702. *Polypeptides. Part IV.* The Self-condensation of the Esters of Some Peptides of Glycine and Proline.*

By H. N. Rydon and P. W. G. Smith.

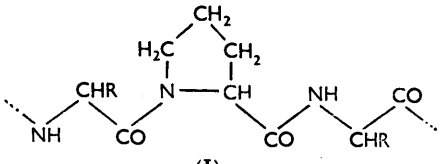
The ethyl esters of the two dipeptides, the three tripeptides, and two tetrapeptides containing glycine and one proline residue have been synthesised. On self-condensation the dipeptide esters give the anhydride (IV) as the major product, this being formed much more readily from the ester of glycylproline than from that of prolylglycine; this difference is ascribed to the more favourable conformation of the side-chain and the greater degree of fixation of the atoms in the former peptide ester. The self-condensation of the tripeptide esters gives complex mixtures in which both the anhydride (IV) and piperazine-2 : 5-dione are present. L-Prolyldiglycylglycine ethyl ester is resistant to self-condensation.

The introduction into a peptide chain of a residue of proline, or hydroxyproline, has a profound effect on its geometry owing to the fixation of the two atoms of the pyrrolidine ring and the two atoms adjacent to it in the peptide chain; thus, for example, a proline-containing polypeptide (I) cannot adopt the fully extended conformation (II) available to a proline-free peptide. The effect of introducing such residues into a peptide chain has been discussed in general terms by Edsall,[1] who points out that they not only influence the geometry of the molecule, interfering with the attainment of helical (and likewise, necessarily, other) conformations and serving even to bend the chain back on itself, but also with the potentialities of the molecule for hydrogen-bonding; these considerations are of particular importance for the conformation of the molecules of the proline-rich fibrous proteins of the

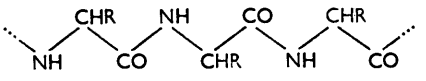
* Part III, Heaton, Rydon, and Schofield, *J.*, 1956, 3157.

[1] Edsall, *J. Polymer Sci.*, 1954, **12**, 253.

collagen–gelatin group.[2] In dipeptides the presence of proline residues results [3,4] in remarkably ready cyclisation to anhydrides (piperazine-2 : 5-diones) under conditions which leave other dipeptides quite unaffected. It may also be of significance that such naturally occurring cyclic oligopeptides as gramicidin-S and the tyrocidines [5] and phalloidine [6] contain residues of proline or hydroxyproline.



(I)

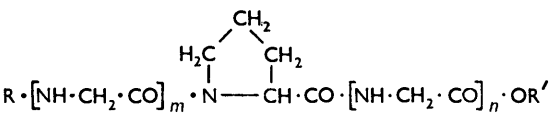


(II)

In this paper we describe the extension of our earlier study [7] of the self-condensation of the esters of glycine peptides to the esters of a representative series of peptides containing also proline, *viz.*, glycylproline, prolylglycine, diglycylproline, glycylprolylglycine, prolylglycylglycine and prolyldiglycylglycine.

Bergmann and his colleagues prepared glycyl-L-proline [8] and glycyl-DL-proline [9] by coupling benzyloxycarbonylglycyl chloride with proline; in our hands this procedure was unsatisfactory, as also was its obvious modification employing proline ethyl ester. Neither the thiothiazolidone method of Cook and Levy,[10] which was so successful in the preparation of polyglycine esters,[7] nor the mixed anhydride procedure of Boissonnas [11,12] was satisfactory when applied to proline or its ethyl ester. Finally, the method of Wieland, Schäfer, and Bokelmann [13] was adopted and proved very satisfactory; coupling of *N*-benzyloxycarbonylglycine thiophenyl ester with L- and DL-proline afforded the benzyloxycarbonyl-peptides (III; $m = 1$, $n = 0$, R = Ph·CH_2·O·CO, R′ = H) in good yield; hydrogenolysis, followed by esterification,[14] then afforded the required L- and DL-glycylproline ethyl esters (III; $m = 1$, $n = 0$, R = H, R′ = Et), isolated as their hydrochlorides.

By contrast, the Boissonnas procedure [11,12] proved entirely satisfactory in the isomeric series, condensation of the mixed anhydrides from ethyl chloroformate and L- and DL-benzyloxycarbonylproline with glycine ethyl ester yielding benzyloxycarbonyl-dipeptide esters (III; $m = 0$, $n = 1$, R = Ph·CH_2·O·CO, R′ = Et) in excellent yield. Direct hydrogenation of these derivatives gave rather impure products and it was found best to hydrolyse them first to the corresponding acids which were then hydrogenolysed to the free peptides



(III)

(III; $m = 0$, $n = 1$, R = R′ = H), these finally being re-esterified to the required L- and DL-prolylglycine ethyl esters (III; $m = 0$, $n = 1$, R = H, R′ = Et), isolated as their hydrochlorides.

