. Elementary analysis confirmed the presence of one mole of methanol as a methanol of crystallization.

Initially we tried to synthesize a *p*-nitrophenyl ester of carbobenzoxy-L-alanyl-(glycyl)$_3$ through a free acid, XXVIII, but the ethyl ester, XXII, showed a striking resistance towards hydrolysis under usual conditions, in great contrast with the other cases.[21] For instance, when treated with 1.3 molar sodium hydroxide in methanol, analogous ethyl esters, III, XIII, and XVIII, were hydrolyzed completely within 20 min. On the contrary, 90% or more of XXII was present even after 48 hr under the same conditions (as confirmed by an infrared spectral analysis). Also, the isobutyl ester, XXVII, was not hydrolyzed. In these cases, the prolonged treatment of the XXII or XXVII esters with alkali or treatment under more drastic conditions resulted in the simultaneous elimination of the carbobenzoxy group. The effort to obtain a *t*-butyl ester of XXVIII from VIII and *t*-butyl glycinate[22] failed; the oily crude material resisted further purification. A *p*-toluenesulfonic acid-catalyzed ester-interchange reaction between XXII or XXVII and a large excess of *p*-nitrophenol in

14) a) D. F. DeTar, W. Honsberg, U. Honsberg, A. Wieland, M. Gouge, H. Bach, A. Tahara, W. S. Brinigar and F. F. Rogers, Jr., *J. Am. Chem. Soc.*, **85**, 2873 (1963). b) S. M. Bloom, S. K. Dasgupta, R. P. Patel and E. R. Blout, *ibid.*, **88**, 2035 (1966). c) R. D. B. Fraser, B. S. Harrap, T. P. MacRae, F. H. C. Stewart and E. Suzuki, *J. Mol. Biol.*, **14**, 423 (1965). d) R. D. B. Fraser, T. P. MacRae and F. H. C. Stewart, *ibid.*, **19**, 580 (1966).

15) J. Kovacs, R. Giannotti and A. Kapoor, *J. Am, Chem. Soc.*, **88**, 2282 (1966).

16) E. Schröder and K. Lübke, "The Peptides," Vol. I, Academic Press, N. Y. (1965), p. 325.

17) S. M. Beaumont, B. D. Handford, J. H. Jones and G. T. Young, *Chem. Commun. (London)*, **1965**, 53.

18) B. F. Erlanger and E. Brand, *J. Am. Chem. Soc.*, **73**, 3508 (1951).

19) E. Fischer and E. Forneau, *Chem. Ber.*, **34**, 2868 (1901) (J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley, N. Y. (1961), p. 803).

20) M. Bergmann, L. Zervas, J. S. Fruton, F. Schneider and H. Schleich, *J. Biol. Chem.*, **109**, 325(1935).

21) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley, N. Y. (1961), p. 1158.

22) E. Taschner, A. Chimiak, B. Bator and T. Sokotowska, *Ann.*, **646**, 134 (1961).

