30. *The Structure of Native Poly-D-glutamic Acid. Part III.**

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Poly-D-glutamic acid, secreted by *B. subtilis* in the culture medium, was isolated as the acid sodium salt and thence converted directly into the polymethyl ester. Conversion of this into the polyamide followed by Hofmann degradation and acid hydrolysis, gave β-formylpropionic acid but no detectable quantities of αγ-diaminobutyric acid. The quantity of β-formylpropionic acid isolated as its *p*-nitrophenylhydrazone suggests that probably only γ-glutamyl bonds are present in the polyacid, since no evidence has been found for α-glutamyl bonds.

In Part I (*J.*, 1952, 4255; see also Kovács and Bruckner, *Research*, 1952, **5**, 194) it was reported that the poly-D-glutamic acid secreted in the culture medium by *B. subtilis*, and of molecular weight *ca.* 6400, contains mainly γ-glutamyl bonds. This conclusion was based on the absence of αγ-diaminobutyric acid, which could readily have been identified (Kovács, Bruckner, and Kovács, Part II *), from the products of hydrolysis of the poly-glutamylhydrazine obtained from the acid *via* the methyl ester. Since, however, the yield of β-formylpropionic acid, isolated as its *p*-nitrophenylhydrazone from the hydrolysate was only 14·5% of that to be expected from a pure hydrazide, we sought to obtain a better yield by degradation of the polyacid amide by Hofmann's method.

For this purpose we used poly-D-glutamic acid produced by a strain of *B. subtilis* apparently identical with that used by Bovarnick (*J. Biol. Chem.*, 1942, **145**, 415). In view of Hanby and Rydon's suggestion (*Biochem. J.*, 1946, **40**, 297) that the acid used by Ivánovics and Bruckner (*Z. Immunforsch.*, 1937, **90**, 304; **91**, 175; *Naturwiss.*, 1937, **25**, 250) had been partly degraded by too long contact with acid, we took precautions to minimise this. From the copper salt of the acid, we prepared the sodium salt, and thence the polymethyl ester (I). This was not quite homogeneous and was divided into two fractions, one containing a trace of ash, and the other ash-free. The latter had a molecular weight of *ca.* 10,000, as calculated from its amino-nitrogen content (0·14%), corresponding to 9000 for the free poly-D-glutamic acid. Although this is higher than the values, 6400—7100, found for preparations isolated by Ivánovics and Bruckner's method (*loc. cit.*), it is much lower than the maximum values obtained by Hanby and Rydon (*loc. cit.*) for the acid obtained from the capsules of *B. anthracis*, *viz.*, 45,300. The methoxyl contents of the two fractions indicated 94 and 89% esterification of the carboxyl groups.

Both polymethyl ester fractions were readily converted into the amide (II) by liquid ammonia in a sealed tube at 25—30°, but the nitrogen contents of the amide were slightly low (20·6 and 21·5. Calc.: 21·8%). Fraenkel-Conrat, Olcott, and Cooper (*J. Amer. Chem. Soc.*, 1945, **67**, 950) prepared the polyamide by a similar method and concluded, from analyses for total and amido-nitrogen, that only 80% of the ester groups had undergone amidation, but in our opinion such analyses do not give a reliable measure of the degree of amidation since some of the original carboxyl groups escape esterification and are converted into ammonium salts. As calculated from our esterification values (above), amidation appears to give polyamide of *ca.* 90% purity.

$$\underset{\text{(I; R = CO}_2\text{Me)}}{\text{NH}_2\cdot\text{CHR}\cdot[\text{CH}_2]_2\cdot\text{CO}\cdot[\text{NH}\cdot\text{CHR}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CO}]_n\cdot\text{NH}\cdot\text{CHR}\cdot[\text{CH}_2]_2\cdot\text{R}}$$
$$\text{(II; R = CO}\cdot\text{NH}_2\text{)} \qquad \text{(III; R = NH}_2\text{)}$$

$$\text{(III)} \longrightarrow \underset{\text{(IV)}}{(n+1)\text{OHC}\cdot[\text{CH}_2]_2\cdot\text{CO}_2\text{H}} + \underset{\text{(V)}}{\text{OHC}\cdot[\text{CH}_2]_2\cdot\text{NH}_2}$$

The polyamide was degraded by Hofmann's method, the resulting solution hydrolysed by hydrochloric acid, and β-formylpropionic acid (IV) precipitated as its *p*-nitrophenylhydrazone. αγ-Diaminobutyric acid could not be detected even by flavianic acid. No

* Part II, preceding paper.

reaction could be obtained for β-aminopropaldehyde (V) which would be formed from the terminal unit in (III) on hydrolysis, but as this substance is rather unstable and would be formed in only very small amount, this failure is not regarded as significant.

