Polypeptides. Part I. The Condensation-polymerisation of Some Polyglycine Esters and Azides.

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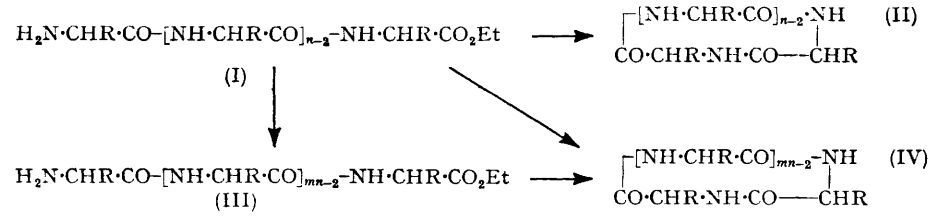
The condensation-polymerisation of the ethyl esters of the series of polyglycines from glycylglycine to pentaglycylglycine has been studied. In the solid state, the di- and the tri-peptide polymerise much more readily than the others, polymerising completely at temperatures which bring about little or no polymerisation of the higher peptide esters. In *m*-cresol solution stepwise degradation occurs, pairs of glycine residues being split off as piperazine-2 : 5-dione.

The derived peptide azides polymerise in aqueous solution with the formation of polyglycines of high molecular weight.

The condensation-polymerisation of esters, and other derivatives, of peptides can give rise to both open-chain and cyclic products; thus, intramolecular condensation-polymerisation of the peptide ester (I) would lead to the cyclic peptide (II) while the corresponding

intermolecular reaction could give rise to both the open-chain peptides (III) and the cyclic peptides (IV). The proportions in which these products are formed will depend on both



the length and the conformation of the peptide chain in both the original peptide (I) and its linear polymers (III), these conformations being, in turn, dependent on the nature of the side chain R and the stereochemical configuration of the individual amino-acid residues in (I). The three controllable structural variations in (I) which must be taken into account in any general study of the reaction are thus (*a*) the nature of R, (*b*) the stereochemical configuration of the amino-acid residues, and (*c*) the value of n. In order to simplify matters in this initial study by eliminating (*a*) and (*b*), we chose to work with polyglycine esters (I; R = H); values of n up to 6 appeared sufficient in the light of both the known influence of chain-length on the yields of macrocyclic carbon rings from open-chain compounds and the views of Pauling, Corey, and Branson (*Proc. Nat. Acad. Sci.*, 1951, **37**, 206) on the coiling of peptide chains.

Dipeptide esters are known to yield piperazine-2 : 5-diones readily by intramolecular condensation, (I) ⟶ (II); thus, Fischer and Fourneau (*Ber.*, 1901, **34**, 2868) found that piperazine-2 : 5-dione (II; $n = 2$), was rapidly formed at room temperature in aqueous solutions of glycylglycine ethyl ester (I; $n = 2$), although the action of heat on the ester in the absence of solvent yielded, in addition, a small amount of a product giving a positive biuret reaction; dipeptide esters readily cyclise to piperazine-2 : 5-diones on treatment with alcoholic ammonia (Fischer, *Ber.*, 1906, **39**, 467, 2893; Fischer and Reif, *Annalen*, 1908, **363**, 118; Butenandt, Karlson, and Zillig, *Z. physiol. Chem.*, 1951, **288**, 279). Tripeptide esters, on the other hand, appear to undergo linear polymerisation, (I) ⟶ (III) (for a review, see Katchalski, *Adv. Protein Chem.*, 1950, **6**, 123). The polymerisation of diglycylglycine methyl ester was first studied by Fischer (*Ber.*, 1906, **39**, 471), who obtained 76% of the hexapeptide ester, together with some water-insoluble material considered to be the dodecapeptide ester; this polymerisation has been studied in detail by Pacsu and Wilson (*J. Org. Chem.*, 1942, **7**, 117), who concluded, on the basis of methoxyl analyses, that stepwise dimerisation occurred wth the formation of peptide esters such as (III) with $n = 6$, 12, 24, 48, and 96. The literature on the polymerisation of triglycylglycine esters is conflicting; Curtius (*Ber.*, 1904, **37**, 1300) reported that the methyl ester, on being heated, yielded a water-insoluble product, formulated as " octaglycine anhydride," *i.e.* (IV; R = H; $n = 4$; $m = 2$), but Fischer (*Ber.*, 1906, **39**, 2927) could not detect any polymerisation under similar conditions. While our work was in progress, Sluyterman and Veenendaal (*Rec. Trav. chim.*, 1952, **71**, 137) re-investigated this reaction; at 100°, a prolonged induction period was followed by migration of methyl from oxygen to nitrogen with the formation of *N*-methylglycyldiglycylglycine, $Me{\cdot}NH{\cdot}CH_2{\cdot}CO{\cdot}[NH{\cdot}CH_2{\cdot}CO]_2{\cdot}NH{\cdot}CH_2{\cdot}CO_2H$, *NN*-dimethylglycyldiglycylglycine, $Me_2N{\cdot}CH_2{\cdot}CO{\cdot}[NH{\cdot}CH_2{\cdot}CO]_2{\cdot}NH{\cdot}CH_2{\cdot}CO_2H$, and some triglycylglycine; at higher temperatures condensation-polymerisation occurred, with the formation of polyglycine esters (Sluyterman and Kooistra, *ibid.*, p. 277). More recently, however, Brockmann and Musso (*Chem. Ber.*, 1954, **87**, 581) observed no change on heating triglycylglycine methyl ester at 125° for 4 days, although decomposition, with the formation of the free tetrapeptide and other (unidentified) products, was observed at higher temperatures.