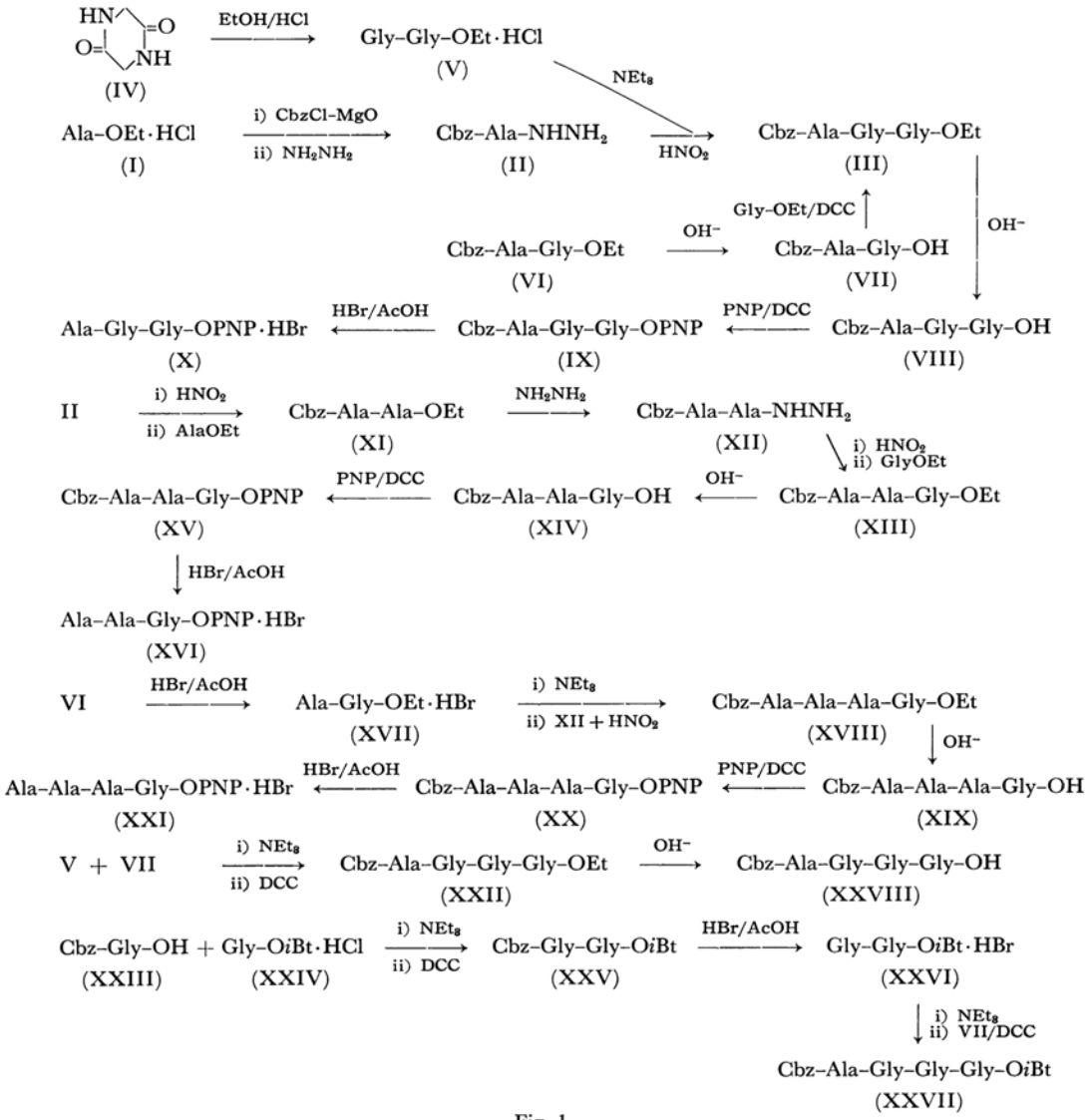

Fig. 1.

tetrahydrofuran also resulted in the accompanying destruction of the carbobenzoxy group.

Polymerization. Polymerization was achieved successfully in dimethyl sulfoxide as the solvent. 30—40% solutions of X, XVI and XXI were reacted with equimolar amounts of triethylamine. The yields of the polymers were in the range of 30—40% of the theory.

Molecular-weight Determinations. Since the polymers dissolved only in dichloroacetic acid or in trifluoroacetic acid, we met very great difficulty in determining the molecular weights of the polymers. Measurements using the ultracentrifuge were impossible because such solvents would corrode the cells.

