632. *Synthetic Polypeptides. Part II. Polyglutamic Acid.*

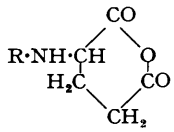
By W. E. Hanby, S. G. Waley, and J. Watson.

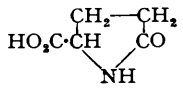
The synthesis of polyglutamic acid has been investigated. Reduction of poly-(γ-benzyl L-glutamate) by phosphonium iodide proved to be the only method of obtaining the polymeric acid optically pure. The structures of this synthetic polypeptide and the poly-D-glutamic acid isolated from *B. anthracis* have been investigated by infra-red spectroscopy; poly-D-glutamic acid has also been synthesised and compared serologically with the naturally occurring material.

All the synthetic polypeptides described in Part I have non-polar side chains. Since proteins contain also amino-acid residues with polar side chains, we have therefore studied the synthesis of an acidic polypeptide, polyglutamic acid (Hanby, Waley, and Watson, *Nature*, 1948, **161**, 132). While we were investigating this, the preparation of both a basic and an acidic

polypeptide was reported (polylysine, Frankel, Grosfeld, and Katchalski, *J. Amer. Chem. Soc.*, 1948, **70**, 2094; polyaspartic acid, Frankel and Berger, *Nature*, 1949, **163**, 213). Another reason for investigating polyglutamic acid was that the capsular substance of *Bacillus anthracis* had been shown to be a polypeptide built up solely of (−)-D-glutamic acid residues (Ivánovics and Brückner, *Z. Immunitäts.*, 1937, **90**, 304; 1938, **93**, 119; Hanby and Rydon, *Biochem. J.*, 1946, **40**, 297).

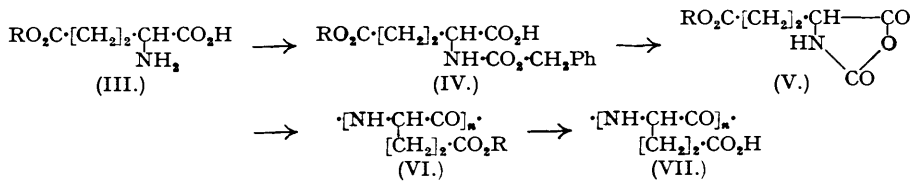
The first method which we tried was reduction of *N*-carbobenzyloxy-L-glutamic acid anhydride (I; R = Ph·CH_2·O_2C). We hoped that the amine (I; R = H) would polymerise to

(I.) 


(II.)

give a chain consisting predominantly of α-peptide with a few γ-peptide links. Although hydrogenation proceeded smoothly, the only compound isolated under a variety of experimental conditions was 2-ketopyrrolidine-5-carboxylic acid (II). It thus appears that the amine (I; R = H) rearranges easily to (II).

We therefore adopted the following route, using the Leuchs polymerisation of *N*-carboxy-amino-acid anhydrides :



In preliminary experiments, treatment of the γ-ethyl *N*-carbobenzyloxyglutamate (IV; R = Et) with phosphorus pentachloride gave γ-ethyl *N*-carboxy-L-glutamate anhydride (V; R = Et), which was polymerised in cold pyridine. Hydrolysis of the polymer (VI; R = Et) with cold dilute alcoholic alkali removed only two-thirds of the ester groups. However the methyl ester group should be more easily removed, and Fraenkel-Conrat and Olcott (*J. Biol. Chem.*, 1945, **161**, 259) state that the methyl ester (VI; R = Me), prepared by esterification of the capsular substance of *Bacillus anthracis*, is readily soluble in water and that the ester is easily hydrolysed. Although difficulty was at first experienced in obtaining consistent yields in the esterification of glutamic acid with methanolic hydrogen chloride, esterification proceeded smoothly when the glutamic acid was added to methanol to which some acetyl chloride had previously been added. The γ-methyl glutamate (III; R = Me) with benzyl chloroformate in aqueous sodium hydrogen carbonate gave γ-methyl *N*-carbobenzyloxy-L-glutamate (IV; R = Me) which with phosphorus pentachloride afforded γ-methyl *N*-carboxy-L-glutamate anhydride (V; R = Me). Polymerisation in pyridine gave the polypeptide (VI; R = Me), which was found to be insoluble in water and alcohols. This discrepancy between the synthetic ester (VI; R = Me) and the ester of the naturally occurring polyglutamic acid (Fraenkel-Conrat and Olcott, *loc. cit.*) led us to attempt esterification of the naturally-occurring polyglutamic acid. We found, however, that our specimen of polyglutamic acid (" H. 230 B "; mol. wt. 21,000; Hanby and Rydon, *loc. cit.*) did not dissolve in methanolic hydrochloric acid even in three days (Fraenkel-Conrat and Olcott report rapid dissolution and nearly complete esterification in 1 day) and contained no methoxyl groups. It is probable that this difference is due to the nature of the polyglutamic acid used.

Since the poly-ester (VI; R = Me) was so sparingly soluble, the pyridine solution resulting from the polymerisation was used directly for the hydrolysis; a potassium salt was precipitated on the addition of alcoholic alkali, and further hydrolysis was effected by dilute aqueous potassium hydroxide. The isolation, after dialysis, of a silver salt (VI; R = Ag) showed that most of the ester groups had been removed. Proteins and peptides are readily racemised by alkali (Neuberger, *Adv. in Protein Chem.*, 1948, **4**, 297) so that it was important to find out whether our product was optically pure.

Racemisation might occur at several stages in the synthesis of a polypeptide, and this problem has not hitherto been examined. The polypeptide must be hydrolysed to the parent amino-acid, and the specific rotation based on the nitrogen content of the hydrolysate.

Determination of the total nitrogen (Kjeldahl) and the amino-nitrogen (Van Slyke) shows whether hydrolysis is complete. If the amino-acid is isolated some of the DL-compound may be lost and the optical purity over-estimated.

Complete hydrolysis of polypeptides frequently needs more vigorous conditions than are customary for the hydrolysis of proteins (cf. Curtius and Sieber, *Ber.*, 1922, **55**, 1543; Frankel, Grosfeld, and Katchalski, *loc. cit.*). Thus we have found that boiling with 20% hydrochloric acid for 24 hours sometimes causes complete hydrolysis, but the use of 47% hydrobromic acid is preferable for several polypeptides.

When the poly-ester (VI; R = Me) was hydrolysed with hydrobromic acid the glutamic acid was racemised to the same extent as L-glutamic acid when treated similarly. The same degree of racemisation was also observed when the polymerisation was carried out in cooled or in boiling pyridine. But after the removal of the ester groups from (VI; R = Me) with alkali, hydrolysis with hydrochloric acid (under conditions which did not racemise L-glutamic acid itself) gave extensively racemised glutamic acid. It seems clear, then, that there is no appreciable loss of optical purity up to the stage of alkaline hydrolysis, but that there is at this last stage. This synthesis has also been carried out by Professor E. Y. Spencer (University of Saskatchewan) (private communication) who has confirmed our result that the polyglutamic acid obtained is extensively racemised; this was measured by estimating the concentration of L-glutamic acid in the hydrolysate by the glutamic acid decarboxylase method.