[2] See, *e.g.*, Astbury, *J. Int. Soc. Leather Chem.*, 1940, **24**, 69; Pauling and Corey, *Proc. Nat. Acad. Sci. U.S.A.*, 1951, **37**, 272; Ramachandran and Kartha, *Nature*, 1954, **174**, 269; 1955, **176**, 593; Ramachandran, *ibid.*, 1956, **177**, 710; Rich and Crick, *ibid.*, 1955, **176**, 915; Cowan, McGavin, and North, *ibid.*, p. 1062.
[3] Abderhalden and Nienburg, *Fermentforsch.*, 1933, **13**, 573.
[4] E. L. Smith and Bergmann, *J. Biol. Chem.*, 1944, **153**, 627.
[5] Reviewed by Synge, *Quart. Rev.*, 1949, **3**, 259.
[6] Lynen and Wieland, *Annalen*, 1938, **533**, 93; Wieland and Witkop, *ibid.*, 1940, **543**, 171.
[7] Rydon and P. W. G. Smith, *J.*, 1955, 2542.
[8] Bergmann, Zervas, Schleich, and Leinert, *Z. physiol. Chem.*, 1934, **212**, 72.
[9] Bergmann and Fruton, *J. Biol. Chem.*, 1937, **117**, 189.
[10] Cook and Levy, *J.*, 1950, 646.
[11] Boissonnas, *Helv. Chim. Acta*, 1951, **34**, 874.
[12] Vaughan, *J. Amer. Chem. Soc.*, 1951, **73**, 3547.
[13] Wieland, Schäfer, and Bokelmann, *Annalen*, 1951, **573**, 99.
[14] Synge, *Biochem. J.*, 1948, **42**, 99.

In the tripeptide series, coupling of the mixed anhydride from benzyloxycarbonylglycine and ethyl chloroformate with L- and DL-prolylglycine ethyl esters yielded the benzyloxycarbonyl esters (III; $m = n = 1$, R = Ph·CH_2·O·CO, R′ = Et) which were then hydrogenolysed to the required L- and DL-glycylprolylglycine ethyl esters (III; $m = n = 1$, R = H, R′ = Et), isolated as their hydrochlorides. L-Prolylglycylglycine ethyl ester (III; $m = 0$, $n = 2$, R = H, R′ = Et) was synthesised similarly from benzyloxycarbonyl-L-proline and glycylglycine ethyl ester. A different method was used for the synthesis of diglycylproline ethyl ester. Condensation of benzyloxycarbonylglycylglycine thiophenyl ester with L-proline gave an excellent yield of the benzyloxycarbonyl-tripeptide (III; $m = 2$, $n = 0$, R = Ph·CH_2·O·CO, R′ = H); hydrogenolysis, followed by esterification, afforded the required diglycyl-L-proline ethyl ester (III; $m = 2$, $n = 0$, R = H, R′ = Et).

The Boissonnas mixed-anhydride procedure [11,12] was used for the synthesis of the tetrapeptide esters. Coupling of the mixed anhydride from benzyloxycarbonylglycylglycine and ethyl chloroformate with L- and DL-glycylproline ethyl esters afforded the benzyloxycarbonyl-tetrapeptide esters (III; $m = 3$, $n = 0$, R = Ph·CH_2·O·CO, R′ = Et), hydrogenolysis of which gave the required L- and DL-triglycylproline ethyl esters (III; $m = 3$, $n = 0$, R = H, R′ = Et), isolated as their hydrobromides. L-Prolyldiglycylglycine ethyl ester (III; $m = 0$, $n = 3$, R = H, R′ = Et) was prepared similarly from benzyloxycarbonyl-L-proline and diglycylglycine ethyl ester. In view of the poor yield obtained in the Boissonnas synthesis of triglycyl-L-proline ethyl ester the thiophenyl ester route [13] to this compound was also explored; triglycyl-L-proline (III; $m = 3$, $n = 0$, R = R′ = H) was obtained in very satisfactory yield by condensing benzyloxycarbonylglycylglycine thiophenyl ester with glycyl-L-proline and hydrogenolysing the product, but unexpectedly proved very resistant to esterification.

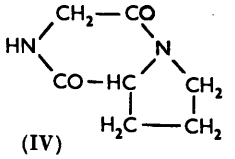
The principal product of the self-condensation of both glycylproline and prolylglycine ethyl esters was glycylproline anhydride (3 : 6-dioxo-1 : 2-pyrrolidinopiperazine) (IV), irrespective of whether the reaction was brought about by heating without solvent or by the action of ethanolic triethylamine. There was, however, a remarkable difference in the ease of cyclisation of the two esters, that from glycylproline undergoing this reaction very much faster than its isomeride, cyclisation being complete in a few hours at room temperature; as was to be expected there was no detectable difference between the self-condensation of the L- and the DL-esters to form the anhydride (IV) which contains only one asymmetric carbon atom. It is also noteworthy that, whereas the anhydride (IV) is the sole product of the self-condensation of glycylproline ethyl ester, it is accompanied in the case of prolylglycine ethyl ester by other products which, although not closely investigated, are almost certainly open-chain condensation polymers.

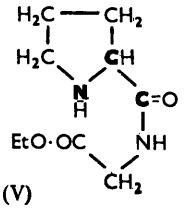
The greater ease of cyclisation of glycylproline ethyl ester as compared with prolylglycine ethyl ester is surprising, since the probable basic strengths of the amino- and imino-groups involved (pK_a between 7·6 and 8·4, and about 9·7, respectively [15]) are such that the latter ester might have been expected to be the more readily cyclised. Clearly this is a case in which geometrical and stereochemical factors take control; two such can be recognised. First, in prolylglycine ethyl ester the peptide linkage will presumably adopt the generally preferred *trans*-conformation [16] and energy will be required to convert this into the *cis*-conformation (V) necessary for cyclisation; no such reluctance to take up the necessary conformation (VI) is to be expected in the case of glycylproline ethyl ester. Secondly, in glycylproline ethyl ester four of the six atoms involved in the final piperazinedione ring are rigidly fixed by virtue of their occurrence in, or direct attachment to, the pyrrolidine ring, whereas only three atoms are so fixed in prolylglycine ethyl ester [atoms fixed in this way are shown in (V) and (VI) in heavy type]; this will result in a considerably greater probability of the molecule's taking up the conformation required for cyclisation in the case of glycylproline ethyl ester.