If we assume the Doty's viscosity-molecular-weight relationships[23] which were obtained in the case of poly-benzyl-L-glutamate in dichloroacetic acid, to be valid in our case also, we can estimate the order of the molecular weights of our polymers by measuring the intrinsic viscosity in dichloroacetic acid. Such measurements give the values of 30000, 35000, and 20000 as the molecular weights for poly-Ala-Gly-Gly, poly-Ala-Ala-Gly, and poly-Ala-Ala-Ala-Gly respectively. There must be, of course, some slight modifications of the coefficient appearing in Doty's viscosity-molecular weights relationship, and the relationship remains to be established for other peptides, but the results obtained above will

23) P. Doty, J. H. Bradbury and A. M. Holtzer, *J. Am. Chem. Soc.*, **78**, 947 (1956).

be not so incredible as a first-order approximation if we remember that most polypeptides exist in the form of a random-coil conformation in dichloroacetic acid (except in the case of poly-L-leucine).[24] For instance, Kovacs calculated the molecular weight of his poly-Glu-Ala-Glu to be 25000 by the sedimentation velocity method using the ultracentrifuge. On the other hand, his sample showed an intrinsic viscosity of 0.3 dl/g in dichloroacetic acid, a value which corresponds to MW 40000 if we apply Doty's relationship. DeTar[14a] also showed that the values obtained by this relationship for the various polymers are at least roughly comparable with those obtained by the Archibald technique or by end-group assays using the DNP method.

Problem of Racemization. It is essential to know whether a racemization reaction has occurred or not. We measured the optical rotatory dispersion curves for the acid hydrolysates of polymers and compared them with that of L-alanine treated under the same hydrolytic conditions. The results showed that the racemization was less than 5%, if it occurred at all.

Experimental

The recorded melting points are determined on a microhotstage and are uncorrected. The infrared spectra were taken with a JASCO DS-402G grating-type spectrometer or with a JASCO IR-S spectrometer with a NaCl prism mounted. The measurements were made with KBr discs. The optical rotatory dispersion measurements were made with a JASCO ORD/UV 5 recording spectropolarimeter. The azide coupling reactions were carried out in a cold room (at 2—3°C). Viscosity measurements were made at 22°C with an Ostwald type viscometer whose flow time for solvent dichloroacetic acid was about 8 min. Elementary micro analysis was carried out at the Faculty of Agriculture in this university, and the mean values of two or three times analysis are reported. The amino acids analysis was carried out with a Beckman-Spinco amino acid autoanalyzer, Model 120, using a 90 cm column. Commercially available dimethyl sulfoxide was distilled under reduced pressure, 90—92°C fraction at 28 mm/Hg was used. L-Alanine was kindly gifted from Ajinomoto Co. Inc., by the courtesy of Professor Y. Hirata.

Ethyl Carbobenzoxy-L-alanyl-glycyl-glycinate (III). *a) Azide Method.* Five grams of carbobenzoxy-L-alanine hydrazide[18] was dissolved in the mixture of 10 m*l* of 5N hydrochloric acid and 100 m*l* of water and cooled to −10°C. A cold solution of sodium nitrite (1.65 g) was added to it, then the oily azide precipitated was extracted with diethyl ether. After washing the extracts with water and 3% sodium bicarbonate and drying over sodium sulfate for a short time, the cold solution of ethyl glycyl-glycinate, which was prepared from 4.2 g of ethyl glycyl-glycinate hydrochloride (V) and 2.75 g of triethylamine in 100 m*l* of diethyl ether and applied filtration of precipitated amine hydrochloride, was added to it. The reaction mixture was stood for overnight at 2—3°C, then washed with 0.5 N hydrochloric acid and 3% sodium bicarbonate solution. The solvent was removed *in vacuo* to leave white crystalline III, which was recrystallized from ethanol. Yield, 4.2 g mp 134—136°C.

Found: C, 56.06; H, 6.11; N, 11.63%. Calcd for $C_{17}H_{23}O_6N_3$: C, 55.88; H, 6.35; N, 11.50%.

IR: 1743 (ester), 1699 (carbobenzoxy) cm^{-1}.