We next set out to prepare the poly-benzyl ester (VI; R = CH_2Ph), so that the ester groups could be removed by reduction. Preliminary attempts to prepare it from the poly-methyl ester by ester exchange were unsuccessful, and the exchange reaction was no more successful with the carbobenzyloxy-ester (IV; R = Me). Frankel and Berger (*loc. cit.*) have prepared β-benzyl *N*-carbobenzyloxy-L-aspartate (VIII) by half-hydrolysis of dibenzyl *N*-carbobenzyloxy-

$CH_2Ph{\cdot}O_2C{\cdot}CH_2{\cdot}CH(CO_2H){\cdot}NH{\cdot}CO_2{\cdot}CH_2Ph$ (VIII.)

$CH_2Ph{\cdot}O_2C{\cdot}CH_2{\cdot}CH(CO_2{\cdot}CH_2Ph){\cdot}NH{\cdot}CO_2{\cdot}CH_2Ph$ (IX.)

L-aspartate (IX) with potassium hydroxide in benzyl alcohol. However, dibenzyl *N*-carbobenzyloxy-L-glutamate (X) [prepared from the di-silver salt of (IV; R = H) and benzyl bromide] with one molecular equivalent of potassium hydroxide in benzyl alcohol gave a mixture of products, from which the pure γ-ester (IV; R = CH_2Ph) could not be isolated. Hydrolysis was not selective, since the acid (IV; R = H) could be isolated; some of the γ-ester (IV; R = CH_2Ph) was formed, as shown by formation of γ-benzyl α-*p*-bromophenacyl *N*-carbobenzyloxy-L-glutamate (XI) on treatment with *p*-bromophenacyl bromide. Since the crude

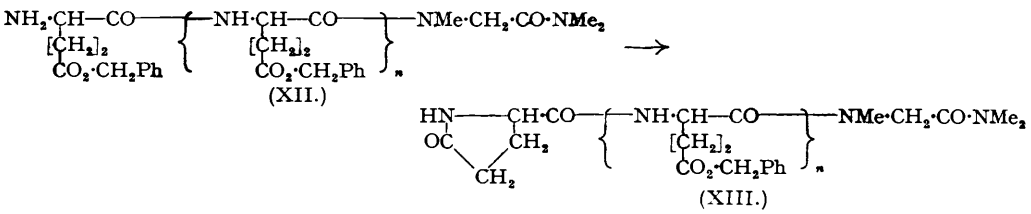
$CH_2Ph{\cdot}O_2C{\cdot}[CH_2]_2{\cdot}\underset{NH{\cdot}CO_2{\cdot}CH_2Ph}{CH}{\cdot}CO_2{\cdot}CH_2Ph$ (X.)

$CH_2Ph{\cdot}O_2C{\cdot}[CH_2]_2{\cdot}\underset{NH{\cdot}CO_2{\cdot}CH_2Ph}{CH}{\cdot}CO_2{\cdot}CH_2{\cdot}CO{\cdot}C_6H_4Br$ (XI.)

product obtained by the action of alkali on the dibenzyl ester (X) was unsatisfactory for cyclisation to the anhydride (V; R = CH_2Ph), this synthetic route was abandoned.

It was then found that γ-benzyl L-glutamate (III; R = CH_2Ph) could be prepared from L-glutamic acid by two methods. The copper complex of glutamic acid was treated with benzyl chloride, and the complex of the benzyl ester decomposed with hydrogen sulphide. This method of protecting the α-carboxyl (or amino-)group of an α-amino-acid has been used in the preparation of derivatives of lysine (Neuberger and Sanger, *Biochem. J.*, 1943, **37**, 515) and ornithine (Synge, *ibid.*, 1948, **42**, 99; Kurtz, *J. Biol. Chem.*, 1949, **180**, 1253), but has not previously been used for glutamic acid. The second method, which was more satisfactory, was direct esterification of glutamic acid with benzyl alcohol which proceeded at room temperature in the presence of constant-boiling hydriodic acid. Although concentrated hydrochloric acid gave equally good yields, heat was required, as one of the main factors controlling this reaction is the solubility of the salt of glutamic acid in the reaction medium. The ester obtained is contaminated with about 30% of glutamic acid, but this is readily removed because the benzyl ester is sparingly soluble in water and crystallises from a solution containing sufficient sodium hydrogen carbonate to combine with the acid. The low solubility of the benzyl ester made it necessary to effect acylation with benzyl chloroformate at 60°, but at this temperature satisfactory yields of γ-benzyl *N*-carbobenzyloxy-L-glutamate (IV; R = CH_2Ph) were obtained, and this with phosphorus pentachloride in ether gave γ-benzyl *N*-carboxy-L-glutamate anhydride (V; R = CH_2Ph). This anhydride was polymerised in nitrobenzene, by using sarcosine dimethylamide as initiator (see Part I); in this case, however, there is a complication since

terminal amino-groups may be lost by the cyclisation (XII) ⟶ (XIII) which was shown to occur by following the disappearance of basic groups in a low polymer (XII; $n \approx 5$) in dioxan



(XII.)

(XIII.)

solution; there is thus, in this case, a termination reaction in the polymerisation. This cyclisation also prevents amino-nitrogen estimations being used to determine the molecular weight of the polymer.

The three methods of removing protecting benzyl ester groups (catalytic hydrogenation, sodium and liquid ammonia, and phosphonium iodide) have been investigated for the polybenzyl ester (VI; R = CH_2Ph). γ-Benzyl glutamate (III; R = CH_2Ph) and *N*-carbobenzyloxy-glutamate (IV; R = CH_2Ph) and the anhydride (V; R = CH_2Ph) were readily hydrogenated in presence of a palladium charcoal catalyst. Hydrogenation of a specially low polymer (XIII; $n = 4$) in acetic acid was much slower than that of the simpler benzyl esters mentioned above. Hydrogenation of the higher polymers was unsuccessful. The difficulty of hydrogenating linear high polymers seems to be general, since Frankel, Grosfeld, and Katchalski (*loc. cit.*) could not hydrogenate poly(carbobenzyloxylysine). The poly-benzyl ester (VI; R = CH_2Ph) was reduced by sodium in liquid ammonia; we have found it advantageous to add ammonium iodide (rather than other ammonium salts) at the end of the reduction, since both ammonium and sodium iodides, but not the reduction product, are readily soluble in ethanol. Although there is normally no loss of optical purity in the reduction of oligopeptides by this method, the polyglutamic acid obtained was considerably racemised. Phosphonium iodide has been used for the reduction of both poly(carbobenzyloxylysine) (Frankel, Grosfeld, and Katchalski, *loc. cit.*) and poly(benzyl aspartate) (Frankel and Berger, *loc. cit.*). In the latter case the extent of reduction has not yet been reported. When poly-(γ-benzyl L-glutamate) (VI; R = CH_2Ph) was reduced with phosphonium iodide in acetic acid, an acidic product was obtained which could be dissolved in aqueous potassium hydrogen carbonate and, after dialysis was reprecipitated by acid. The equivalent weight and the mean residue weight of the polymer showed that about 90% of the carbobenzyloxy-groups had been converted into carboxy-groups, so that the product has essentially structure (VII). The low solubility of this product in the reaction mixture probably prevents complete reduction. The polyglutamic acid obtained by this method is not appreciably racemised, for both the poly-acid and the poly-benzyl ester give hydrolysates with the same specific rotation.