In this paper we describe the polymerisation, alone and in solvents, of the series of polyglycine ethyl esters ranging from glycylglycine ethyl ester (I; R = H; $n = 2$) to pentaglycylglycine ethyl ester, (I; R = H; $n = 6$). With the exception of the first-mentioned, these peptide esters were all prepared by the thiothiazolidone method of Cook

and Levy (*J.*, 1950, 646); it was later found more convenient to prepare pentaglycylglycine ethyl ester by heating diglycylglycine ethyl ester. The polymerisation products were investigated qualitatively by paper chromatography; in the early stages of the work the usual ninhydrin spraying method was used to locate the spots, but this was later replaced by the chlorine–starch–iodide method (Rydon and Smith, *Nature*, 1952, **169**, 922), which is more sensitive and has the added advantage of revealing cyclic peptides (but see footnote, p. 2545). In many cases the polymerisation products were also studied quantitatively, paper chromatography being followed by elution and colorimetric estimation with ninhydrin (Moore and Stein, *J. Biol. Chem.*, 1948, **176**, 367); similar methods have been described by Fowden (*Biochem. J.*, 1951, **48**, 327) and Boissonnas (*Helv. Chim. Acta*, 1950, **33**, 1975). It was also possible, by elution, hydrolysis, and colorimetric estimation of the glycine so produced, to obtain an independent check on the chain-lengths of the products, which could also be inferred from the R_F values. In certain cases molecular weights were estimated colorimetrically by using the *N*-2 : 4-dinitrophenyl derivatives; details of the method are given in the Experimental section.

The results of the experiments in which the peptide esters were heated *in vacuo* in the solid state are summarised in Table 1; only the di- and the tri-peptide ester underwent appreciable reaction, the others being recovered largely unchanged. The formation of small amounts of free peptide is no doubt due to hydrolysis owing to the presence, in materials dried *in vacuo* at room temperature, of small amounts of tenaciously held adsorbed water (cf. Mellon, Korn, and Hoover, *J. Amer. Chem. Soc.*, 1947, **69**, 827; 1948, **70**, 3040).

Glycylglycine ethyl ester (I; R = H; $n = 2$) yielded piperazine-2 : 5-dione (II; R = H; $n = 2$) and triglycylglycine ethyl ester (III; R = H; $m = 2$; $n = 2$) as the sole products, there being no trace of higher polymers; this is borne out by the results of heating the tetrapeptide ester itself. Clearly, triglycylglycine ethyl ester does not react under these conditions with piperazine-2 : 5-dione, although the latter has been shown to react with glycine peptides in aqueous solution (Meggy, *J.*, 1953, 851); this result renders unlikely the suggestion (Carothers, *Chem. Rev.*, 1931, **8**, 389) that piperazine-2 : 5-dione is an intermediate in the thermal polymerisation of glycine.