The compound V was prepared by the restricted ethanolysis of diketopiperazine (IV) in the presence of hydrogen chloride.[19]

b) Dicyclohexylcarbodiimide Method. Ethyl carbobenzoxy-L-ananyl-glycinate[18,20] (30 g) was hydrolyzed in 20% aqueous methanol with 130 m*l* (1.2 eq.) of 0.95 N sodium hydroxide. 23 g of the free acid (VII) was obtained as white massive crystals, and recrystallized from methanol-water, mp 129—134°C. 3.8 g of the free acid VII was dissolved in the mixture of 1.9 g of ethyl glycinate hydrochloride and 1.75 g of triethylamine in 40 m*l* of dimethylformamide. 2.8 g of dicyclohexylcarbodiimide in 5 m*l* of dimethylformamide was then added dropwise under stirring with external cooling. The solution was stirred for 1 day at room temperature. The dicyclohexylurea was filtered off and the solvent was removed at 1 mm/Hg. The resultant solid was washed with water and recrystallized from ethanol to give the tripeptide ester III, mp 133—135°C, which was undepressed on admixture with a sample prepared by method a).

Carbobenzoxy-L-alanyl-glycyl-glycine (VIII). Ethyl carbobenzoxy-L-alanyl-glycly-gylcinate III, 10 g, was hydrolyzed by 37.4 m*l* of 0.95 N sodium hydroxide in 100 m*l* of 90% aqueous methanol. The solution was stood for 20 min and filtered. Acidification of the filtrate with 5 N hydrochloric acid to pH 1 and concentration under reduced pressure gave a solid of free acid (VIII), which was recrystallized from water. An analytical sample showed mp 163°C.

Found: C, 53.43; H, 5.61; N, 12.54%. Calcd for $C_{15}H_{19}O_6N_3$: C, 53.40; H, 5.68; N, 12.46%.

1725 (COOH), 1680 (sh. carbobenzoxy) cm^{-1}.

***p*-Nitrophenyl Carbobenzoxy-L-alanyl-glycyl-glycinate (IX).** To a cooled mixture of carbobenzoxy-L-alanyl-glycyl-glycine (VIII, 5 g), *p*-nitrophenol (2.2 g), and dimethylformamide (90 m*l*), 3.7 g of dicyclohexylcarbodiimide in 10 m*l* of dimethylformamide was added dropwise with magnetically stirring. The stirring was continued at 0°C overnight. The dicyclohexylurea was filtered off and the solvent was removed at 1 mm/Hg. On addition of diethyl ether (50 m*l*) and storage in the refrigerator, *p*-nitrophenyl ester (IX) crystallized, 4 g, mp 147—150°C. The compound was crystallized from ethanol, mp 149—152°C.

Found: C, 55.03; H, 4.81; N, 12.26%. Calcd for $C_{21}H_{22}O_8N_4$: C, 55.02; H, 4.84; N, 12.22%.

1770 (ester), 1700(carbobenzoxy), 1495 and 1355-(NO_2)cm^{-1}.

***p*-Nitrophenyl L-Alanyl-glycyl-glycine Hydrobromide (X).** The compound (IX), 3 g, was dissolved at room temperature in 5 m*l* of acetic acid which was half saturated with hydrogen bromide. After 30 min excess hydrogen bromide was replaced by a nitrogen stream, then the solvent was removed under reduced

24) G. D. Fasman, in "Polyamino Acids, Polypeptides, and Proteins," ed. by M. A. Stahmann, the University of Wisconsin Press, Madison (1962), p. 221.

pressure at 45°C. The resultant oil was washed thoroughly with dry diethyl ether, and dissolved in isopropyl alcohol. On adding dry ether and storage in the refrigerator, the desired ester hydrobromide (X) appeared as fine crystals, 1.5 g, mp 180°C dec., slightly hygroscopic. Further purification of X was carried out with isopropyl alcohol and diethyl ether. The compound X was stored in a dark place, since the compound colored in a brighter place.