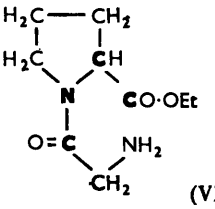
Very complex mixtures were obtained when the three tripeptide esters were heated alone or in ethanol, and only the cyclic products formed were isolated and identified. All

[15] Cohn and Edsall, "Proteins, Amino-acids and Peptides as Ions and Dipolar Ions," Reinhold, New York, 1943, Chapter 4.
[16] Corey and Pauling, *Proc. Roy. Soc.*, 1953, *B*, **141**, 10.

three esters gave glycylproline anhydride (IV), the yield of this product being higher from the esters of diglycylproline and glycylprolylglycine than from that of prolylglycylglycine. This is a further example of the similar degradation to piperazine-2 : 5-dione observed with polyglycine esters,[7] although the formation of the anhydride (IV) from diglycylproline ethyl ester shows that the reaction is not restricted to the amino-end of the molecule.


(IV)


(V)


(VI)

Piperazine-2 : 5-dione was also present in the products from diglycylproline and glycylprolylglycine ethyl esters; in the former case this product, too, can arise by the same degradative reaction but in the latter case it must be formed by dimerisation of glycine ethyl ester, produced as the other product in the cyclisation to the anhydride (IV). The lower yield of the anhydride (IV) obtained from prolylglycylglycine ethyl ester, as compared with its isomerides, is ascribed to the operation of the stereochemical factors considered to govern the cyclisation of the dipeptide esters.

The tetrapeptide ester, prolyldiglycylglycine ethyl ester (III; $m = 0$, $n = 3$, R′ = H, R′ = Et), resembled triglycylglycine ethyl ester [7] in being very resistant to self-condensation, both under the influence of heat and in ethanolic triethylamine.

Experimental

Preparations

M. p.s (uncorrected) were determined in a bath preheated to 15° below the m. p. The purity of all products was checked by paper chromatography.

DL-Proline was synthesised by Albertson and Fillman's method;[17] L-proline was isolated (13% yield) from gelatin by the rhodanilate method,[18] without prior removal of arginine.

Derivatives of Glycyl-DL-proline.—*N*-Benzyloxycarbonylglycine thiophenyl ester [13] (9·5 g.), in tetrahydrofuran (50 ml.), was treated with a solution of DL-proline hydrochloride (4·6 g.) in 2N-sodium hydroxide (31 ml.). After addition of methanol until homogeneous, the mixture was heated under reflux at 60—65° for 4 hr. The volatile solvents were then removed under reduced pressure and the residue, diluted with a little water, was extracted with ether to remove thiophenol. Acidification (1 : 1 hydrochloric acid; Congo-red) precipitated an oil (7·7 g., 80%) which rapidly crystallised when seeded; recrystallisation from ethyl acetate yielded *N*-benzyloxycarbonylglycyl-DL-proline as plates, m. p. 123° (Found: C, 59·3; H, 6·0. Calc. for $C_{15}H_{18}O_5N_2$: C, 58·8; H, 5·9%) (lit.,[9] m. p. 129—130°). Very much poorer yields (22%, 18%, and 7%, respectively) were obtained by the procedures of Bergmann and Fruton,[9] Boissonnas,[11] and Vaughan.[12]

This benzyloxycarbonyldipeptide (6 g.) was kept overnight in methanol (100 ml.) over Raney nickel (1 g.). After addition of water (2 ml.) and acetic acid (2 ml.) to the filtered solution the mixture was shaken in a current of hydrogen in the presence of palladium black [19] (0·35 g.). Evolution of carbon dioxide ceased after 3 hr., then the catalyst was removed by filtration; careful addition of ether then precipitated glycyl-DL-proline (3 g., 89%) as a slightly hygroscopic microcrystalline powder, m. p. 186° (decomp.) (Found: C, 48·4; H, 7·2; N, 16·7. Calc. for $C_7H_{12}O_3N_2$: C, 48·8; H, 7·0; N, 16·3%) (Bergmann and Fruton [9] do not record a m. p.).

The dipeptide (1·7 g.) was kept overnight in anhydrous ethanolic N-hydrogen chloride (11 ml.). The product was evaporated to dryness under reduced pressure and the whole procedure repeated twice. *Glycyl-DL-proline ethyl ester hydrochloride* (2·25 g., 96%), recrystallised from ethanol–ether, had m. p. 137° (decomp.) (Found: C, 46·25; H, 7·4. $C_9H_{17}O_3N_2Cl$ requires C, 45·7; H, 7·2%).

[17] Albertson and Fillman, *J. Amer. Chem. Soc.*, 1949, **71**, 2818.
[18] Bergmann, *J. Biol. Chem.*, 1955, **110**, 473.
[19] Willstätter and Waldschmidt-Leitz, *Ber.*, 1921, **54**. 123.