Best yields of β-formylpropionic acid were obtained by use of the theoretical quantity of sodium hypochlorite and then brief hydrolysis with 10% hydrochloric acid: 50 mg. of polyamide gave 38 mg. (41%) of β-formylpropionic acid *p*-nitrophenylhydrazone. However, in control experiments with β-formylpropionic acid only 47—54% yields of the *p*-nitrophenylhydrazone were obtained, so, as our polyglutamide was only 90% pure, we regard the above results as indicating the presence in poly-D-glutamic acid of γ-glutamyl bonds almost exclusively, with little if any α-glutamyl bonds.

The degradation product (III) appears to undergo slight hydrolysis in the alkaline hypochlorite solution since towards the end of the reaction ammonia can be smelled. This fact would account for at least part of the slight deficiency of the *p*-nitrophenylhydrazone in the actual as compared with the control experiments.

Experimental

Methyl Poly-D-glutamate.—*B. subtilis* strain No 16, of the Institute of Medical Chemistry of the University of Budapest, was used, and was found to be identical with strain No. 712 of the Department of Agriculture, U.S.A., used by Bovarnick (*loc. cit.*). The medium was worked up according to her method, and the polypeptide precipitated as its copper salt. This was dissolved in 1·5N-hydrochloric acid, and the solution dialysed first against a citrate buffer (0·5M, pH 5·0), and then, after removal of copper ions, against distilled water and freeze-dried until the volume was about 30 ml. Methanol–ether (100 + 20 ml.) precipitated the acid sodium salt of D-polyglutamic acid. The supernatant liquid was removed, and the sticky precipitate washed in the centrifuge four times with methanol, and dried ($CaCl_2$) in a vacuum-desiccator. The product was then dissolved in a small quantity of water, and the solution evaporated by freeze-drying. The residue was dried at 60°/0·001 mm (P_2O_5) to an almost colourless powder (5 g.). A suspension of this substance (4 g.) in absolute methanol (100 ml.) was mixed with 2 ml. of acetyl chloride. On shaking, within a few minutes, almost complete dissolution occurred with slight evolution of heat. The solution was kept at room temperature for 12 days with exclusion of atmospheric moisture, after which any precipitated material (mostly inorganic) was removed on the centrifuge. Absolute ether (35 ml.) precipitated from the remaining solution a copious solid (no longer sticky) which was isolated by centrifuge, and washed with a small amount of absolute methanol. These washings and the original mother-liquor were mixed and retained (solution A), and the precipitate was further washed three times with 15 ml. of water on the centrifuge, the washings being collected (solution B). Only 0·2 g. remained undissolved.

(*a*) Mixture A was mixed with 2 vols. of absolute ether. A gum appeared on the sides of the flask; this was dissolved in 40 ml. of methanol, and reprecipitated by absolute ether. It was then freed from solvents on the centrifuge and washed three times with small amounts of water. The aqueous washings (C) were added to solution B. The water-insoluble polymethyl ester was dried *in vacuo* (P_2O_5), powdered, and again dried (P_2O_5) for 4 hours at 100°/1 mm. The resulting product (0·6 g.) was ash-free [Found: MeO, 20·3; total N, 9·5. Calc. for $(C_6H_9O_3N)_n$: MeO, 21·7; total N, 9·8%]. The amino-N was 0·14%.

(*b*) The combined liquors B and C were evaporated *in vacuo* at room temperature, and the residue washed three times on the centrifuge with water (3 × 5 ml.) and then dried in a vacuum-desiccator. The product was powdered, dissolved in warm methanol, and mixed with an equal volume of water, the solution freeze-dried, and the remaining product dried for 4 hours (P_2O_5) at 100°/1 mm., giving an almost colourless substance of loose, cottony appearance (0·9 g.) (Found: ash, 0·8; MeO, 19·3; total N, 9·7; amino-N, 0·17%). The last value indicates a molecular weight of 8240, corresponding to *M* 7430 for the free polyacid.