Diglycylglycine ethyl ester (I; R = H; $n = 3$) yielded almost exclusively the hexapeptide ester (III; R = H; $m = 2$; $n = 3$) and this reaction is convenient for preparative purposes; a small amount of the nonapeptide ester (III; R = H; $m = 3$; $n = 3$) was

TABLE 1. *Products formed by heating polyglycine esters.*

Peptide ester *	Temp. and time of heating	Products
G_2Et	78° (20 hrs.)	G_2Et (25%) †; G_4Et (25%); piperazine-2 : 5-dione (50%).
	Room temp. (18 months)	G_4Et; piperazine-2 : 5-dione.
G_3Et	135° (12 hrs.)	G_6Et (83%); G_9Et (9%); higher polymers (trace).
	105° (1 month)	G_6Et; high polymer (main product).
G_3Me	108° (20 hrs.)	G_6Me; G_9Me; higher polymers.
	105° (1 month)	High polymers.
G_4Et	135° (12 (hrs.)	G_4Et (83%); G_4H (9%).
	124° (9 hrs.) + 184° (7 hrs.)	G_4Et; G_4H; piperazine-2 : 5-dione (3%).
G_5Et	124° (17·5 hrs.)	G_5Et (86%); G_5H (trace).
	163° (17 hrs.)	G_5Et (80%); G_5H (4%).
G_6Et	124° (17 hrs.)	G_6Et; higher polymers (trace); G_6H (trace).
	163° (37 hrs.)	G_6Et; higher polymers (trace); G_6H (trace).

* In this Table, and elsewhere, G_nEt denotes (I; R = H; $n = n$), G_nMe the corresponding methyl ester, and G_nH the corresponding free peptide.
† Sublimed unchanged.

also formed. Ethyl esters of amino-acids are known to polymerise less readily than methyl esters (Abderhalden and Suzuki, *Z. physiol. Chem.*, 1928, **176**, 101; Frankel and Katchalski, *J. Amer. Chem. Soc.*, 1942, **64**, 2264) and we have observed the same difference with peptide esters; thus, diglycylglycine ethyl ester, sealed *in vacuo* over silica gel and heated at 105° for a month, yielded a product containing a considerable amount of the hexapeptide ester, whereas diglycylglycine methyl ester, treated similarly, yielded a water-insoluble polymer

which did not move from the starting-line on paper chromatography. Our results with the tri- and the hexa-peptide ethyl ester are notably different from those obtained by Pacsu and Wilson (*loc. cit.*) with the methyl esters; however, these authors relied solely on methoxyl analyses of the products, and the methyl migration observed by Sluyterman and Veenendaal (*loc. cit.*) in the case of the tetrapeptide methyl ester renders their interpretation of their results of doubtful significance.

In none of our experiments was there evidence of the formation of cyclic peptides (other than piperazine-2 : 5-dione); this suggests that these esters have the fully extended β-conformation which has been established for polyglycine by *X*-ray crystallographic methods (Meyer and Go, *Helv. Chim. Acta*, 1934, **17**, 1488; Astbury, Dalgleish, Darmon, and Sutherland, *Nature*, 1948, **162**, 596). The observed restriction of linear polymerisation to the di- and the tri-peptide ester in these solid-state reactions is no doubt due to a favourable orientation of the molecules within the crystal lattice in these two cases.

The results of heating 5% solutions of the peptide esters in *m*-cresol are summarised in Table 2. In every case piperazine-2 : 5-dione was a major component of the products and

TABLE 2. *Products formed from polyglycine ethyl esters at* 100° *in* 5% *solution in* m-*cresol.*

Peptide ester	Period of heating (hr.)	GEt	G_2Et	G_3Et	G_4Et	G_5Et	G_6Et	Higher polymers	Piperazine-2 : 5-dione
G_3Et ...	96	Trace	—	Trace	Trace	Trace	—	—	++
G_4Et ...	165	Trace	Trace	—	Trace	—	—	—	++
G_5Et ...	192	Trace	—	++	Trace	++	Trace	Trace	++
G_6Et ...	216	—	+	Trace	++	Trace	++	+	++

the results show clearly that, under these conditions, the peptide esters undergo degradation by the stepwise removal of pairs of glycine residues from the amino-end of the molecule, thus :

$$H_2N{\cdot}CH_2{\cdot}CO{\cdot}[NH{\cdot}CH_2{\cdot}CO]_{n-2}{\cdot}NH{\cdot}CH_2{\cdot}CO_2Et \longrightarrow$$

$$HN\begin{matrix}CO—CH_2\\ \diagdown\quad\diagup\\ CH_2—CO\end{matrix}NH + + H_2N{\cdot}CH_2{\cdot}CO{\cdot}[NH{\cdot}CH_2{\cdot}CO]_{n-4}{\cdot}NH{\cdot}CH_2{\cdot}CO_2Et$$