The low solubility of this synthetic polyglutamic acid in water and aqueous ethanol is in striking contrast to the high solubility of the naturally occurring polyglutamic acid. While this difference may be partly accounted for by the residual carbobenzyloxy-groups in the synthetic material, the main reason is probably that the naturally occurring acid contains both α- and γ-glutamyl residues (Hanby and Rydon, *loc. cit.*). Thus the *X*-ray powder photograph of the naturally occurring material shows an almost complete lack of crystallinity.

The structural differences between the natural and the synthetic polymers are discussed in detail in the Appendix by Mr. E. J. Ambrose. The infra-red data show the considerable difference between the arrangement of the glutamic acid residues in the two substances.

Despite these differences it was clearly important to prepare poly-D-glutamic acid for serological tests. Addition of acrylonitrile to ethyl acetamidocyanoacetate gave a 90% yield of ethyl α-acetamido-$\alpha\gamma$-dicyanobutyrate $CN{\cdot}[CH_2]_2{\cdot}C(CN)(NHAc){\cdot}CO_2Et$, which was hydrolysed by hydrobromic acid to DL-glutamic acid. This method compares favourably with other recent syntheses of DL-glutamic acid (Dunn and Rockland, *Adv. in Protein Chem.*, 1947, **3**, 295). In the acylation with benzyl chloroformate the magnesium oxide used by Bergmann and Zervas (*Ber.*, 1932, **65**, 1192) could conveniently be replaced by hot aqueous sodium hydroxide. In resolution by the method of Fruton, Irving, and Bergmann (*J. Biol. Chem.*, 1940, **133**, 703) in which *N*-carbobenzyloxy-L-glutamic acid reacts preferentially with aniline in the presence of papain, we found that the troublesome purification of papain was unnecessary. Removal of the *N*-carbobenzyloxy-group by 5N-hydrochloric acid was more convenient on a large scale than the hydrogenation procedure described by Fruton, Irving, and Bergmann (*loc. cit.*). D-Glutamic

acid was converted into the benzyl ester, and the poly-D-glutamic acid synthesised in the same way as the poly-L-glutamic acid.

When tested serologically by Dr. G. P. Gladstone of the Sir William Dunn School of Pathology, Oxford, this poly-D-glutamic acid was found not to have the haptenic activity shown by the naturally occurring poly-D-glutamic acid.

Experimental.

Ethyl α-Acetamido-αγ-dicyanobutyrate.—Ethyl acetamidocyanoacetate (102 g.) was added to sodium (0·3 g.) in ethanol (150 c.c.); the suspension was vigorously stirred and acrylonitrile (45 c.c.) added dropwise, the temperature being kept below 20°. After an hour, the *adduct* was collected and washed with ethanol (30 c.c.); a further amount crystallised from the mother-liquors, the total yield being 115—120 g. (86—90%), and the m. p. 101—102°, not raised by recrystallisation from *iso*propanol (Found: C, 54·2; H, 5·7; N, 19·1. $C_{10}H_{13}O_3N_3$ requires C, 53·85; H, 5·8; N, 18·8%).

DL-*Glutamic Acid.*—This adduct (55 g.) was boiled under reflux with hydrobromic acid (*d* 1·48; 150 c.c.) for 4 hours, the solution evaporated, and the residue freed from hydrobromic acid by repeated addition of water and evaporation. The residue was suspended in methanol (450 c.c.) and treated with pyridine (50 c.c.). After 16 hours at 0° the DL-glutamic acid was collected, washed with methanol and ether, and recrystallised from 50% aqueous ethanol (yield, 27 g., 75%) (Found: C, 40·8; H, 6·1; N, 9·5. Calc. for $C_5H_9O_4N$: C, 40·8; H, 6·2; N, 9·5%).

N-*Carbobenzyloxy*-DL-*glutamic Acid.*—DL-Glutamic acid (60 g.) in 4N-sodium hydroxide (208 c.c.) was stirred vigorously while benzyl chloroformate (84 c.c.) and 4N-sodium hydroxide (120 c.c.) were added in small portions during 20 minutes. The mixture was then at 60°, and was stirred at 50—60° for 15 minutes, further 4N-sodium hydroxide (25 c.c.) was added, and the mixture stirred for another 15 minutes. The cooled mixture was extracted with ether, the aqueous layer acidified with concentrated hydrochloric acid, and the oil isolated with ethyl acetate. The product (106 g., 93%) crystallised under light petroleum and was pure enough for the next stage.

D-*Glutamic Acid Hydrochloride.*—Papain (Hopkin and Williams, Ltd.) (22 g.) was extracted with water (500 c.c.), and the filtered solution added to *N*-carbobenzyloxy-DL-glutamic acid (152 g.) in 4N-sodium hydroxide (135 c.c.). Aniline (104 c.c.), L-cysteine hydrochloride (7 g.) and citrate buffer (450 c.c.; pH 5) were added, and the total volume was made up to 2·2 l. The mixture was set aside at 40° for 96 hours, the pH being kept at 5 by occasional additions of citric acid, and then filtered to remove *N*-carbobenzyloxy-L-glutamic acid anilide. The filtrate was concentrated to 400 c.c., made acid to Congo-red, and extracted with ethyl acetate. Removal of the solvent at 15 mm. gave *N*-carbobenzyloxy-D-glutamic acid as a colourless oil which was hydrolysed by boiling under reflux with 5N-hydrochloric acid (220 c.c.) for 2 hours. After cooling, the hydrolysate was extracted with ether to remove benzyl alcohol, then saturated with hydrogen chloride at 10°. The optically pure D-glutamic acid hydrochloride was collected, washed with concentrated hydrochloric acid, and dried *in vacuo*; yield, 40 g. (80%); $[\alpha]_D^{19}$ −25·0° (*c*, 3·445 in N-hydrochloric acid).

Reduction of N-*Carbobenzyloxy*-L-*glutamic Acid Anhydride* (I; R = $CH_2Ph{\cdot}O_2C$).—*N*-Carbobenzyloxy-L-glutamic acid anhydride (Harington and Mead, *Biochem. J.*, 1935, **29**, 1602) was recrystallised from ethyl acetate–light petroleum (charcoal). This material (2 g.) in purified acetic acid (30 c.c.) was reduced in a stream of hydrogen in the presence of 10% palladised charcoal (0·5 g.). The evolution of carbon dioxide had ceased after 1½ hours, and after 3 hours the mixture was filtered and the filtrate evaporated. The residue was dissolved in water and the solvent distilled off; the oil crystallised on storage, and was extracted with ethyl acetate (Soxhlet), giving 2-ketopyrrolidine-5-carboxylic acid (II) (0·45 g.), m. p. and mixed m. p. with an authentic sample, 160—161°, $[\alpha]_D^{25}$ −11·8° (*c*, 1·336 in water) (Found: equiv., 127. Calc. for $C_5H_7O_3N$: equiv., 129). The acid was also obtained when ethyl acetate or dioxan was used as solvent for the reduction.