Derivatives of Glycyl-L-proline.—*N*-Benzyloxycarbonylglycyl-L-proline, prepared in 68% yield by condensing *N*-benzyloxycarbonylglycine thiophenyl ester with L-proline as described for the DL-compound, had m. p. 155° (lit.,[8] m. p. 156°); the procedures of Bergamnn *et al.*[8] and Boissonnas [11] gave inferior yields (37% and 20%, respectively). Hydrogenolysis gave glycyl-L-proline, m. p. 184° (lit.,[8] m. p. 185°), in 85% yield; esterification afforded the ethyl ester hydrochloride as a brittle gum which resisted all attempts at crystallisation.

Derivatives of DL-*Prolylglycine.*—*N*-Benzyloxycarbonyl-DL-proline [3] (9·0 g.), dissolved in dry chloroform (25 ml.) containing dry redistilled triethylamine (5 ml.), was cooled to 0° while redistilled ethyl chloroformate (3·5 ml.) was added rapidly with shaking. After 15 min. at 0°, a cold mixture of glycine ethyl ester hydrochloride (5·1 g.), triethylamine (5 ml.), and chloroform (25 ml.) was added, with stirring, to the semi-solid mass. The mixture was kept at room temperature for 30 min. and then at 50° for 10 min., and then washed successively with water (25 ml.), N-hydrochloric acid (15 ml.), 0·5N-sodium hydrogen carbonate (2 × 15 ml.), and water (15 ml.). Evaporation of the dried solution under reduced pressure afforded a gum (11·3 g., 94%), which crystallised slowly but completely (m. p. 54—56°); *N-benzyloxycarbonyl-*DL*-prolylglycine ethyl ester*, crystallised from ether–light petroleum (b. p. 40—60°), had m. p. 59—60° (Found: C, 61·1; H, 6·9. $C_{17}H_{22}O_5N_2$ requires C, 61·1; H, 6·6%).

This ester (8·2 g.) was kept at room temperature for an hour with N-sodium hydroxide (26 ml.) and acetone (10 ml.). Concentration, acidification, extraction with ethyl acetate, and evaporation of the dried extract gave an oil (6 g., 80%) which solidified (m. p. 123—124°) on being rubbed with ether; *N-benzyloxycarbonyl-*DL*-prolylglycine* crystallised from ethyl acetate in needles, m. p. 125° (Found: C, 58·75; H, 5·7. $C_{15}H_{18}O_5N_2$ requires C, 58·8; H, 5·9%). Hydrogenation of this derivative (3 g.), over palladium black (0·3 g.) in 50% aqueous methanol (100 ml.) containing acetic acid (1·5 ml.), yielded DL-prolylglycine (1·45 g., 86%), m. p. 223—224° (decomp.) (from aqueous methanol) (lit.,[3] m. p. 225—227°). Esterification as usual with ethanolic hydrogen chloride gave an 85% yield of *DL-prolylglycine ethyl ester hydrochloride*, hygroscopic needles, m. p. 95—96° (from ethanol–ether containing a little hydrogen chloride) (Found: C, 45·3; H, 7·3. $C_9H_{17}O_3N_2Cl$ requires C, 45·7; H, 7·2%).

Derivatives of L-*Prolylglycine.*—*N*-Benzyloxycarbonyl-L-proline, prepared in 90% yield by the procedure described by Abderhalden and Nienburg [3] for the DL-compound and recrystallised from ether–light petroleum (b. p. 40—60°), had m. p. 75° (Found: C, 62·6; H, 6·05. Calc. for $C_{13}H_{15}O_4N$: C, 62·7; H, 6·05%) (lit.,[20] m. p. 76—77°) and was converted, by the procedures described for the DL-compounds, into (i) *N*-benzyloxycarbonyl-L-prolylglycine ethyl ester (98% yield), an uncrystallisable oil, (ii) *N-benzyloxycarbonyl-*L*-prolylglycine* (65% yield), needles, m. p. 125° (from water) (Found: C, 59·2; H, 5·9. $C_{15}H_{18}O_5N_2$ requires C, 58·8; H, 5·9%) (described as a syrup by Abderhalden and Nienburg [3]), (iii) L-prolylglycine monohydrate (84% yield), m. p. 236° (decomp.), $[\alpha]_D^{17}$ −19·8° (*c* 4·0 in H_2O) (lit.,[3] m. p. 236°, $[\alpha]_D^{20}$ −22·8°), and (iv) *L-prolylglycine ethyl ester hydrochloride* (87% yield), hygroscopic needles, m. p. 119—120° (from ethanol–ether), $[\alpha]_D^{22}$ −39·5° (*c* 2·4 in H_2O) (Found: C, 45·8; H, 7·5. $C_9H_{17}O_3N_2Cl$ requires C, 45·7; H, 7·2%).

*Derivatives of Diglycyl-*L*-proline.*—*N*-Benzyloxycarbonylglycylglycine [21] (2·66 g.), in dry tetrahydrofuran (10 ml.) containing *N*-ethylpiperidine (1·4 ml.), was treated at 0° with ethyl chloroformate (1 ml.). After 15 min. at 0°, thiophenol (1 ml.) was added to the semisolid mass, which was then kept at room temperature for 4 hr. Base hydrochloride was then filtered off and washed with a little tetrahydrofuran. The solid product, obtained by evaporation of the filtrate and washings to dryness under reduced pressure, was recrystallised from benzene (100 ml.); *N-benzyloxycarbonylglycylglycine thiophenyl ester* (2·5 g., 70%) had m. p. 117° (Found: C, 60·7; H, 5·3. $C_{18}H_{18}O_4N_2S$ requires C, 60·3; H, 5·05%).