A similar degradation of certain tripeptides on being heated in β-naphthol has been observed by Lichtenstein (*J. Amer. Chem. Soc.*, 1938, **60**, 560). This type of degradation may well be responsible for the formation of dioxopiperazines by the mild hydrolysis of certain proteins (cf., *e.g.*, Abderhalden, *Z. physiol. Chem.*, 1923, **128**, 119; Abderhalden and Komm, *ibid.*, 1924, **132**, 1). This mechanism should lead exclusively to odd peptide esters from odd peptide esters and to even peptide esters from even peptide esters. The formation of traces of even from odd peptide esters (G_4Et from G_3Et; G_4Et and G_6Et from G_5Et) is no doubt to be ascribed to direct polymerisation of the glycine ethyl ester which is the end product of the degradation of any odd peptide ester by the above route. The formation of traces of odd peptide esters from even (G_3Et and G_5Et from G_6Et) is, however, anomalous; a possible mechanism for their formation involves the removal of sets of three glycine residues as *cyclo*triglycine, but no trace of such a product could be detected in the reaction mixtures.* In the case of the penta- and the hexa-peptide ester polymerisation accompanied the degradation but no evidence could be obtained for the formation of cyclic peptides, other than piperazine-2 : 5-dione, although the possibility of the presence of small amounts of such products cannot be excluded.

Magee and Hofmann (*J. Amer. Chem. Soc.*, 1949, **71**, 1515) obtained a polyglycine by the polymerisation of diglycylglycine azide in aqueous solution and this reaction, carried out at high dilution, has very recently been used by Sheehan and Richardson (*ibid.*, 1954, **76**,

* The assumption that cyclic peptides would be revealed on chromatograms by the chlorination method was based on the positive reaction given by piperazine-2 : 5-dione (Rydon and Smith, *loc. cit.*). It has recently been found by one of us (P. W. G. S.) that cyclic tripeptides do not give the reaction at all readily; *cyclo*triglycine, if present in these reaction mixtures, would, therefore, probably not have been detected.

6329) for the preparation of *cyclo*triglycine (II; R = H; $n = 3$); we have made a preliminary investigation of the applicability of this reaction to higher peptide azides. The crude hydrazides prepared from di-, tri-, and tetra-glycylglycine ethyl ester were treated at 0° with aqueous nitrous acid; basification of the resulting solutions of the azides with sodium hydroxide resulted in every case in the precipitation of polyglycine. The products from the tri- and the tetra-peptide azide were insoluble, white, granular solids, similar to the polyglycine described by Magee and Hofmann (*loc. cit.*); that from the pentapeptide azide was a hard translucent solid and probably a higher polymer than the others. There is no doubt that the reaction is a general one, suitable for the preparation of polypeptides of high molecular weight (and probably also of *cyclo*peptides) containing repeating units made up of amino-acid residues with a pre-determined arrangement.

Experimental

Preparations.

Polyglycine Esters.—These were prepared and stored as their hydrochlorides (chromatographically pure) which were converted into the free esters immediately before use.

Glycylglycine ethyl ester. The hydrochloride, m. p. 181° (Schott, Larkin, Rockland, and Dunn, *J. Org. Chem.*, 1947, **12**, 490), was converted into the free ester, m. p. 85—86°, in 74% yield by Fischer and Fourneau's method (*loc. cit.*).

Diglycylglycine ethyl ester. The hydrochloride, m. p. 215—216° (decomp.), was prepared in 25-g. batches in 82% yield by Cook and Levy's method (*loc. cit.*). This hydrochloride (1·5 g.) was suspended in ethanol (15 ml.) and cooled to 0° while ethanolic sodium ethoxide (from sodium, 135 mg., and ethanol, 4·3 ml.) was added dropwise with shaking; the product was evaporated to dryness at 30° under reduced pressure and the solid residue extracted with chloroform (3 × 5 ml.) at 40°. Addition of dry ether to the cooled, filtered extract precipitated the ester (900 mg., 70%), m. p. 107° (in bath at 102°).