Found: C, 38.32; H, 4.40; N, 14.06%. Calcd for $C_{13}H_{16}O_6N_4$: C, 38.53; H, 4.23; N, 13.83%.

1778 (ester), 1361 (NO_2) cm^{-1}.

Ethyl Carbobenzoxy-L-alanyl-L-alanyl-glycinate (XIII). Ethyl carbobenzoxy-L-alanyl-L-alaninate (XI) was derived from carbobenzoxy-L-alanine hydrazide (II) and ethyl L-alaninate hydrochloride (I) by an azide method, and recrystallized from ethyl acetate–*n*-hexane, mp 115—117°C (lit.[18,25] mp 116°C). The compound (XI) was reacted with hydrazine in ethanol to afford carbobenzoxy-L-alanyl-L-alanine hydrazide (XII), which was recrystallized from ethanol, mp 211—213°C (lit.[18] mp 209°C). 10 g of XII was dissolved in the mixture of 5 N hydrochloric acid (15.5 m*l*) and water (300 m*l*), and cooled to −10°C. A solution of 2.7 g of sodium nitrite in 10 m*l* of water was then added to it, and the resultant precipitate was extracted with chloroform (150 m*l* × 2). The combined extracts were washed with water and 3% sodium bicarbonate solution and reacted with ethyl glycinate which was prepared from 7 g of ethyl glycinate hydrochloride and 6.7 g of triethylamine in 100 m*l* of chloroform. After standing overnight at 2—3°C, the reaction mixture was washed thoroughly with 0.5 N hydrochloric acid and 3% sodium bicarbonate solution. The solvent was removed under reduced pressure to give a crystalline tripeptide ethyl ester (XIII), 9.2 g, mp 170°C. Recrystallization twice from ethanol raised the mp to 177—179°C.

Found: C, 57.03; H, 6.41; N, 11.13%. Calcd for $C_{18}H_{25}O_6N_3$: C, 56.98; H, 6.64; N, 11.08%.

IR: 1742 (ester), 1683 (carbobenzoxy) cm^{-1}.

Carbobenzoxy-L-alanyl-L-alanyl-glycine (XIV). Ethyl ester XIII was hydrolyzed in aqueous methanol with 1.3 equivalent of sodium hydroxide as described in the preparation of VIII. It was recrystallized from water or dimethylformamide-chloroform, mp 205—206°C. Yield was about 85%.

Found: C, 54.64; H, 6.05; N, 11.91%. Calcd for $C_{16}H_{21}O_6N_3$: C, 54.69; H, 6.02; N, 11.96%.

IR: 1730 (broad, COOH), 1670 (carbobenzoxy) cm^{-1}.

***p*-Nitrophenyl Carbobenzoxy-L-alanyl-L-alanyl-glycinate (XV).** Five grams of dicyclohexylcarbodiimide in 10 m*l* of chloroform was added dropwise to the mixture of 5 g of the compound XIV, 2.26 g of *p*-nitrophenol, and 90 m*l* of dimethylformamide with magnetically stirring at 0°C. The reaction mixture was stirred overnight at room temperature. A half milliliters of acetic acid was added to it to destroy an excess carbodiimide and stirring was continued for another 2 hr. Dicyclohexylurea was filtered off and the solvent dimethylformamide was removed at 1 mm/Hg until a few milliliters of dimethylformamide remained. On adding 20 m*l* of chloroform and standing for a few hours, the solution separated further amounts of dicyclohexylurea. This step was repeated several times to eliminate cyclohexylurea as possible. The syrup remained was dissolved in a few milliliters of chloroform and kept in the refrigerator to give crystalline XV. Yield, 6.0 g (90% of theory). Crude material was recrystallized from methanol, mp 192—193°C.

Found: C, 55.54; H, 5.15; N, 11.94%. Calcd for $C_{22}H_{24}O_8N_4$: C, 55.93; H, 5.12; N, 11.86%.