γ-*Ethyl* L-*Glutamate* (III; R = Et).—L-Glutamic acid (10 g.) was added to ethanol (100 c.c.) containing hydrogen chloride (2·73 g.), and the mixture kept for 3 days. Pyridine (8 c.c.) was added and the ester collected and washed with ethanol (yield, 8·3 g.; m. p. 190°). Recrystallisation from moist ethanol gave 6·3 g., m. p. 194° (Bergmann and Zervas, *Z. physiol. Chem.*, 1933, **221**, 51, give m. p. 194°). Consistent yields could not be obtained by the methods of Bergmann and Zervas (*loc. cit.*), Abderhalden and Nienburg (*Z. physiol. Chem.*, 1933, **219**, 155), or Nienburg (*Ber.*, 1935, **68**, 2232).

γ-*Ethyl* N-*Carbobenzyloxy*-L-*glutamate* (IV; R = Et).—The following modification of Abderhalden and Nienburg's method (*loc. cit.*) was preferred. Benzyl chloroformate (4 c.c.) was added to γ-ethyl glutamate (4 g.) in water (60 c.c.) containing sodium hydrogen carbonate (4 g.). The mixture was stirred at 0° to −5° for 3 hours, then at room temperature for 2 hours, and extracted with ether, and the aqueous layer acidified. The product was isolated with ether and after two recrystallisations from carbon tetrachloride had m. p. 87° (4·7 g.) (Found: C, 58·2; H, 6·3; N, 4·8. Calc. for $C_{15}H_{19}O_6N$: C, 58·2; H, 6·2; N, 4·5%).

γ-*Ethyl* N-*Carboxy*-L-*glutamate Anhydride* (V; R = Et).—Phosphorus pentachloride (3·75 g.) was added to γ-ethyl *N*-carbobenzyloxy-L-glutamate (5·29 g.) in dry ether (100 c.c.) at 0°. After 1 hour, the mixture was boiled under reflux for a further hour. The ether was distilled off, and the residue triturated under light petroleum and then recrystallised from chloroform–carbon tetrachloride. The *anhydride* crystallised in colourless prisms, m. p. 71—72° (2·4 g., 70%) (Found: N, 7·1; OEt, 21·7. $C_8H_{11}O_5N$ requires N, 7·0; OEt, 22·3%).

Polymerisation of γ-*Ethyl* N-*Carboxy*-L-*glutamate Anhydride.*—The anhydride (2·4 g.) in dry pyridine (10 c.c.) was set aside overnight, and the viscous solution treated with 0·835N-ethanolic potassium

hydroxide (15 c.c.). After 2 hours the solid was collected, and washed with ethanol and ether. Analysis indicated that two-thirds of the ester groups had been removed (Found: N, 8·3; OEt, 9·1; K, 16·7. Calc. for $C_{22}H_{28}O_9N_3K_2$: N, 8·55; OEt, 9·2; K, 15·9%).

γ-Methyl L-*Glutamate* (III; R = Me).—L-Glutamic acid (36·8 g.) was added to a cooled solution prepared by adding acetyl chloride (20 c.c.) to methanol (250 c.c.), the mixture shaken vigorously for a few minutes until most of the solid had dissolved, and then set aside for 20 hours. Pyridine (25 c.c.) was added; after 48 hours at room temperature the *methyl* ester was collected and washed with ethanol and ether; it (17—22 g.) had m. p. 182° (decomp.). Recrystallisation from 70% methanol gave colourless leaflets, m. p. unchanged (Found: C, 44·3; H, 6·8; N, 8·4. $C_6H_{11}O_4N$ requires C, 44·7; H, 6·8; N, 8·7%). It is unnecessary for further work to recrystallise the ester if it contains less than 1% of free glutamic acid, as shown by alkali titration with bromothymol-blue as indicator.

γ-Methyl N-*Carbobenzyloxy-*L-*glutamate* (IV; R = Me).—γ-Methyl L-glutamate (26·7 g.) in water (360 c.c.) containing sodium hydrogen carbonate (29·2 g.) was stirred at 0—5° while benzyl chloroformate (30 c.c.) was added during 15 minutes. The mixture was stirred at this temperature for 1½ hours, and then stirred for a further 2 hours while warming to room temperature. The solution was extracted twice with ether and acidified, and the oil isolated with ethyl acetate. The *carbobenzyloxy*-compound crystallised on trituration under light petroleum, and, recrystallised from carbon tetrachloride, had m. p. 72—73° (40 g.) (Found: C, 56·5; H, 5·9; N, 5·0. $C_{14}H_{17}O_6N$ requires C, 56·9; H, 5·8; N, 4·7%). This compound had zero rotation in ethyl acetate solution, but in 1·4N-potassium hydrogen carbonate solution (*c*, 7·456) had $[\alpha]_D^{21}$ −15·3°. The α-p-*bromophenacyl* ester, recrystallised from aqueous ethanol, had m. p. 114—116° (Found: C, 53·4; H, 4·7; OMe, 6·5. $C_{22}H_{22}O_7NBr$ requires C, 53·7; H, 4·5; OMe, 6·3%).

γ-Methyl N-*Carboxy-*L-*glutamate Anhydride* (V; R = Me).—Phosphorus pentachloride (15·3 g.) was added to the *N*-carbobenzyloxy-ester (19·7 g.) in dry ether (350 c.c.) and after 1¼ hours the mixture was refluxed for 1¼ hours. The ether was distilled off and the residue triturated under light petroleum. After two recrystallisations from ethyl acetate the *anhydride* (7·5 g.) had m. p. 99—100° (decomp.) $[\alpha]_D^{28}$ −24·0° (*c*, 7·57 in ethyl acetate) (Found: C, 45·1; H, 5·0; N, 7·6. $C_7H_9O_5N$ requires C, 44·9; H, 4·9; N, 7·5%).