Diglycylglycine methyl ester. 2-Thiothiazolid-5-one (2·5 g.) (Cook and Levy, *loc. cit.*; Pollock, Thesis, London, 1949) was added to glycylglycine methyl ester hydrochloride (3·5 g.) in methanol (25 ml.) and triethylamine (3·8 g.). Acidification with methanolic hydrogen chloride precipitated the ester hydrochloride (3·8 g., 82%), m. p. 197° after recrystallisation from methanol. The free ester, m. p. 110° (in bath at 108°), was liberated, in 64% yield, by treatment with methanolic sodium methoxide as described by Pacsu and Wilson (*loc. cit.*).

Triglycylglycine ethyl ester. The hydrochloride, m. p. 212° (decomp.), was prepared in 84% yield by Cook and Levy's method (*loc. cit.*). This compound (2 g.), in water (5 ml.), was treated dropwise, with shaking, at 0° with triethylamine (0·8 g.). After 5 min., the semisolid mass was diluted with ethanol (10 ml.), filtered, and washed with ethanol; after trituration with more ethanol (10 ml.), the ester (1·3 g., 74%), no definite m. p., was dried *in vacuo* over silica gel.

Tetraglycylglycine ethyl ester. The hydrochloride, m. p. 233—234° (decomp.), prepared in 78% yield by Cook and Levy's method (*loc. cit.*), was converted into the free ester (78% yield) as described for the tetrapeptide ester.

Pentaglycylglycine ethyl ester. (*a*) Tetraglycylglycine ethyl ester hydrochloride (2 g.), in water (12·5 ml.), was treated with triethylamine (1·1 g.) in ethanol (17·5 ml.). A suspension of 2-thiothiazolid-5-one (0·74 g.) in ethanol (25 ml.) was immediately added and the mixture vigorously shaken for 15 min., most of the solid dissolving. 5N-Ethanolic hydrogen chloride (2·5 ml.) was added and the solution cooled to 0°. The solid which separated on scratching was recrystallised (charcoal) from aqueous ethanol containing a little hydrogen chloride, affording *pentaglycylglycine ethyl ester hydrochloride* (0·8 g., 35%), m. p. 239—240° (decomp.) (Found: C, 39·0; H, 6·0; OEt, 9·7. $C_{14}H_{25}O_7N_6Cl$ requires C, 39·6; H, 5·9; OEt, 10·6%). This hydrochloride (800 mg.), in warm water (4 ml.), was treated with triethylamine (200 mg.); ethanol (10 ml.) was added and the precipitate isolated by centrifugation, washed thrice with ethanol, and dried *in vacuo* over phosphoric oxide. The free *ester* (660 mg., 90%) was an amorphous solid with no definite m. p. (Found: OEt, 10·9. $C_{14}H_{24}O_7N_6$ requires OEt, 11·6%).

(*b*) Diglycylglycine ethyl ester (14 g.; in two batches) was heated at 130°/1 × 10^{-3} mm. for 12 hr. The product was warmed with 2N-hydrochloric acid and diluted with water (60 ml.); some yellow gelatinous insoluble matter was removed by filtration, after addition of charcoal and Supercel, and the filtrate diluted with ethanol (500 ml.) and kept at 0° overnight. One further recrystallisation from aqueous ethanol yielded the pure ester hydrochloride (8 g., 58%), m. p. 250° (decomp.) (Found: C, 39·7; H, 6·2%).

2 : 4-*Dinitrophenyl Derivatives*.—The following were prepared by shaking the peptide or peptide ester (2·5 mmole), in water (5 ml.) containing sodium hydrogen carbonate (5 mmole), with 1-fluoro-2 : 4-dinitrobenzene (2·75 mmole) in ethanol (10 ml.). Derivatives, as follows, of the esters crystallised directly from the reaction mixture; those of the free peptides were precipitated by acidification of the concentrated reaction mixture after extraction with ether.