IR: 1760 (ester), 1685 (carbobenzoxy), 1345 (NO_2) cm^{-1}.

***p*-Nitrophenyl L-Alanyl-L-alanyl-glycinate Hydrobromide (XVI).** The compound XV was dissolved in 6 m*l* of acetic acid saturated with hydrogen bromide. After the reaction had proceeded for 2 hr, excess hydrogen bromide and acetic acid were removed under reduced pressure at 40°C. The gummy residue was washed thoroughly with dry diethyl ether to be free from the traces of acid. On addition of small amount of methanol, tripeptide ester hydrobromide XVI crystallized out soon, 1.3 g, mp 172—184°C with decomposition. For the polymerization, the material was recrystallized from methanol three times, mp 180—185°C with decomposition.

Found: C, 39.79; H, 4.71; N, 11.91%. Calcd for $C_{14}H_{18}O_6N_4 \cdot HBr \cdot CH_3OH$: C, 39.92; H, 5.14; N, 12.42%.

IR: 1764 (ester), 1492 and 1355 (NO_2) cm^{-1}.

Ethyl Carbobenzoxy-L-alanyl-L-alanyl-L-alanyl-glycinate (XVIII). Ten grams of carbobenzoxy-L-alanyl-L-alanine hydrazide XII was reacted with ethyl L-alanyl-glycinate in the same condition as mentioned in the synthesis of the compound XIII. Ethyl L-alanyl-glycinate was prepared from triethylamine (5.1 g) and XVII (13 g), which in turn was obtained by the deacylation of ethyl carbobenzoxy-L-alanyl-glycinate, VI, in acetic acid saturated with hydrogen bromide. After standing for 24 hr at room temperature, the reaction mixture separated the crystalline XVIII. Filtration and recrystallization from ethanol-water gave a pure product, 9.5 g, mp 235—237°C.

Found: C, 56.03; H, 6.88; N, 12.38%. Calcd for $C_{21}H_{30}O_7N_4$: C, 55.99; H, 6.71; N, 12.44%.

IR: 1748 (ester), 1695 (carbobenzoxy) cm^{-1}.

Carbobenzoxy-L-alanyl-L-alanyl-L-alanyl-glycine (XIX). Tripeptide ethyl ester (XVIII, 7 g) was hydrolyzed with 31 m*l* of 1 N sodium hydroxide in 40% aqueous methanol. On acidification with 7 m*l* of 5 N hydrochloric acid, free acid (XIX) crystallized out as fine needles, 2.6 g, mp 225°C. It was crystallized from ethanol-water, the sample for analysis had mp 232—234°C. The same pure sample was obtained by reprecipitation (after dissolving the material in 1 N sodium hydroxide, the required amount of 5 N hydrochloric acid was added).

Found: C, 54.01; H, 6.31; N, 13.29%. Calcd for $C_{19}H_{26}O_7N_4$: C, 54.02; H, 6.20; N, 13.26%.

IR: 1720 (COOH), 1697 (carbobenzoxy) cm^{-1}.

***p*-Nitrophenyl Carbobenzoxy-L-alanyl-L-alanyl-L-alanyl-glycinate (XX).** 2.1 g of the tetrapeptide free acid XIX was reacted with *p*-nitrophenol (0.85 g) and dicyclohexylcarbodiimide (1.25 g) in 30 m*l* of dimethylformamide. The *p*-nitrophenyl ester XX was crystallized from dioxane-water or ethanol-water. The material from dioxane-water showed slightly higher mp (240°C with decomposition), than that from ethanol-water (mp 236—237°C with decomposition).

25) W. H. Stein, S. Moore and M. Bergmann, *J. Biol. Chem.*, **154**, 191 (1944).

Found: C, 55.46; H, 5.00; N, 12.62%. Calcd for $C_{25}H_{29}O_9N_5$: C, 55.24; H, 5.38; N, 12.89%.

