29. *The Structure of Native Poly-*D*-glutamic Acid. Part II.* Synthesis of α-Poly-*L*-glutamic Acid Hydrazide and the Curtius Degradation thereof.*

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Methyl α-poly-L-glutamate was converted into the polyhydrazide, which was degraded by the Curtius method. Hydrolysis then gave αγ-diaminobutyric acid. This corroborates the previous conclusion (Part I*) that in native poly-D-glutamic acid the glutamyl units are, predominantly at least, in bonds other than α, since the analogous degradation and acid hydrolysis of the polyhydrazide prepared from native poly-D-glutamic acid affords no detectable quantity of αγ-diaminobutyric acid.

IT was reported (Kovács and Bruckner, *Research*, 1952, **5**, 194; Part I*) that poly-D-glutamic acid (molecular weight 6400) secreted by *B. subtilis* (Ivánovics and Bruckner, *Naturwiss.*, 1937, **25**, 250; *Z. Immunforsch.*, 1937, **90**, 304; **91**, 175; Bruckner, Ivánovics, and Kovács Oskolás, *Magyar Chem. Fol.*, 1939, **45**, 131; *Chem. Abs.*, 1940, **34**, 3766; Bovarnick, *J. Biol. Chem.*, 1942, **145**, 415) yields, on Curtius degradation of its polyhydrazide and subsequent hydrolysis, β-formylpropionic acid. This suggested the predominance of γ-peptide bonds (I), since αγ-diaminobutyric acid could not be isolated from the hydrolysate.

According to Hanby and Rydon (*Biochem. J.*, 1946, **40**, 297), poly-D-glutamic acid of lower molecular weight (*e.g.*, 6400)—resulting from partial hydrolysis of the original polymeric material of *B. anthracis* capsules during isolation—did not contain γ-glutamyl bonds. This would seem to be inconsistent with above statements, since the identity of the poly-D-glutamic acids of *B. subtilis* and *B. anthracis* of lower molecular weight (*e.g.*, 6400) appears to be established (Ivánovics and Bruckner, *loc. cit.*).

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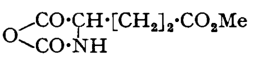
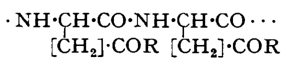
* Part I, *J.*, 1952, 4255.

$$\cdots NH{\cdot}CH(CO_2H){\cdot}[CH_2]_2{\cdot}CO{\cdot}NH{\cdot}CH(CO_2H){\cdot}[CH_2]_2{\cdot}CO \cdots \quad (I)$$



$$O\begin{matrix} \diagup CO{\cdot}\underset{|}{C}H{\cdot}[CH_2]_2{\cdot}CO_2Me \\ \diagdown CO{\cdot}NH \end{matrix}$$

(II)



$$\cdots NH{\cdot}\underset{[CH_2]{\cdot}COR}{CH}{\cdot}CO{\cdot}NH{\cdot}\underset{[CH_2]{\cdot}COR}{CH}{\cdot}CO \cdots$$

(III; R = OMe) (IV; R = NH·NH$_2$)

We now have investigated the analogous degradation of methyl α-poly-L-glutamate (III). This was prepared from 4-2′-carbomethoxyethyloxazolid-2 : 5-dione (II) by Hanby, Waley, and Watson (*J.*, 1950, 3239), using pyridine, and by Coleman (*J.*, 1950, 2294) who used dry pyridine, dioxan, ethyl acetate, or chloroform in the presence of the sodium salt of phenylalanine as initiator. Following the same route, except that water was preferred as initiator, we obtained a readily filtrable, colourless methyl α-poly-L-glutamate in almost quantitative yield.

The polyester readily formed a polyhydrazide (IV), with about 75% of the theoretical nitrogen content. It appears that the polyhydrazide undergoes partial hydrolysis of the hydrazide groups during its isolation. (The biuret reaction of this product is positive and is accompanied by slight reduction of the reagent.) In dilute alkaline solution the optical rotation shows the rapid decrease characteristic of α-polypeptides (Dakin, *J. Biol. Chem.*, 1912, **13**, 357; Levene and Bass, *ibid.*, 1929, **82**, 171; Levene, Bass, and Steiger, *ibid.*, p. 167; Dakin and Dudley, *ibid.*, 1913, **15**, 263; Bovarnick and Clarke, *J. Amer. Chem. Soc.*, 1938, **60**, 2426).