Polymerisation of *γ-Methyl* N-*Carboxy-*L-*glutamate Anhydride*.—The anhydride (1 g.) was added to pyridine (5 c.c.) and the solution kept for 2½ days. The *polymer* (VI; R = Me) was precipitated with ether and dried at 80° (Found: C, 49·3, 49·5; H, 6·2, 6·25. $C_6H_9O_3N$ requires C, 50·3; H, 6·3%). It is sparingly soluble in most solvents, but dissolved in dichloroacetic acid and in concentrated sulphuric acid. A portion of the polymer was boiled with constant-boiling hydrobromic acid (7·5 c.c.) for 24 hours, and the cooled, filtered solution made up to 15 c.c. with water. The rotation of this solution was determined, and on the assumption that a Kjeldahl nitrogen estimation gives the glutamic acid concentration, gave $[\alpha]_D^{20}$ +25·4°. The rotation of pure L-glutamic acid in constant-boiling hydrobromic acid diluted with an equal volume of water was $[\alpha]_D^{20}$ +30·2° (*c*, 2·99). For comparison a solution of L-glutamic acid was heated with hydrobromic acid under the same conditions; the rotation was $[\alpha]_D^{20}$ +26·0°. The anhydride was also polymerised in pyridine at −10°, and the polymer hydrolysed; $[\alpha]_D^{20}$ was +25·6°; similarly, when polymerisation was conducted in boiling pyridine the rotation was the same: $[\alpha]_D^{20}$ +25·5°.

The solution obtained by decomposing the anhydride (1 g.) with pyridine (5 c.c.) for 2½ days was treated with 0·85N-ethanolic potassium hydroxide (8 c.c.). After 3 hours the precipitated potassium salt was collected and dissolved in water (6 c.c.), and the filtered solution treated with 0·94N-aqueous potassium hydroxide (2·5 c.c.). A portion of this solution was dialysed for 4 days and then converted into the silver salt (VI; R = Ag) (Found: Ag, 40·7; N, 5·8; OMe, 0·35. $C_5H_6O_3NAg$ requires Ag, 45·8; N, 5·9; OMe, 0%). The rest of the solution was hydrolysed by boiling it with an equal volume of concentrated hydrochloric acid for 24 hours; the rotation of the glutamic acid was $[\alpha]_D^{20}$ +17·0°. For comparison L-glutamic acid, boiled with 6N-hydrochloric acid for 24 hours, was found to have $[\alpha]_D^{17}$ +30·0°.

Dibenzyl N-*Carbobenzyloxy-*L-*glutamate* (X).—*N*-Carbobenzyloxy-L-glutamic acid (28 g.) in N-sodium hydroxide (200 c.c.) was added to silver nitrate (34 g.) in water (150 c.c.). The silver salt was collected, washed with water, dried, and finely powdered; the yield was 46 g. (93%). This silver salt (40·5 g.), benzyl bromide (21 c.c.), and dry ether (350 c.c.) were boiled under reflux with stirring for 24 hours. The mixture was filtered, and the filtrate concentrated to low bulk and diluted with light petroleum. The *dibenzyl* ester (22 g.) had m. p. 67—69°. Further dilution of the mother-liquors with light petroleum gave impure material, which was extracted with aqueous sodium carbonate, and the residue was dissolved in ether. Dilution of the dried ethereal solution with light petroleum gave further ester (4 g.). Further recrystallisation from ether–light petroleum raised the m. p. to 71—73° (Found: C, 69·9; H, 5·8; N, 3·2. $C_{27}H_{27}O_6N$ requires C, 70·3; H, 5·85; N, 3·0%).

Hydrolysis of *Dibenzyl* N-*Carbobenzyloxy-*L-*glutamate*.—Water (4·5 c.c.) and benzyl alcohol (300 c.c.) were added to potassium hydroxide (16 g.); this solution was 0·78N. The dibenzyl ester (23·057 g.) was added to this solution (64·1 c.c.) and the whole kept overnight. After dilution with 5 volumes of ether the solution was extracted thrice with water, and the aqueous solution extracted with ether and acidified. The oil was isolated with ether, and thoroughly dried by adding carbon tetrachloride and distilling off the solvent; the yield was 15·8 g. (85%). The pure γ-benzyl ester could not be isolated, but esterification with *p*-bromophenacyl bromide gave *γ-benzyl* α-p-*bromophenacyl* N-*carbobenzyloxy-*L-*glutamate* (XI), which, after crystallisation from aqueous methanol, melted at 107—109° (Found: C, 58·75; H, 4·5; N, 2·5. $C_{28}H_{26}O_7NBr$ requires C, 59·2; H, 4·6; N, 2·5%).

γ-Benzyl L-*Glutamate* (III; R = CH_2Ph).—(*a*) L-Glutamic acid (150 g.) was added to benzyl alcohol (1 l.) containing purified constant-boiling hydriodic acid (250 c.c.), and the mixture kept overnight and

then diluted with ethanol (2 l.) and treated with pyridine (300 c.c.). After 24 hours at 0°, the solid was collected and washed with ethanol and ether. The glutamic acid content of this solid was estimated by titrating a portion against alkali with bromothymol-blue as indicator. The bulk of the solid was then recrystallised from water (2 l.) containing 1 mol. of sodium hydrogen carbonate for each mol. of glutamic acid found by titration. The *γ-benzyl* ester crystallised in colourless plates, m. p. 169—170°, $[\alpha]_D^{25}$ +18·7° (*c*, 7·162 in acetic acid) (Found: C, 60·5; H, 6·6; N, 6·3. $C_{12}H_{15}O_4N$ requires C, 60·8; H, 6·3; N, 5·9%). γ-Benzyl D-glutamate, similarly prepared, had $[\alpha]_D^{25}$ −19·1° (*c*, 4·67 in acetic acid).

(*b*) Benzyl chloride (138 c.c.) and ethanol (150 c.c.) were added to L-glutamic acid (147 g.) and basic copper carbonate (55 g.) in water (220 c.c.) containing potassium hydrogen carbonate (130 g.), and the mixture was stirred under reflux for 6 hours. The next day, the solid copper complex was collected, washed with water, and dried (yield, 105 g.). This complex (20 g.) was suspended in N-hydrochloric acid (120 c.c.) and saturated with hydrogen sulphide. After removal of copper sulphide, the filtrate was neutralised; the γ-benzyl L-glutamate (2·9 g.), collected and recrystallised from water, had $[\alpha]_D^{24}$ +18·6° (*c*, 5·58 in acetic acid).

Reduction of γ-Benzyl L-Glutamate.—The benzyl ester (1 g.) was suspended in water (60 c.c.) and hydrogenated at one atmosphere in the presence of 10% palladised charcoal (0·2 g.). When no more hydrogen was absorbed the solution was filtered and evaporated to dryness, and the residual glutamic acid washed with ether. In 6N-hydrochloric acid this product had $[\alpha]_D^{25}$ +30·1° (*c*, 2·094) [Found: N (Kjeldahl), 9·45. Calc. for $C_5H_9O_4N$: N, 9·5%].

Acid Hydrolysis of γ-Benzyl L-Glutamate.—The benzyl ester was boiled under reflux with constant-boiling hydrobromic acid (10 c.c.) in an atmosphere of nitrogen for 6 hours, and the solution extracted with benzene (to remove benzyl alcohol) and diluted to 25 c.c. with water. The rotation was determined in a 4-dm. tube and the solution analysed: α_D + 1·683°; Kjeldahl-N, 1·51 mg./c.c.; van Slyke-N, 1·50 mg./c.c. Thus, if the analytical figures are used to estimate the concentration of glutamic acid, this sample had $[\alpha]_D^{25}$ +26·4°.