N-2 : 4-*Dinitrophenylglycylglycine hemihydrate*, plates, m. p. 197° (decomp.), from aqueous methanol (Found : C, 38·9, 39·0; H, 3·9, 3·7; N, 17·3, 17·9. $C_{10}H_{10}O_7N_4,\frac{1}{2}H_2O$ requires C, 39·1; H, 3·6; N, 18·2%). N-2 : 4-*Dinitrophenylglycylglycylglycine*, m. p. 206—207° (decomp.), from methanol (Found : C, 41·0; H, 3·4; N, 19·6. $C_{12}H_{13}O_8N_5$ requires C, 40·6; H, 3·7; N, 19·7%); *ethyl ester*, m. p. 190° (decomp.), from aqueous ethanol (Found : C, 44·0; H, 4·5; N, 18·0. $C_{14}H_{17}O_8N_5$ requires C, 43·9; H, 4·5; N, 18·3%). N-2 : 4-*Dinitrophenylglycyldiglycylglycine ethyl ester*, m. p. 218° (decomp.), from water or methanol (Found : C, 43·5; H, 4·4; N, 19·3. $C_{16}H_{20}O_9N_6$ requires C, 43·6; H, 4·6; N, 19·1%). N-2 : 4-*Dinitrophenylglycyltriglycylglycine ethyl ester*, needles, m. p. 234—235° (decomp.), from water (Found : C, 43·5; H, 4·7; N, 20·3. $C_{18}H_{23}O_{10}N_7$ requires C, 43·5; H, 4·6; N, 19·7%).

Analytical Methods.

Qualitative.—Peptide mixtures from the polymerisation experiments were chromatographed on Whatman No. 1 paper, *n*-butanol-pyridine–water (65 : 35 : 65) (upper phase) being used as the developing solvent; the spots were located by spraying with ninhydrin (0·1% in *n*-butanol) or by the chlorine–starch–iodide method (Rydon and Smith, *loc. cit.*). Typical R_F values for the various polyglycine esters, applied as their hydrochlorides, are :

Peptide ester	G_2Et	G_3Et	G_4Et	G_5Et	G_6Et
R_F	0·49	0·40	0·34	0·29	0·23

Quantitative.—The ninhydrin reagent used was the following modification of that of Moore and Stein (*loc. cit.*), which was found to be unstable on keeping. Solution A : Ninhydrin (2 g.), dissolved in redistilled, peroxide-free, ethylene glycol monomethyl ether (50 ml.) and citrate buffer (40 ml.; citric acid monohydrate, 21·008 g., in N-sodium hydroxide, 200 ml., made up to 500 ml. with water). Solution B : 0·8% hydrated stannous chloride in citrate buffer; stored under liquid paraffin. For use, solution B (1 ml.) was added to solution A (9 ml.); fresh reagent was made up at least monthly.

Nine 8·79-µl. drops of a solution of the material for analysis (each containing *ca.* 0·3 µmole of amino-nitrogen) were placed, by means of a Blodgett pipette (cf. Harkins and Anderson, *J. Amer. Chem. Soc.*, 1937, **59**, 2193) on a sheet of Whatman No. 1 paper (24 × 24 in.) marked in 9 vertical strips. After drying, the chromatogram was developed for 15—20 hr. with the upper phase of *n*-butanol–pyridine–water (65 : 35 : 65) in a cabinet saturated with vapour from the lower phase of this mixture. After the paper had dried overnight at 30°, the centre strip, carrying the chromatogram of the fifth spot, was cut out and examined qualitatively as described above. The peptide spots on the other strips were then located precisely by viewing them in ultraviolet light, using the sprayed strip as a guide. The peptide spots on strips 1, 3, 7, and 9 were cut out, allowing *ca.* 7 mm. clearance, and each paper disc was then pushed to the bottom of a test-tube (17 × 1·5 cm.), treated with the ninhydrin reagent (1 ml.), and heated for exactly 20 min. in a vigorously boiling water-bath. After cooling, the contents of the tube were diluted with 50% aqueous *n*-propyl alcohol (9·1 ml.). The colour density of the filtered solution was then determined, against a reagent blank, in a Spekker Photoelectric Absorptiometer, with the Ilford Spectrum Yellow-Green Filter, No. 605. After correction for adsorbed ammonia (see below), the amino-nitrogen content was read off from a reference curve constructed from standard solutions of the appropriate peptide ester; all the peptide esters gave identical reference curves, within the limits of experimental error; fresh reference curves were constructed for each new batch of ninhydrin.

The amino-nitrogen content of the original solution was determined directly, on standard 8·79-µl. drops, by the above method.