This ester (3·6 g.), in tetrahydrofuran (40 ml.), was treated with a solution of L-proline (1·15 g.) in N-sodium hydroxide (10 ml.) and heated under reflux to 65° for 4 hr. after homogenisation with methanol. Concentration under reduced pressure, removal of thiophenol by extraction with ether, and acidification precipitated an oil (3 g., 82%) which crystallised on being seeded; *N-benzyloxycarbonylglycylglycyl-*L*-proline* crystallised from ethyl acetate in short rods, m. p. 137° (Found: C, 56·4; H, 5·9. $C_{17}H_{21}O_6N_3$ requires C, 56·2; H, 5·8%). This compound (3·8 g.), in methanol (50 ml.), was kept overnight with Raney nickel (0·6 g.). Acetic acid (1·5 ml.) was added to the filtered solution which was then shaken in a current of hydrogen over palladium black [19] (0·17 g.); hydrogenolysis was complete in 2 hr. A little water was added to redissolve some product which had crystallised and the mixture was then filtered and

[20] Berger, Kurtz, and Katchalski, *J. Amer. Chem. Soc.*, 1954, **76**, 5552.
[21] Bergmann and Zervas, *Ber.*, 1932, **65**, 1192.

concentrated under reduced pressure to a syrup. Next morning methanol (80 ml.) was added and the first crop of product collected by filtration; re-concentration of the filtrate and further treatment with methanol gave a second crop. Diglycyl-L-proline (2·3 g., 96%) crystallised from aqueous ethanol as the *monohydrate*, large plates, m. p. 205—206° (decomp.), $[\alpha]_D^{22}$ −92·9° (*c* 2·1 in H_2O) (Found: C, 43·7; H, 7·1; N, 16·8. $C_9H_{15}O_4N_3,H_2O$ requires C, 43·8; H, 6·9; N, 17·0%) (E. L. Smith and Bergmann [4] record $[\alpha]_D^{26}$ −101·5° for a product analysing for a hemihydrate but do not give a m. p.). Esterification in the usual manner gave *diglycyl-L-proline ethyl ester hydrochloride* (87% yield), m. p. 112—114° (from ethanol–ether) (Found: C, 43·8; H, 6·6; N, 13·7. $C_{11}H_{20}O_4N_3Cl,0·5H_2O$ requires C, 43·7; H, 6·95; N, 13·9%).

Derivatives of Glycyl-L-prolylglycine.—*N*-Benzyloxycarbonylglycine [21] (2·09 g.), in dry chloroform (10 ml.) containing triethylamine (1·35 ml.), was treated at 0° with ethyl chloroformate (1 ml.). After 10 min. a solution of L-prolylglycine ethyl ester hydrochloride (2·36 g.) in chloroform (10 ml.) containing triethylamine (1·35 ml.) was added. After 2 hr. at room temperature, the product was worked up in the usual manner, yielding *N*-benzyloxycarbonylglycyl-L-prolylglycine ethyl ester as an uncrystallisable oil (3·8 g., 90%); saponification of a portion gave *N*-benzyloxycarbonylglycyl-L-prolylglycine, m. p. 143—144° (from ethyl acetate) (Found: C, 55·9; H, 5·6. Calc. for $C_{17}H_{21}O_6N_3$: C, 56·2; H, 5·8%) (Simmonds and Fruton [22] give m. p. 136—137°; Davies and E. L. Smith [23] m. p. 144—145°).

The oily benzyloxycarbonyl-ester (3·37 g.) in ethanol (60 ml.) containing 10N-hydrochloric acid (1·5 ml.), was hydrogenolysed over palladium black [19] (0·2 g.). After an hour, water (20 ml.) was added to redissolve the precipitate and hydrogenolysis continued for a further hour. After filtration, the solution was evaporated to dryness under reduced pressure; the residue was taken up in ethanol and again evaporated, this process being then twice repeated. *Glycyl-L-prolylglycine ethyl ester hydrochloride* (2·25 g., 89%), recrystallised from ethanol, had m. p. 214° (decomp.), $[\alpha]_D^{25}$ −104·0° (*c* 1·52 in H_2O) (Found: C, 45·3; H, 6·9; N, 14·4. $C_{11}H_{20}O_4N_3Cl$ requires C, 45·0; H, 6·8; N, 14·3%).

Derivatives of Glycyl-DL-prolylglycine.—*N*-Benzyloxycarbonylglycyl-DL-prolylglycine ethyl ester was prepared as a gum (75—80% yield) by the procedure described for the L-compound and was converted by saponification into N-*benzyloxycarbonylglycyl-DL-prolylglycine* (70% yield), m. p. 133·5° (from ethyl acetate) (Found: C, 55·85; H, 6·15. $C_{17}H_{21}O_6N_3$ requires C, 56·2; H, 5·8%), hydrogenolysis of which gave *glycyl-DL-prolylglycine* (85% yield), which crystallised from aqueous methanol as the dihydrate, m. p. 245° (decomp.; previous sintering) (Found: C, 40·85; H, 7·15; N, 16·25. $C_9H_{15}O_4N_3,2H_2O$ requires C, 40·75; H, 7·2; N, 15·85%) (Davies and E. L. Smith [23] describe a sesquihydrate).