IR: 1672(ester), 1705(sh. carbobenzoxy), 1353(NO_2) cm^{-1}.

p-Nitrophenyl L-Alanyl-L-alanyl-L-alanyl-glycine Hydrobromide (XXI). The compound XX was deacylated in hydrogen bromide-saturated acetic acid as described in the case of XVI. The resulting oily product was crystallized and recrystallized from isopropyl alcohol and ethyl ether in about 70% yield, mp 153°C (decomposition). This material was highly hygroscopic and labile to light.

Found: C, 41.78; H, 5.91; N, 14.16%. Calcd for $C_{17}H_{23}O_7N_5 \cdot HBr$: C, 41.64; H, 4.93; N, 14.28%.

IR: 1680 (ester), 1360 (NO_2) cm^{-1}.

Ethyl Carbobenzoxy-L-alanyl-glycyl-glycyl-glycinate (XXII). Twenty-two grams of carbobenzoxy-L-alanyl-glycine was dissolved in the mixture of ethyl glycyl-glycinate hydrochloride (19 g), triethylamine (9.8 g), and dimethylformamide (200 m*l*). To this turbid solution, 18 g of dicyclohexylcarbodiimide in dimethylformamide (50 m*l*) was added portionwise under cooling. After stirring for 24 hr at room temperature, the product ester was obtained by the usual manner, and crystallized from ethanol, 21.5 g, mp 168—169°C. Analytical sample which was recrystallized twice from ethanol showed mp 173—175°C.

Found: C, 54.21; H, 6.13; N, 13.29%. Calcd for $C_{19}H_{26}O_7N_4$: C, 54.02; H, 6.20; N, 13.26.

IR: 1740 (ester) 1696 (carbobenzoxy) cm^{-1}.

Attempted Hydrolysis of Tetrapeptide Ester XXII. a) The peptide ester XXII was treated with 1.3 equivalent molar of sodium hydroxide in 50% aqueous methanol for 48 hr at room temperature. On acidifying and concentrating the reaction mixture, the resultant products contained 90% or more of the starting ester XXII (from an infrared spectra). Tetrahydrofuran, in place of methanol, was also used as solvent, but showed no significant effects.

b) The peptide ester XXII was boiled for 15 min under the same medium described as above. The infrared spectra of the products thus obtained showed a significant relative decrease of the peak 1695 cm^{-1} (carbonyl group of benzyloxycarbonyl moiety). Furthermore, the products was oily and resisted towards further attempts to purify them. The same destruction of benzyloxycarbonyl group was observed in the trial for ester interchange reaction. In that case, the peptide ester XXII was refluxed with large amount of *p*-nitrophenol in tetrahydrofuran in the presence of *p*-toluenesulfonic acid.

Isobutyl Glycyl-glycinate Hydrobromide (XXVI). Isobutyl glycinate hydrochloride (XXIV) was prepared in the usual manner from isobutyl alcohol, glycine, and hydrogen chloride. XXIV was coupled with carbobenzoxy-glycine using dicyclohexylcarbodiimide to afford isobutyl carbobenzoxy-glycyl-glycinate (XXV) in 91% yield.

Thirty five grams of XXV was dissolved in 50 m*l* of acetic acid saturated with hydrogen bromide. After 30 min, the separated crystalline hydrobromide XXVI was filtered. Filtrate gave further crop of XXVI on concentration under reduced pressure. The combined material was recrystallized from isobutyl alcohol, 24.2 g (83% of theory), mp 184—188°C.

Found: C, 35.67; H, 6.19; N, 9.99%. Calcd for $C_8H_{16}O_3N_2 \cdot HBr$: C, 35.69; H, 6.36; N, 10.41%.

IR: 1743(ester) cm^{-1}.