For the determination of the ammonia blank, which was minimised by working in an isolated room reserved for the purpose, the peptide spots on strips 2 and 8 were cut out and each disc placed in the centre compartment of a Conway dish (Conway, " Microdiffusion Analysis and Volumetric Error," Lockwood, London, 1939); the outer compartment of the dish contained the ninhydrin reagent (2 ml.). Saturated potassium carbonate solution (1 ml.) was added to the centre compartment and the dish lid immediately put in place and sealed with Vaseline. The

whole was rocked at room temperature for 5 hr., 1 ml. was removed from the outer compartment, and the colour developed and measured as described above, a blank similarly prepared being used.

The peptide spots on strips 4 and 6 were cut out similarly and subjected to extraction and hydrolysis. The apparatus consisted of a test-tube (20 × 3·5 cm.) to which a cold-finger condenser was attached by means of a ground-glass joint; just below the bottom of the condenser and 8 cm. from the bottom of the test-tube, a small funnel was supported by means of indentations in the tube wall; a small glass bead was placed in the tube to prevent bumping. The paper disc was folded between clean sheets of filter-paper and placed in the funnel; it was then moistened with water containing a little 2N-hydrochloric acid and pushed into position with a glass rod, which was finally washed down with water (2 ml.). Extraction was carried out at gentle reflux for 6 hr., after which the funnel and paper were removed. Concentrated hydrochloric acid (3 ml.) was added and the solution refluxed for a further 24 hr., after which it was evaporated to dryness overnight in a vacuum-desiccator over potassium hydroxide. The residue was dissolved in water (1 ml.), and aliquot parts (0·2 ml.) were pipetted into test-tubes (17 × 1·5 cm.). 1% Methanolic potassium hydroxide (0·2 ml.) was added to each tube and the contents were again evaporated to dryness in a vacuum-desiccator over phosphoric oxide (6 hr.). 1·25% Citric acid (0·2 ml.) and ninhydrin reagent (1 ml.) were added and the colour was developed as usual. After addition of 50% aqueous *n*-propyl alcohol (8·9 ml.) the colour density of the cooled solution was measured against a similarly prepared blank, and the glycine content determined by means of a reference curve. The glycine content of the original solution was similarly determined after hydrolysis of a standard drop with 20% (w/v) hydrochloric acid.

Colorimetric Determination of Molecular Weights of N-2 : 4-*Dinitrophenyl Derivatives.*—The colour densities of solutions of *N*-2 : 4-dniitrophenylglycylglycine, *N*-2 : 4-dinitrophenylglycylglycylglycine and its ethyl ester, *N*-2 : 4-dinitrophenylglycyldiglycylglycine ethyl ester, and *N*-2 : 4-dinitrophenylglycyltriglycylglycine ethyl ester in mixtures of formamide and 1% aqueous sodium hydrogen carbonate were determined by using the Spekker Photoelectric Absorptiometer and Ilford Spectrum Violet Filters, No. 601; the colour values for equimolar solutions were all the same, conforming to a curve drawn through the points :

Concn. (mmolar)	0·05	0·10	0·15	0·20
Spekker reading	0·133	0·250	0·350	0·442

Molecular weights of glycine peptides and peptide esters may thus be determined by treatment with 1-fluoro-2 : 4-dinitrobenzene in aqueous-ethanolic sodium hydrogen carbonate, as described on p. 2547, dissolution of a weighed amount of the resulting *N*-2 : 4-dinitrophenyl derivative in a known volume of a suitable mixture of formamide and 1% aqueous sodium hydrogen carbonate, and determination of the colour density. The reaction with fluorodinitrobenzene should be repeated until the washed derivative no longer gives a positive ninhydrin reaction. This method gave, for authentic pentaglycylglycine ethyl ester, *M*, 414 (Calc. for $C_{14}H_{24}O_7N_6$: *M*, 388).

Polymerisations.

Heat-polymerisation.—The freshly prepared esters were dried in a vacuum-desiccator over phosphoric oxide and then under a high vacuum (10^{-3}—10^{-4} mm.) at room temperature for 2—3 hr. They were then heated, in a suitable vapour-bath, at 10^{-3}—10^{-4} mm. for the times specified in Table 1. The details of a typical experiment (line 3 of Table 1) follow :

Diglycylglycine ethyl ester was heated at 135° (refluxing "Ethylcellosolve") for 12 hr.; the loss in weight was 13·4% (Calc. for loss of 1 mol. of ethanol from 2 mols. of ester : 10·6%). The product (103·0 mg.) was dissolved in water (10 ml.) containing 2N-hydrochloric acid (0·2 ml.) and this solution was employed for analysis (p. 2547) with the following results

		Amino-N (μmole)	Glycine after hydrolysis (μmole)	Mean chain-length (glycine residues)
Total polymer		0·211	1·305	6·2
Isolated peptides	R_F 0·26	0·175 (83%)	1·100	6·3
	R_F 0·04	0·019 (9%)	0·162	8·5

The chlorine–starch–iodide method revealed a trace of material, R_F 0, in addition to the spots with R_F 0·26 and 0·04; this is probably higher polymer.