Poly-D-glutamic Acid Amide.—(*a*) The ash-free polyester (0·5 g.) was kept in liquid ammonia (distilled from sodium) (30 ml.) in a sealed tube at room temperature for 48 hours, the ammonia evaporated, and the residue dissolved in water. Some insoluble substance was removed by filtration, and the filtrate freeze-dried. The colourless, cottony residue (0·4 g.) was dried at 78°/0·001 mm. for 12 hours [Found: total N, 20·6; amido- + ammonium-N, 10·0; MeO, 0·3. Calc. for $(C_5H_8O_2N_2)_n$: total N, 21·8; amido-N, 10·9; MeO, 0·0%].

(*b*) The other polyester (*b*) (380 mg.) was similarly treated, but the final solid was washed

on the centrifuge three times with 3 ml. of cold water, then dried first by freeze-drying and later at 100°/1 mm. (P_2O_5) (15 hours); the yield was 185 mg. (Found: total N, 21·5; amido- + ammonium-N, 10·7; ash, 0·5; MeO, 0·2%).

Degradation of the Polyamide.—(*a*) Polyamide (*a*) (50 mg.) was dissolved at room temperature in 1·4% sodium hypochlorite solution (2·1 ml.); dissolution occurred within a few minutes save for a few flocks, and after 1 hour the smell of ammonia appeared. When the mixture was immersed for 10 minutes in a water-bath at 50°, the flocks disappeared. Then the mixture was cooled to room temperature, acidified with concentrated hydrochloric acid (10 ml.), and refluxed for 30 minutes. The solution was evaporated *in vacuo*, water added, and the solution evaporated to dryness. The remaining solid was mixed with 12 drops of a freshly prepared solution of *p*-nitrophenylhydrazine in N-hydrochloric acid, put for a few seconds into boiling water, and cooled again under the tap. The yellow crystalline solid was collected, washed on the filter with a total of 3 ml. of water, and dried *in vacuo* (P_2O_5); the yield was 32 mg. (*i.e.*, 38·4% of theory for a 90% pure polyamide); the m. p. was 170—172°, raised by one recrystallisation from water to 178°, undepressed by authentic β-formylpropionic acid *p*-nitrophenylhydrazone.

(*b*) Polyamide (*b*) (50 mg.) was similarly treated, the addition of hydrochloric acid being adjusted to give a 10% concentration in 5 ml. of solution, which was refluxed for 30 minutes and worked up as under (*a*); 38 mg. of *p*-nitrophenylhydrazone were obtained (*i.e.*, 45·6% on the basis of a 90% pure polyamide); m. p., etc., as in (*a*).

(*c*) Polyamide (*a*) (50 mg.) was degraded as under (*a*), and the alkaline mixture acidified and evaporated to dryness, the residue being redissolved in concentrated hydrochloric acid (10 ml.) and refluxed for 4 hours. After removal of the acid by distillation, the residue was again dissolved in water, but αγ-diaminobutyric acid could not be detected either as dipicrate (Kovács and Bruckner, *loc. cit.*) or even with flavianic acid.

Control Experiments.—(i) From 36 mg. of freshly prepared β-formylpropionic acid (the amount which would be expected to be formed on degradation from 50 mg. of 90% pure γ-polyglutamic amide), b. p. 132°/14 mm., in a series of experiments (precipitation by 12 drops of the usual reagent, filtration, repeated washings with a total of 3 ml. of water, and drying), the yield of *p*-nitrophenylhydrazone, m. p. 172—173°, was 71—77 mg. (85·3—92·5%). (ii) A solution of 36 mg. of freshly prepared β-formylpropionic acid in 5 ml. of 10% hydrochloric acid was refluxed for 30 minutes, and the solution worked up as for the hydrolysate in the degradation experiments; the yield of *p*-nitrophenylhydrazone, m. p. 172—173° was 39—45 mg. (46·6—53·8%). (iii) When 36 mg. of β-formylpropionic acid were kept in 1·5—2·1 ml. of 8·5% sodium hydroxide solution for 1 hour at room temperature, and 10 minutes at 50°, then treated with hydrochloric acid, as in the methods described earlier, and refluxed for 30 minutes, the residue obtained in the usual way no longer gave detectable quantities of the *p*-nitrophenylhydrazone. (iv) Since sodium hypochlorite degrades glutamic acid to β-formylpropionic acid (Langheld, *Ber.*, 1909, **42**, 2360), we ran controls in order to study the alkaline hydrolysis of poly-D-glutamic acid under the conditions of the Hofmann degradation of the polyamide. We found that in alkaline (8·5%) solutions of the polyacid the amino-N, 0·22, increased only to 0·29, showing that no appreciable hydrolysis occurs under the conditions of the Hofmann degradation.

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