Curtius degradation of the polyhydrazide and subsequent acid hydrolysis afforded αγ-diaminobutyric acid, isolated as diflavianate and also as dipicrate. The assay gave positive results even when no more polyhydrazide was degraded (under exactly the same conditions) than the quantity of native poly-D-glutamic acid polyhydrazide (80—100 mg.) used in our former degradations. This further supports our opinion (Part I) that even *B. subtilis* poly-D-glutamic acids of low molecular weight cannot contain α-glutamyl bonds in appreciable proportion.

The yield of the αγ-diaminobutyric acid obtained as diflavianate from the hydrolysate was only 38·4%, being decreased by the following factors: (*a*) the polyhydrazide used as starting material was only about 75% pure (see above); (*b*) precipitation of the polyhydrazide starts before all hydrazide groups have reacted. For these reasons, no doubt, glutamic acid was always found in the hydrolysate.

EXPERIMENTAL

α-Methyl L-glutamate hydrochloride was prepared by a modification of Coleman's method (*loc cit.*): Finely powdered L-glutamic acid hydrochloride (15 g.) was shaken with anhydrous methanol (60 ml.) containing hydrogen chloride (0·8 g.) for 30 min. The solution, filtered from a trace of undissolved material, was quickly evaporated under reduced pressure at 40°. The solid residue was redissolved in hot dry methanol (16 ml.), mixed with dry ether (75 ml.), and set aside in the ice box; colourless prisms were formed, m. p. 154° (11·2 g.). With large quantities the yield was lower. Purification of large quantities of the crude material is preferably done by suspension in dry acetone, washing on the filter with the same solvent, and suction until dry.

4-2′-Carbomethoxyethyloxazolid-2 : 5-dione (II) was prepared by slight modification of Coleman's method (*loc. cit.*): γ-Methyl L-glutamate hydrochloride (100 g.), suspended in anhydrous dioxan (500 ml.), was saturated under vigorous stirring with carbonyl chloride (5 hours) at 40°. After most of the hydrochloride had dissolved, the mixture was filtered and evaporated under reduced pressure at 50°. The oily residue crystallized from anhydrous chloroform (100 ml.) on addition of 1 ml. of light petroleum, forming colourless crystals (45 g.), m. p. 99° (cf. Hanby, Waley, and Watson, *loc. cit.*; Coleman, *loc. cit.*).

Methyl α-Poly-L-glutamate (III).—The dione (II) is readily polymerised in moist dioxan or ethyl acetate. The following method was satisfactory: To (II) (33 g.) in anhydrous ethyl acetate (200 ml.) were added 5 drops of water; evolution of carbon dioxide set in and lasted for about 4 hours. After 2 days, the colourless solid polyester was collected, washed on the filter with hot water until the filtrate no longer gave the biuret reaction, and dried at 80°/1 mm. over P_2O_5 (yield 25 g.) [Found: N, 9·8; OMe, 20·7. Calc. for $(C_6H_3O_3N)_n$: N, 9·8; OMe, 21·7%].

(The water-soluble material was probably low polymers.) Similar treatment of (II) (55 g.) in dioxan (200 ml.) afforded polymer (40 g.) with a slight yellow tinge.

*α-Poly-*L*-glutamic Acid Hydrazide* (IV).—Finely powdered polyester was ground with 10 times its weight of anhydrous hydrazine and almost completety dissolved on gentle boiling. The solution was cooled, centrifuged, and evaporated at 0·001 mm., and the residue ground with water and freeze-dried. Drying at 100°/1 mm. (P_2O_5) then gave the ***hydrazide*** as an almost colourless amorphous powder in nearly quantitative yield [Found: OMe, 0·1—0·5; N, 24·1—25·7. $(C_5H_9O_2N_3)_n$ requires N, 29·35%]. The product is readily soluble in dilute hydrochloric acid, and slightly soluble in dilute alkali. From dilute acid solutions flavianic acid precipitates the practically insoluble polyflavianate. When the polyhydrazide was dissolved in the minimum of N-hydrochloric acid, and the solution evaporated *in vacuo*, it afforded a polyhydrochloride readily soluble in water. This procedure, however, caused perceptible hydrolysis of the hydrazide groups, as indicated by the distinct increase of the solubility in N-alkalis.