Polymerisation in m-*Cresol.*—(a) *Diglycylglycine ethyl ester.* This (500 mg.) was heated at 100° in *m*-cresol (10 ml.) in a sealed tube for 96 hr.; the amino-nitrogen content of the final

solution had fallen to about 40% of its original value. The solution was diluted with dry ether (400 ml.), and the precipitated buff solid collected; chromatography showed the major component (53%) of this solid to be piperazine-2 : 5-dione, the other components being G_3Et, G_4Et, G_5Et, and glycine. The ethereal filtrate was concentrated under reduced pressure; chromatography showed the presence of G_3Et, glycine ethyl ester, and piperazine-2 : 5-dione.

(b) *Triglycylglycine ethyl ester.* This (500 mg.), in *m*-cresol (10 ml.), was heated similarly for 165 hr.; the amino-nitrogen content of the final solution was about 40% of the original. Precipitation with ether (400 ml.) yielded a solid (300 mg.), containing 47% of piperazine-2 : 5-dione together with G_4Et, G_2Et, and glycine; chromatography of the ethereal filtrate revealed only a little glycine ethyl ester.

(c) *Tetraglycylglycine ethyl ester.* This (500 mg.) in *m*-cresol (10 ml.) was heated at 100° for 192 hr. in an evacuated sealed tube; the amino-nitrogen content of the final solution was about 70% of the original. Chromatography showed the presence of the products indicated in Table 2, together with a trace of glycine.

(d) *Pentaglycylglycine ethyl ester.* This (500 mg.) in *m*-cresol (10 ml.) was heated at 100° for 216 hr. in an evacuated sealed tube; the amino-nitrogen content of the final solution was about 70% of the original. Chromatography showed the presence of the products indicated in Table 2, together with a trace of glycine.

Polymerisation of Polyglycine Azides.—(a) *Diglycylglycine azide.* Diglycylglycine ethyl ester hydrochloride (2·5 g.), suspended in ethanol (20 ml.), was treated with hydrazine hydrate (0·75 g.). After 4 days at room temperature, the solid (1·9 g.) was filtered off. This crude hydrazide (1·5 g.) was dissolved in N-hydrochloric acid (16 ml.) and cooled to 0°; 25% aqueous sodium nitrite was added until an immediate positive starch–iodide reaction was obtained. After 5 min. at 0°, the solution was brought to pH 10 with 2N-sodium hydroxide; gas was evolved and a white precipitate formed, which was collected by filtration after being kept at room temperature overnight. The product (0·75 g.) was an amorphous granular solid, not completely soluble in water; it gave an orange-brown colour, rapidly changing to violet, when boiled with ninhydrin, and a positive biuret reaction; chromatography showed the presence of a little diglycylglycine and much polymer; acid hydrolysis yielded only glycine.

(b) *Triglycylglycine azide.* Triglycylglycine ethyl ester (0·5 g.) was triturated with hydrazine hydrate (0·3 g.); the excess of hydrazine was removed in a vacuum-desiccator over concentrated sulphuric acid. The crude ethanol-washed product (0·4 g.) was dissolved in 2N-hydrochloric acid, treated with sodium nitrite, and basified as described in (a). The product (0·3 g.) was a granular white powder, difficultly soluble in hot water; it gave a positive ninhydrin reaction; chromatography revealed only polymeric material; acid hydrolysis yielded only glycine.

(c) *Tetraglycylglycine azide.* Tetraglycylglycine ethyl ester hydrochloride (3·7 g.), suspended in ethanol (25 ml.), was treated with hydrazine hydrate (1 g.); after 3 days at room temperature, the crude hydrazide (3·8 g.) was filtered off. This compound (2·1 g.) was treated with nitrous acid and basified, as described in (a). The product (360 mg.) was a hard translucent ninhydrin-positive solid.

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