Hydrogenolysis of the gummy benzyloxycarbonyl-ester, as described for the L-compound, afforded *glycyl-DL-prolylglycine ethyl ester hydrochloride* (91% yield), m. p. 213° (decomp.) (from ethanol) (Found: C, 45·2; H, 6·6. $C_{11}H_2O_4N_3Cl$ requires C, 45·0; H, 6·8%).

Derivatives of L-Prolylglycylglycine.—*N*-Benzyloxycarbonyl-L-proline (8·6 g.) was treated as usual with ethyl chloroformate (3·3 ml.) in chloroform (25 ml.) containing triethylamine (4·7 ml.). Condensation of the resulting mixed anhydride with glycylglycine ethyl ester hydrochloride (6·8 g.) in chloroform (25 ml.) containing triethylamine (4·7 ml.) afforded N-*benzyloxycarbonyl-L-prolylglycylglycine ethyl ester* (11·8 g., 87%), which crystallised from chloroform–ether or water in needles, m. p. 120° (Found: C, 58·4; H, 6·5; N, 10·8. $C_{19}H_{25}O_5N_3$ requires C, 58·3; H, 6·4; N, 10·7%). This compound (1·95 g.) was hydrogenolysed over palladium black [19] (0·3 g.) in ethanol (75 ml.) containing 10N-hydrochloric acid (1 ml.). The product was dried by repeated dissolution in ethanol and evaporation under reduced pressure and finally precipitated from ethanol with anhydrous ether as a gum which solidified to a very hygroscopic solid when rubbed with ether. This ester hydrochloride (1·4 g.), in chloroform (10 ml.), was treated with 2N-ammonia in chloroform (3·5 ml.); filtration and evaporation of the filtrate under reduced pressure gave *L-prolylglycylglycine ethyl ester* (1·1 g., 86%), m. p. 109° after repeated precipitation from chloroform with light petroleum (b. p. 60—80°) (Found: C, 50·1; H, 7·4; N, 16·2. $C_{11}H_{19}O_4N_3$ requires C, 51·3; H, 7·4; N, 16·3%).

The benzyloxycarbonyl-tripeptide ester (2 g.), in ethanol (40 ml.), was treated with hydrazine hydrate (100%; 0·5 ml.). Next day, the solution was evaporated to dryness under reduced pressure and the residue treated with light petroleum (b. p. 60—80°). The gelatinous product (1·9 g., 96%) was recrystallised from chloroform-ether; N-*benzyloxycarbonyl-L-prolylglycylglycine hydrazide* so prepared had m. p. 117—118° (indefinite; opaque melt) (Found: C, 52·6, 52·65; H, 6·2, 6·15; N, 18·6. $C_{17}H_{23}O_5N_5,0·5H_2O$ requires C, 52·85; H, 6·25; N, 18·15%). This

[22] Simmonds and Fruton, *J. Biol. Chem.*, 1948, **174**, 705.
[23] Davies and E. L. Smith, *ibid.*, 1953, **200**, 373.

hydrazide (3·8 g.), in ethanol (80 ml.) containing water (10 ml.) and 10N-hydrochloric acid (2 ml.), was hydrogenated for 5 hr. over palladium black (0·4 g.). The hydrazide hydrochloride, isolated by evaporation of the filtered solution, was converted into the free hydrazide by passage of a 10% aqueous solution through a column of De-acidite FF and elution with water; two crystallisations from ethanol afforded *L-prolylglycylglycine hydrazide*, m. p. 184—187° after decomp. at 135—140° (Found : N, 28·25. $C_9H_{17}O_3N_5$ requires N, 28·8%).

Derivatives of Triglycyl-L-proline.—*N*-Benzyloxycarbonylglycylglycine thiophenyl ester (3·6 g.), in tetrahydrofuran (40 ml.), was treated with glycyl-L-proline (1·7 g.) in N-sodium hydroxide (10 ml.); methanol was added until the mixture was homogeneous, then it was heated under reflux at 60—65° for 4 hr. Volatile solvents were removed under reduced pressure; the residue was extracted with ether, acidified, and finally evaporated to dryness under reduced pressure. Extraction with acetone afforded *N*-benzyloxycarbonylglycyldiglycyl-L-proline (4·2 g., 100%) as an uncrystallisable gum. Hydrogenolysis by the usual procedure afforded triglycyl-L-proline which crystallised from aqueous methanol as the *hemihydrate* (1·6 g., 54%), hexagonal plates, m. p. 246° (decomp.), $[\alpha]_D^{24}$ −78·5° (*c* 1·2 in H_2O) (Found : C, 45·05; H, 6·5; N, 19·1. $C_{11}H_{18}O_5N_4,0{\cdot}5H_2O$ requires C, 44·75; H, 6·45; N, 19·0%).

N-Benzyloxycarbonyldiglycylglycine [24] (3·2 g.), suspended in chloroform (100 ml.) containing triethylamine (1·35 ml.), was treated at 0° with ethyl chloroformate (1 ml.). The resulting solution was treated with L-proline ethyl ester hydrochloride (1·8 g.) in chloroform (20 ml.) containing triethylamine (1·35 ml.). Next day unchanged benzyloxycarbonyldiglycylglycine (1·3 g.) was filtered off and the filtrate worked up in the usual manner, giving *N*-benzyloxycarbonyltriglycyl-L-proline ethyl ester (2·8 g., 62%) as a gum. Hydrogenolysis in a N-solution of hydrogen bromide in acetic acid (20 ml.), followed by filtration and addition of ether, precipitated *triglycyl-L-proline ethyl ester hydrobromide* (0·4 g., 16%) which, after crystallisation from slightly aqueous ethanol and two recrystallisations from aqueous ethanol–ether, had m. p. 179—181° (decomp.), $[\alpha]_D^{25}$ −60·0° (*c* 2·2 in H_2O) (Found : C, 37·4; H, 5·8. $C_{13}H_{23}O_5N_4Br,H_2O$ requires C, 37·8; H, 6·1%). The same compound was obtained in 14% overall yield by a similar Boissonnas coupling of *N*-benzyloxycarbonylglycylglycine and glycyl-L-proline ethyl ester, followed by hydrogenolysis.