γ-Benzyl N-Carbobenzyloxy-L-glutamate (IV; R = CH_2Ph).—γ-Benzyl L-glutamate (23·7 g.) and sodium hydrogen carbonate (20 g.) were dissolved in water (1 l.) at 65°, and the solution stirred vigorously while benzyl chloroformate (20 c.c.) was added quickly. Stirring was continued while the reaction mixture cooled to 50° (50 minutes). The mixture was then cooled, extracted with ether, filtered, and acidified. The oil was extracted with ether, the ethereal solution washed with water containing 1·4N-potassium hydrogen carbonate (7 c.c.) and dried, and the solvent evaporated. The residue was dissolved in carbon tetrachloride and the solvent distilled off till the volume was reduced to about 150 c.c., and the residue kept overnight. The *carbobenzyloxy*-ester (19·8 g.) crystallised in colourless needles, m. p. 76—78° (Found: C, 64·6; H, 5·6; N, 3·9. $C_{20}H_{21}O_6N$ requires C, 64·7; H, 5·7; N, 3·8%). This substance had zero rotation in ethyl acetate, but in N-potassium hydrogen carbonate (*c*, 6·30) had $[\alpha]_D^{22}$ −23°. γ-Benzyl *N*-carbobenzyloxy-D-glutamate had $[\alpha]_D^{25}$ +24° (*c*, 5·84 in N-potassium hydrogen carbonate).

γ-Benzyl N-Carboxy-L-glutamate Anhydride (V; R = CH_2Ph).—Powdered phosphorus pentachloride (23·5 g.) was added to a cooled solution of γ-benzyl *N*-carbobenzyloxy-L-glutamate (35 g.) in dry ether (200 c.c.), and the mixture shaken vigorously until most of the solid had disappeared, and then decanted. After 30 minutes at 0° the *anhydride* was collected, washed with dry ether and recrystallised twice from carbon tetrachloride containing 5% of ethyl acetate (charcoal), giving colourless needles (14 g.), m. p. 96—97° (sealed capillary; decomp.), $[\alpha]_D^{23}$ −11·2° (*c*, 3·78 in ethyl acetate) (Found: C, 59·35; H, 5·0; N, 5·5. $C_{13}H_{13}O_5N$ requires C, 59·3; H, 4·9; N, 5·3%).

Polymerisation of γ-Benzyl N-Carboxy-L-glutamate Anhydride in Dioxan.—This experiment was carried out to investigate the loss of terminal amino-groups from the poly-(γ-benzyl L-glutamate). γ-Benzyl *N*-carboxy-L-glutamate anhydride (1·2 g.) was added to a 0·0845N-solution of sarcosine dimethylamide in pure dioxan (10 c.c.). Carbon dioxide evolution stopped after 30 minutes. Aliquots (1 c.c.) were then withdrawn at intervals, diluted with dioxan (40 c.c.), and titrated with 0·01N-hydrochloric acid with 2-*p*-benzylideneaminophenylquinoline (Waley and Watson, *Proc. Roy. Soc., A*, 1949, **199**, 499) as indicator. The following results show that nearly all the amino-groups disappear in several days.

Time, hours	1	2·3	21·25	42·75	68·16	91·16	140·0
0·01N-HCl, c.c.	7·60	7·70	4·90	3·20	1·95	1·30	0·80

Poly-(γ-benzyl L-Glutamate) (VI; R = CH_2Ph).—The anhydride (13·7 g.) was added to a 0·0085N-solution of sarcosine dimethylamide in nitrobenzene (35 c.c.). After 5 days at 30° the polymer was precipitated with light petroleum, collected, and washed with acetone. As thus obtained, in nearly quantitative yield, it was a colourless, amorphous solid, very soluble in chloroform, readily soluble in dioxan or pyridine, and slightly soluble in hot acetic acid. A 5% solution in " AnalaR " chloroform had a specific viscosity of 9·33 at 25° (we are indebted to Dr. B. A. Toms for this measurement) and $[\alpha]_D^{25}$ +14° (*c*, 0·954 in chloroform).

Acid Hydrolysis of Poly-(γ-benzyl L-Glutamate).—Poly-(γ-benzyl L-glutamate) (0·5596 g.) in constant-boiling hydrobromic acid (10 c.c.) was boiled under reflux in an atmosphere of nitrogen for 6 hours, and the solution diluted with water, extracted with benzene to remove benzyl alcohol, and made up to 25 c.c. with water. The rotation was determined in a 4-dm. tube and the solution analysed: α_D +1·607°; Kjeldahl-N, 1·46 mg./c.c.; van Slyke-N, 1·47 mg./c.c. When the analytical figures are used to estimate the concentration of glutamic acid, this sample has $[\alpha]_D^{25}$ +26·2°.

Hydrogenation of Poly-(γ-benzyl L-Glutamate).—γ-Benzyl *N*-carboxy-L-glutamate anhydride (1·5 g.) was added to a solution of sarcosine dimethylamide (0·1 c.c.) in nitrobenzene (5·3 c.c.). After 3 days, the product was precipitated with light petroleum and purified by dissolution in warm ethyl acetate and reprecipitation with light petroleum. This product (0·3 g.), assumed to have the structure (XIII;

$n = 4$), in acetic acid (30 c.c.) was hydrogenated in the presence of 10% palladised charcoal (0·1 g.). After 24 hours, the uptake of hydrogen was complete, the mixture was filtered and evaporated, and the residue extracted with hot ethyl acetate. The residual colourless amorphous solid (0·195 g.) was insoluble in water, but dissolved readily in aqueous sodium hydrogen carbonate. The equivalent weight, determined by dissolution in standard sodium hydroxide and back-titration with standard hydrochloric acid, was 204; the value, calculated for complete reduction of (XIII; $n = 4$), is 190.

Reduction of Poly-(γ-benzyl L-Glutamate) with Sodium in Liquid Ammonia.—The poly(benzyl ester) (13·4 g.) was finely powdered and placed in the reduction vessel; liquid ammonia (*ca.* 60 c.c.) was distilled from sodium on to the polymer, the mixture cooled to −75°, and sodium (0·8 g.) added in portions with stirring. After 1½ hours, ammonium iodide (3 g.) was added, and the ammonia allowed to evaporate. The product was collected, washed with ethanol until free from halide ion, and dried *in vacuo*, giving 2 g. of a hygroscopic colourless solid *sodium* salt (Found: C, 40·6; H, 4·7; N, 9·5. $C_5H_6O_3NNa$ requires C, 39·75; H, 3·9; N, 9·3%). A portion was converted into the sparingly soluble silver salt (Found: C, 26·15; H, 3·3; N, 6·2; Ag, 39·6. $C_5H_6O_3NAg$ requires C, 25·4; H, 2·5; N, 5·9; Ag, 45·8%). Another portion of the sodium salt was hydrolysed to glutamic acid by boiling with constant-boiling hydrobromic acid (7·5 c.c.) for 16 hours; the solution, made up to 15 c.c. with water, had α +0·84° ($l = 2$ dm.; 2·52 mg. of N/c.c.), giving $[\alpha]_D^{20}$ +15·9°. After L-glutamic acid had been boiled with hydrobromic acid in the same manner, it had $[\alpha]_D^{20}$ +26·3°.