Isobutyl Carbobenzoxy-L-alanyl-glycyl-glycyl-glycinate (XXVII). To the cold suspension of carbobenzoxy-L-alanyl-glycine (VII, 10 g), isobutyl glycyl-glycinate hydrobromide (XXVI, 11.5 g) and triethylamine (4.33 g) in dimethylformamide (100 m*l*), 8.1 g of dicyclohexylcarbodiimide in 30 m*l* of dimethylformamide was added dropwise at 0°C with magnetically stirring. Stirring was continued for 24 hr at room temperature. Following filtration through Hyflo "Super-Cel", solvent was evaporated under reduced pressure at 45°C. Scratching and trituration with water induced crystallization. The product (XXVII) was filtered off and recrystallized from ethyl acetate or aqueous tetrahydrofuran. There were slight differences in the fingerprint region of infrared spectra between the product crystallized from ethyl acetate and that from tetrahydrofuran and water; the former had higher mp (141—145°C), the latter lower mp (122—128°C). Elementary analysis was taken with the product from ethyl acetate.

Found: N, 12.01%. Calcd for $C_{21}H_{30}O_7N_4$: N, 12.44%.

IR: 1719 (ester), 1699 (carbobenzoxy) cm^{-1}.

The isobutyl ester, XXVII, was not hydrolyzed under the condition shown above.

Polymerization of X, XVI and XXI. Each compound was dissolved in dimethyl sulfoxide to give a 30—40% solution. With vigorous stirring, an equimolar amount of triethylamine was added to it in a portion. Stirring was continued, but after about 30 min, the reaction mixture solidified. After 1 day, the completely solidified mixutre was crushed and washed with dimethyl sulfoxide (5 times, applying a centrifugation) and methanol. The resultant pale yellow powder was dissolved in a few milliliters of dichloroacetic acid, filtered with a glass-filter. Polymer was reprecipitated from this solution with methanol or chloroform. Amino acid analysis: (a) Poly-(L-Ala-Gly_2), Ala : Gly=0.99 : 2; (b) Poly-(L-Ala_2-Gly), Ala : Gly=2.01 : 1; (c) Poly-(L-Ala_3-Gly), Ala : Gly=2.98 : 1.

Elementary analysis: (a) Poly-(L-Ala-Gly_2). Found: C, 45.49; H, 5.79; N, 22.67%. Calcd for $(C_7H_{11}O_3N_3)_n$: C, 45.40; H, 5.99; N, 22.69%. (b) Poly-(L-Ala_2-Gly). Found: C, 47.21; H, 6.27; N, 19.98%. Calcd for $(C_8H_{13}O_3N_3)_n$: C, 48.23; H, 6.58; N, 21.10%. (b) Poly-(L-Ala_3-Gly). Found: C, 47.32; H, 6.80; N, 19.52%. Calcd for $(C_{11}H_{18}O_4N_4)_n$: C, 48.88; H, 6.71; N, 20.73%.

Viscosities in dichloroacetic acid: (a) Poly-(L-Ala-Gly_2); $\eta_{sp}/c=0.235$ ($c=1\%$), $[\eta]=0.22$. (b) Poly-(L-Ala_2-Gly); $\eta_{sp}/c=0.294$ ($c=1\%$), $[\eta]=0.26$. (c) Poly-(L-Ala_3-Gly); $\eta_{sp}/c=0.170$ ($c=1\%$), $[\eta]=0.15$.

Optical Purity. 0.5% solutions of polymers in 6 N hydrochloric acid were heated in refluxing xylene for 24 hr. Hydrolysates were evaporated to dryness in a vacuum desiccator in which potassium hydroxide was placed, and redissolved in 0.1 N hydrochloric acid to make 1% solutions. L-Alanine itself was also treated under the same condition. Optical rotatory dispersion curve was measured on each sample; the value of molecular rotation in the range of 240 mμ to 600 mμ were identical in each case within 5% error. These values are: 518° at 250 mμ, 135° at 300 mμ, 62° at 350 mμ, and 27° at 400 mμ.

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