Optical rotation. Polyhydrazide (0·504 g.) was dissolved in N-hydrochloric acid (2·6 ml.), the solution evaporated under diminished pressure, and the residue dissolved in water (2 ml.) and N-sodium hydroxide (13 ml.). The initial rotation, α_D −1·20° ($l = 1$), gradually decreased during 10 days to α_D −0·20°. In order to confirm that this decrease was due to partial racemization and not to hydrolytic decomposition, we ran a control as follows. The 10-days old alkaline solution was acidified (5 ml. of concentrated hydrochloric acid) and then evaporated under reduced pressure. The residue was redissolved in concentrated hydrochloric acid (6 ml.) and refluxed for 6 hours, the hydrochloric acid removed by evaporation, and the residue dissolved in water (8·5 ml.). After addition of a minute quantity of charcoal the solution was filtered; it had α_D +0·10° ($l = 1$) whereas a solution of 0·64 g. of L-glutamic acid hydrochloride (equiv. to 0·504 g. of polyhydrazide), 0·35 g. of hydrazine dihydrochloride, and 0·76 g. of sodium chloride in 8·5 ml. of water had α_D +1·49° ($l = 1$). This is considered to prove that the decrease of the rotation of the polyhydrazide in alkaline solution results from racemization.

*Degradation of α-*L*-Polyglutamic Acid Hydrazide.*—(*a*) A solution of polyhydrazide (180 mg.) in N-hydrochloric acid (1·4 ml.) was evaporated under reduced pressure, and the residue dissolved in water (2 ml.). With cooling and stirring, 4·1% sodium nitrite solution (1·94 ml.) was added (10 min.). The gummy precipitate and the turbid solution were warmed over a micro-burner until foaming ceased and the precipitate coagulated (1—2 min.), concentrated hydrochloric acid (20 ml.) was added, and on further warming (10 min.) the precipitate completely dissolved. The solution was evaporated under reduced pressure, the residue redissolved in concentrated hydrochloric acid (10 ml.), and the whole refluxed for 15 hours, and then evaporated to dryness. The residue was dissolved in water (0·6 ml.), saturated aqueous picric acid (10 ml.) added, and the solid (a few mg.) collected next day and washed repeatedly with water (total, 4 ml.). The m. p. (185°) rose after one recrystallization from water (2 ml.) to 188—189° undepressed on admixture with L-αγ-diaminobutyric acid dipicrate, m. p. 187—189° (Found: C, 33·7; H, 3·1. Calc. for $C_{16}H_{16}O_6N_8$: C, 33·3; H, 2·8%). In another experiment the polyhydrazide (77 mg.) was decomposed under the conditions employed in the degradation of native D-polyglutamic acid (83 mg.) (Kovács and Bruckner, *loc. cit.*), and 2 mg. of diaminobutyric acid dipicrate were isolated.

(*b*) To a solution of polyhydrazide (250 mg.) in 0·3N-hydrochloric acid (12 ml.), with stirring and cooling, 5·2% sodium nitrite solution (2·5 ml.) was added dropwise. The mixture was worked up as under (*a*) except that hydrolysis was for only 4 hours. The residue obtained on evaporation was redissolved in hot water (2·5 mm.) in which flavianic acid (1 g.) was then dissolved. On cooling, the solution set to a mass of crystals. The product was collected, and washed with cold water, methanol, and then ether, a yield of 496 mg. (38·4%) of dry flavianate being obtained; m. p., after one recrystallization from water, 239°, as reported for L-αγ-diaminobutyric acid diflavianate (Found: C, 38·2; H, 3·2; N, 11·0. Calc. for $C_{24}H_{22}O_{18}N_6S_2$: C, 38·5; H, 3·0; N, 11·25%). In another experiment the degradation of the polyhydrazide (100 mg.) and the assay of αγ-diaminobutyric acid were carried out under the conditions of our previous degradation of native D-polyglutamic acid (195 mg.) in which no diaminobutyric acid diflavianate could be obtained (from 97·5 mg. of polyhydrazide), but in the present experiment 26·2 mg. of diflavianate were obtained.

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