Derivatives of Triglycyl-DL-proline.—*N*-Benzyloxycarbonylglycylglycine, coupled with glycyl-DL-proline ethyl ester hydrochloride by the usual Boissonnas procedure, afforded *N*-benzyloxycarbonyltriglycyl-DL-proline ethyl ester as an uncrystallisable gum (82% yield); hydrogenolysis in ethanol containing hydrobromic acid afforded *triglycyl-DL-proline ethyl ester hydrobromide* (43% yield), m. p. 241° (decomp.) after recrystallisation from aqueous ethanol–ether (Found : C, 39·4; H, 5·9; Br, 20·3. $C_{13}H_{23}O_5N_4Br$ requires C, 39·5; H, 5·8; Br, 20·25%).

Derivatives of L-Prolyldiglycylglycine.—*N*-Benzyloxycarbonyl-L-proline (5 g.), in chloroform (15 ml.) containing triethylamine (2·7 ml.), was treated at 0° with ethyl chloroformate (2 ml.). After 10 min., diglycylglycine ethyl ester hydrochloride (5·0 g.), suspended in chloroform (60 ml.) containing triethylamine (2·7 ml.), was added with shaking and the mixture kept at room temperature for 30 min. and then at 50° for 10 min. Working up as usual afforded *N-benzyloxycarbonyl-L-prolyldiglycylglycine ethyl ester* (8·6 g., 95%) which had m. p. 108—109° after recrystallisation from ethyl acetate (Found : C, 56·35; H, 6·4. $C_{21}H_{28}O_7N_4$ requires C, 56·25; H, 6·25%). Hydrogenolysis in ethanol containing hydrochloric acid gave *L-prolyldiglycylglycine ethyl ester hydrochloride* (82% yield), m. p. 188° (decomp.) after recrystallisation from ethanol, $[\alpha]_D^{24}$ −14·0° (*c* 1·7 in H_2O) (Found : C, 44·65; H, 6·6; N, 16·0. $C_{13}H_{23}O_5N_4Cl$ requires C, 44·5; H, 6·6; H, 16·0%); treatment with ammoniacal chloroform yielded the free *ester*, m. p. 161—162° after precipitation from chloroform with ether (Found : OEt, 14·0. $C_{13}H_{22}O_5N_4$ requires OEt, 14·3%).

Self-condensation experiments

Paper chromatography was carried out on Whatman No. 1 filter paper, usually with butan-1-ol–pyridine–water (65 : 35 : 65; upper phase); spots were located by spraying with ninhydrin (0·1% in butan-1-ol) and by the chlorine–starch–iodide method.[25]

Glycyl-L-proline Ethyl Ester.—The ester, liberated from the hydrochloride (1·2 g.) with a solution of ammonia in chloroform, solidified overnight in a vacuum-desiccator over phosphoric

[24] Bergmann, Zervas, and Fruton, *J. Biol. Chem.*, 1935, **111**, 225.
[25] Rydon and P. W. G. Smith, *Nature*, 1942, **169**, 922.

oxide. A sample of the solid yielded glycyl-L-proline anhydride, m. p. 213° after recrystallisation from ethanol (lit.,[4] m. p. 213°), R_F 0·55 (ninhydrin-negative; chlorine-positive). When heated at 105°/10^{-3} mm. for 15 hr. the crude solid (669 mg.) gave the same anhydride (370 mg., 46%), m. p. 213°, and no other detectable product, apart from traces of unchanged ester and a little glycyl-L-proline.

Glycyl-DL-proline ethyl ester, liberated from the hydrochloride (1·2 g.), behaved similarly to the L-compound, cyclising rapidly at room temperature and, when heated at 105°/10^{-3} mm. for 16 hr., giving only *glycyl-DL-proline anhydride* (DL-3 : 6-*dioxo*-1 : 2-*pyrrolidinopiperazine*) (680 mg., 85%), rhombs, m. p. 173° (from ethanol) (Found: C, 54·9; H, 6·5; N, 17·8. $C_7H_{10}O_2N_2$ requires C, 54·5; H, 6·5; N, 18·2%), R_F 0·55 (ninhydrin-negative; chlorine positive).

L-*Prolylglycine Ethyl Ester*.—(*a*) The free ester (579 mg.), liberated from the hydrochloride with ammonia in chloroform, was dried at room temperature at 10^{-3} mm. for 3 hr. and then heated at 105°/10^{-3} mm. for 15 hr. Crystallisation began after 2 hr. and a sublimate and an oily distillate collected on the neck of the reaction vessel. Trituration of the product (439 mg.) with ethanol afforded glycyl-L-proline anhydride (250 mg., 56%), m. p. 211—212° after recrystallisation from ethanol, R_F 0·55. Chromatography of the remainder showed the presence of some unchanged dipeptide ester (R_F 0·52) and four other products, two ninhydrin-positive (R_F 0·41 and 0·33) and two, present in only small amount, ninhydrin-negative and chlorine-positive (R_F 0·27 and 0·21).