Reduction of Poly-(γ-benzyl L-Glutamate) with Phosphonium Iodide.—The poly(benzyl ester) (1·3 g.) was powdered and suspended in pure acetic acid (100 c.c.) at 60°; hydrogen was bubbled through the mixture, and re-sublimed phosphonium iodide (2 g.) added during 2 hours. The solution became clear, but 1 hour later a gelatinous precipitate formed. Ethanol (10 c.c.) was added, the solvents were distilled off, then further ethanol (100 c.c.) was added, and the solvents were distilled again. The residue was collected, washed with ether, dissolved in ethanol (20 c.c.), and reprecipitated by ether (200 c.c.). This yellow material (0·58 g.) was dissolved in 0·6N-potassium hydrogen carbonate (8·5 c.c.), and the solution dialysed against distilled water for 2 hours and acidified with dilute hydrochloric acid. The gelatinous precipitate was gently warmed to coagulate it, and then collected, washed with water, and dried *in vacuo*, giving 0·46 g. of a colourless solid (Found: C, 49·2; H, 5·7; N, 9·9. Calc. for a polymer containing one carbobenzyloxy-group per seven free carboxyl groups: C, 48·9; H, 5·5; N, 10·3%). This purified material is very sparingly soluble in water, ethanol, chloroform, acetic acid, *m*-cresol, or dichloroacetic acid, but is readily soluble in aqueous potassium hydrogen carbonate or in dimethylformamide. The equivalent weight of the polymer was determined by dissolution in excess of 0·1N-sodium hydroxide and back-titration with 0·1N-hydrochloric acid (Found: 160, 162). The titration solutions were analysed for nitrogen (Kjeldahl) and the mean residue weight thus determined (Found: 140, 140). The calculated values for a polymer which contains one remaining carbobenzyloxy-group to seven free carboxyl groups are equiv., 160; mean residue wt., 140.

The purified reduction product (0·2575 g.) was hydrolysed to glutamic acid by boiling hydrobromic acid (10 c.c.) during 6 hours; the solution, made up to 25 c.c., had α +1·12° ($l = 4$ dm.; Kjeldahl-N, 1·02 mg./c.c.; van Slyke-N, 1·03 mg./c.c.), giving $[\alpha]_D^{25}$ +26·0°.

Appendix. By E. J. Ambrose.

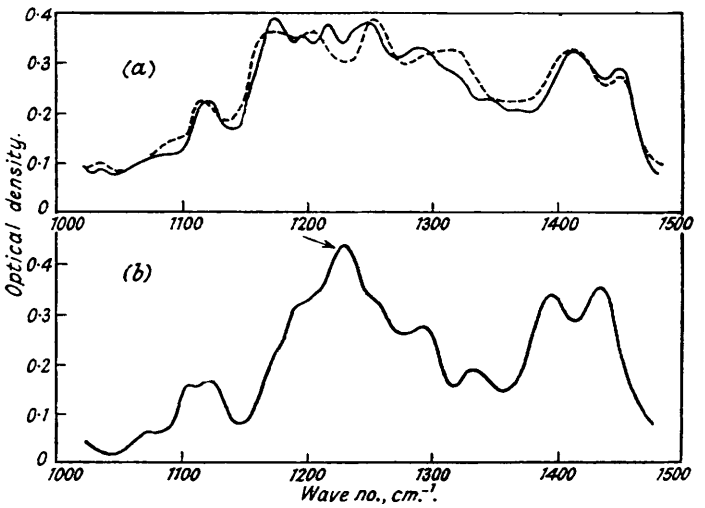
The Structures of Synthetic and Natural Polyglutamic Acids compared by Means of Infra-red Spectroscopy.—A comparison of the physical properties of the synthetic polyglutamic acid described above with those of the material which forms the capsular substance of *B. anthracis* is of interest since these two substances provide an almost direct link between a synthetic and a naturally occurring polypeptide.

Fig. 1 shows the infra-red spectra of these two materials in the spectral region 1000—1500 cm.$^{-1}$. The natural material 1(*b*) was a sample " H. 230 B " prepared as described by Hanby and Rydon (*Biochem. J.*, 1946, **40**, 297). Both polymers are soluble in dimethylformamide and the films were cast from this solvent. The spectral region shown in Fig. 1 is considered to give absorption bands which are characteristic of polypeptide side chains (Astbury, Dalgliesh, Darmon, and Sutherland, *Nature*, 1948, **162**, 596). In the two spectra, identical or almost identical absorption bands appear, either as shoulders or as distinct bands, but the overall contours of the spectra are not the same, indicating that although similar the structures are not identical. The most noticeable difference appears to lie in the presence of a strong absorption band at 1230 cm.$^{-1}$ (shown by an arrow) in the spectrum of the natural material which is not present in that of the synthetic material.

Fig. 2 gives the spectra obtained with oriented films by using polarised infra-red radiation, in the region 1500—1800 cm.$^{-1}$. The band appearing at 1658 cm.$^{-1}$ in the synthetic and at 1655 cm.$^{-1}$ in the natural material is due to a stretching mode of the C=O group in the ·CO·NH· peptide linkage. The band at 1550 cm.$^{-1}$ is due to a deformation mode of the N–H group of this linkage. There is a change in absorption on passing from the parallel to the perpendicular position of the electric vector relative to the chain axis, as shown by the broken and full line curves of Fig. 2. The type of dichroism observed is the same as that already described in the polyglutamic methyl and benzyl esters (Ambrose and Hanby, *Nature*, 1949, **163**, 483; Elliott, and Ambrose, communicated to *Nature*). It has been identified with a folded configuration of

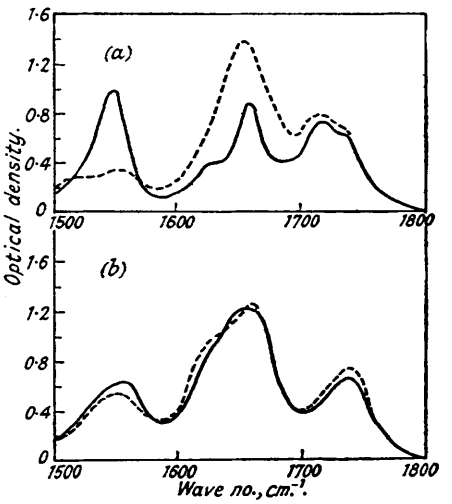
the polypeptide chain, in which the folds are produced by intramolecular hydrogen bonds between CO and NH groups. In this note the folded form is not referred to as the α-form, in order to avoid confusion with the α used to describe α-linked polypeptides. In the β- or extended form the opposite kind of dichroism is observed in both CO and NH bands. Frequency shifts are also observed when folded synthetic polypeptides pass from the folded to the extended

FIG. 1.



– – – – *E vector parallel to chains;* ——— *E vector perpendicular to chains.*
(*a*) *Synthetic polyglutamic acid.* (*b*) "H. 230 B."

FIG. 2.

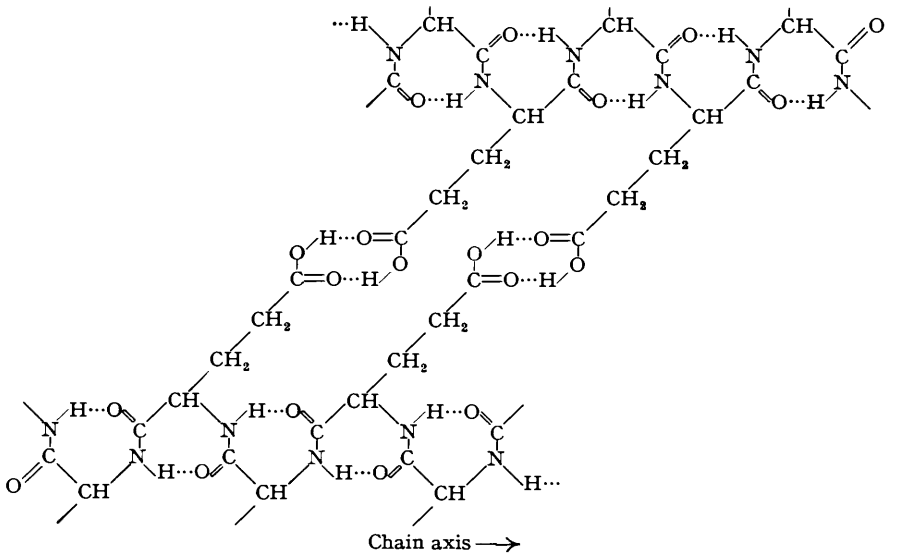


– – – – *E vector parallel to chains;* ——— *E vector perpendicular to chains.*
(*a*) *Synthetic polyglutamic acid.* (*b*) "H. 230 B."

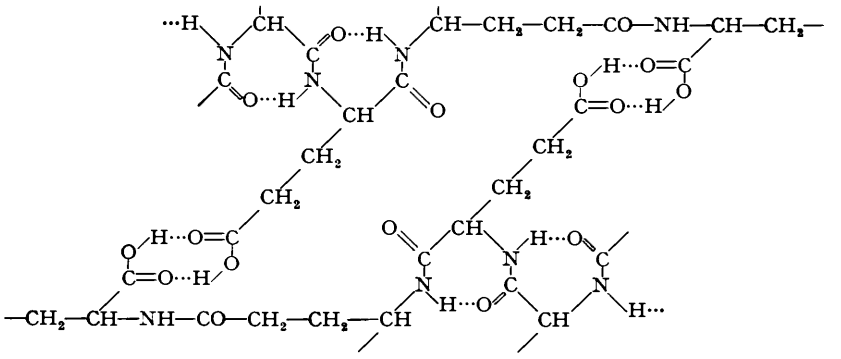
form. The characteristic CO and NH bands of the folded form are at about 1660 and 1550 cm.$^{-1}$ respectively. In the extended form they are at about 1630 and 1520 cm.$^{-1}$.

Although both materials give curves (Fig. 2) which indicate that they are in the folded configuration, the dichroism in the spectra of the natural material is considerably less and there is a pronounced broadening of the CO band on the low-frequency side. This broadening indicates the presence of a considerable proportion of the extended form, which gives a band at 1630 cm.$^{-1}$.

This band can also be seen in the spectrum of the synthetic material in the full-line curve but it is much weaker than in the natural material.



Suggested structure for synthetic polyglutamic acid.



Suggested structure for " H. 230 B."

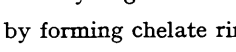
The band at 1720 cm.$^{-1}$ in the synthetic, and at 1738 cm.$^{-1}$ in the natural, material is due to the C=O stretching mode of the carboxyl groups of the side chains. In both materials it shows dichroism, which indicates that these CO groups point in a direction predominantly along the chain axis, an effect that is not observed in the polyglutamic esters.

The general conclusions which can be drawn from the spectra are as follows. The synthetic material, when cast from dimethylformamide is almost completely in the folded configuration. The natural material, when prepared from the same solvent, contains considerable proportions of both the folded and the extended configuration.

This result is consistent with the fact that the synthetic material is a completely α-linked polypeptide (see above) whereas Hanby and Rydon (*Biochem. J.*, 1946, **40**, 297) have shown that the natural products contain a proportion of γ-linked polypeptide chains. The probability of intramolecular ring formation through hydrogen bonds becomes correspondingly less as the size of the rings is increased. In the γ-linked polypeptide the size of the rings is increased by the presence of two CH_2 groups intervening between hydrogen-bonding CO and NH groups. For example, Nylon 66, which contains hydrocarbon chains in the main polyamide chain, is thought to exist only in the extended form.

The above conclusions are supported by observations of the rheological properties of

solutions of the two polymers in dimethylformamide. A synthetic polypeptide, poly-γ-benzyl L-glutamate, shows marked streaming birefringence in a small shear gradient when dissolved in chloroform. Infra-red evidence shows that in this case it is in a completely intramolecularly hydrogen-bonded state in solution (Ambrose and Elliott, communicated to the Royal Society). With the polyglutamic acids in dimethylformamide the synthetic material shows marked streaming birefringence whereas the natural material shows no birefringence. This suggests that in the natural material the presence of the extended form causes frequent cross-linking between chains by means of hydrogen bonds and an anastomosis will be present. It is only in the folded form that the chains are completely intramolecularly hydrogen-bonded and able to slide past one another in such a way as to show streaming birefringence.

The $\cdot CO_2H$ groups attached to the side chains of both polymers will, in the most stable state, satisfy all their hydrogen-bonding attractions. In crystals of straight-chain carboxylic acids this is effected by forming chelate rings of the type  (*e.g.*, in adipic acid, as shown by MacGillavry, *Rec. Trav. chim.*, 1941, **60**, 605). The spectra of the polypeptides are consistent with the existence of this ring structure because there is no sharp O—H stretching frequency. It also appears that rings of this kind provide the only structure which can give sufficient rigidity to the carboxyl groups to account for the dichroism shown by the C=O bond.

The annexed formulæ represent possible structures for the synthetic and the natural polyglutamic acid consistent with the infra-red observations. Where γ-links occur in the natural material an extended chain is present and intermolecular hydrogen bonds occur between molecules in a direction perpendicular to the plane of the paper. In both structures the carboxyl chelate rings are preserved in a tilted position with respect to the chain axis in order to account for the dichroism of the C=O bond. The γ-links in the natural material seem to be very effective in destroying regularity of structure and consequent crystallinity. Not only do they put side chains of neighbouring chains out of step, but they also produce variations in the length of the side chains and therefore in the side-chain spacing. These factors can account for the differences in solubility of the natural and synthetic polymers.

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