(*b*) The ester (from 800 mg. of hydrochloride) was kept at room temperature in N-ethanolic triethylamine (10 ml.). Glycyl-L-proline anhydride (370 mg., 71%), m. p. 208—210°, was deposited over a period of two weeks. Chromatography did not reveal the presence of any ninhydrin-positive material in the supernatant liquid.

DL-*Prolylglycine Ethyl Ester*.—(*a*) The ester (689 mg.), heated at 105°/10^{-3} mm., gave a product (544 mg.) from which glycyl-DL-proline anhydride (350 mg., 70%), rhombs, m. p. 170—171° (from ethanol), R_F 0·55, was isolated; unchanged dipeptide ester (R_F 0·52) and two other ninhydrin-positive products (R_F 0·42 and 0·36) were also present.

(*b*) Self-condensation in N-ethanolic triethylamine afforded glycyl-DL-proline anhydride (75% yield), m. p. 170°; no ninhydrin-positive products were found in the mother-liquor.

Diglycyl-L-proline Ethyl Ester.—(*a*) The ester (708 mg.), heated at 105°/10^{-3} mm. for 15 hr., gave a glass (621 mg.), shown by paper chromatography to contain unchanged ester (R_F 0·37), glycyl-L-proline anhydride (R_F 0·51), and a series of ninhydrin-positive polymers (R_F 0·32, 0·27, 0·22, and 0·19).

(*b*) The ester (from 750 mg. of hydrochloride) was refluxed in ethanol (80 ml.) for 21 days. Chromatography showed the ester to be largely unchanged, although a small amount of polymer was present, together with glycyl-L-proline anhydride and piperazine-2 : 5-dione.

(*c*) The product from (*b*), freed from ethanol under reduced pressure, was heated at 100°, in a sealed tube, for 7 days in *m*-cresol (12 ml.). Removal of the solvent under reduced pressure in an atmosphere of nitrogen and treatment of the residue with ether precipitated piperazine-2 : 5-dione (20 mg.), decomp. 300° after recrystallisation from water, identified by paper chromatography. The ethereal solution was evaporated to dryness; treatment of the residue with cold ethanol afforded a little glycyl-L-proline anhydride (20 mg.), m. p. 205—208° after recrystallisation from ethanol. The mother-liquors contained unchanged tripeptide ester, more glycylproline anhydride and at least three ninhydrin-positive polymers of regularly decreasing R_F value.

Glycyl-L-prolylglycine Ethyl Ester.—The ester (793 mg.) was heated at 105°/10^{-3} mm. for 15 hr. Trituration of the product (612 mg.) with ethanol yielded glycyl-L-proline anhydride (120 mg., 25%), m. p. 212—213°. Chromatography of the remainder showed the presence of unchanged tripeptide ester (R_F 0·35) and several other ninhydrin-positive products (R_F 0·0—0·23), in addition to more anhydride.

Glycyl-DL-prolylglycine Ethyl Ester.—(*a*) Heated for 15 hr. at 100°/10^{-2} mm., this ester gave a mixture similar to that obtained from the L-compound, although less of the more soluble glycyl-DL-proline anhydride (*ca.* 10%), m. p. 170—171°, could be isolated.

(*b*) The ester (670 mg.) was refluxed in ethanol (15 ml.) for 8 days. On cooling, crystals (40 mg.) separated and were shown, by chromatography, to be a mixture of approximately equal amounts of piperazine-2 : 5-dione and glycyl-DL-proline anhydride. Evaporation of the mother-liquor to dryness and trituration of the residue with cold ethanol (3 ml.), afforded more glycyl-DL-proline anhydride (160 mg., 40%), m. p. 171—172° after recrystallisation from ethanol. Paper chromatography of the final mother-liquors showed them to contain a complex mixture of products, including unchanged tripeptide ester and glycyl-DL-proline anhydride; one product

gave a blue-violet spot with ninhydrin, characteristic of polyglycine esters, rather than the yellow, changing to blue-grey of esters containing proline, and may have been diglycylglycine ethyl ester, since it moved at the same rate as a marker spot of this compound.

L-*Prolylglycylglycine Ethyl Ester.*—The ester (964 mg.), heated at 105°/10^{-3} mm. for 15 hr., yielded a hard glass (892 mg.). Chromatography showed the presence of much unchanged tripeptide ester (R_F 0·47) and one other ninhydrin-positive material (R_F 0·33) and of glycyl-L-proline anhydride (R_F 0·59) and two other ninhydrin-negative, chlorine-positive products (R_F 0·40 and 0·27).

L-*Prolyldiglycylglycine Ethyl Ester.*—(*a*) The ester was recovered unchanged (m. p. 159—160°; OEt, 13·7%) after being heated at 95°/10^{-3} mm. for 20 hr.

(*b*) The ester hydrochloride (350 mg.) was refluxed for 10 days with triethylamine (2·7 ml.) in ethanol (200 ml.). Paper chromatography revealed no other product than unchanged ester.

We thank Mr. B. Manohin (Manchester) and Mr. F. H. Oliver (London) for the microanalyses.

College of Technology, University of Manchester,
Manchester, 1.
Imperial College of Science and Technology,
S. Kensington, London, S.W.7.

[*Received, May 18th,* 1956.]