816. *The Structure of Native Poly-D-glutamic Acid. Part I.**

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The poly-D-glutamic acid secreted by *Bacillus subtilis* was converted through the polymethyl ester into the polyhydrazide. After Curtius degradation and subsequent acid hydrolysis of the product, only β-formylpropionic acid (succinic semialdehyde) was found in the hydrolysate, and no αγ-diaminobutyric acid. This suggests that in native poly-D-glutamic acid, γ-glutamyl bonds predominate.

In 1937 Iványovics and Bruckner (*Z. Immunforsch.*, **90**, 304; **91**, 175; *Naturwiss.*, **25**, 250) reported the isolation, from cultures, of the capsular substance responsible for the high virulency of anthrax (Gruber and Futaki, *Münch. Med. Wochenschr.*, 1907, **54**, 249; Preisz, *Zentr. Bakt.*, 1907, **44**, 209); by a technique based on that of Tomcsik and Szongott (*Z. Immunforsch.*, 1932, **76**, 214; 1933, **78**, 86) and of Iványovics and Erdös (*ibid.*, 1937, **90**, 4), they obtained a well-defined substance, the hapten character of which, realised first by Tomcsik and Szongott (*loc. cit.*), made it possible to control further purification by serological reactions. Iványovics (*Zentr. Bakt.*, 1937, **138**, 211) attributed the existing close serological relationship between aërobic sporal microbes and anthrax bacilli to the specific substance of the anthrax capsules, which the microbes of the *subtilis* class convert in the culture medium into a form readily isolable as the insoluble copper salt. The chemical identity of this water-soluble acid substance with the hapten of the anthrax capsules was confirmed by Iványovics and Bruckner (*loc. cit.*) by elementary analysis, neutralisation equivalent, and amino-nitrogen content. The last suggested an approximate molecular weight of 6400—7000 for the *B. subtilis* polypeptide. The identity of the two substances was further demonstrated by acid hydrolysis; in both cases the amino-nitrogen value increased and finally became constant, D-(−)-glutamic acid being the product. The average yield of the hydrochloride was 86% of the value calculated on the assumption that the haptenic polypeptide contains only D-(−)-glutamic acid units, as suggested also by further analytical investigations (Bruckner, Iványovics, and Kovács Oskolás, *Magyar Chem. Foly.*, 1939, **45**, 131; *Chem. Abs.*, 1940, **34**, 3766).

These observations were later confirmed by Bovarnick (*J. Biol. Chem.*, 1942, **145**, 415) and then by Hanby and Rydon (*Biochem. J.*, 1946, **40**, 297), on haptens isolated from *B. subtilis* and from *B. anthracis*, respectively. Hanby and Rydon converted the glutamic acid from acid hydrolysis of *B. anthracis* hapten into pyroglutamic acid, which was quantitatively isolated by chromatography on silica gel. Both the yield and the values obtained from the partition chromatogram of acetylated hydrolysates suggested the presence only of glutamic acid. Although glycine and aspartic acid would remain undetected by this method, the recorded yield of glutamic acid was 99%, proving the absence of other amino-acids. Pongor (*Experientia*, 1950, **6**, 421), using paper chromatography, confirmed this conclusion.

All reports therefore agree that the polypeptide produced by *B. anthracis* and *B. subtilis* is exceptional among the *native* polypeptides in being built up from units of one single amino-acid. Such polypeptides may be termed "monotone polypeptides." (A number of polypeptides of this type have recently been obtained by synthesis; see review by Wieland, *Angew. Chem.*, 1951, **63**, 7.)

Present views on the detailed structure of native poly-D-glutamic acid are conflicting. Iványovics and Bruckner (*loc. cit.*) indicated that the following structural types are to be considered: (*a*) pure α-glutamyl bonds (I*a*), (*b*) pure γ-glutamyl bonds (IV*a*), and (*c*) combinations of both types. Bovarnick (*loc. cit.*) advocated only (IV*a*). Hanby and Rydon (*loc. cit.*) suggested that, in the polyglutamic acid of *B. anthracis*, α-peptide bonds predominate, chains of 50—100 α-bonded D-glutamic acid units being held together by small γ-bonded chains; they noted that the molecular weights of isolated samples were considerably higher (*e.g.*, 53,000) when, in isolation and purification by Iványovics and

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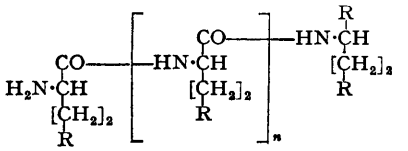
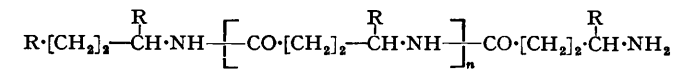
Bruckner's method (*loc. cit.*), the action of acid was kept to a minimum, and they believe that samples of molecular weight 5000—6000, as investigated by Iványovics and Bruckner, are products of degradation occurring during isolation, and contain only α-glutamyl bonds. It is, however, noteworthy that synthetic α-polyglutamic acid (Hanby, Waley, and Watson, *J.*, 1950, 3239; Coleman, *J.*, 1950, 3222) is practically insoluble in water and gives a strongly positive biuret reaction, whereas the native substance is water-soluble and gives no biuret reaction. Also, we find that the optical activity of alkaline solutions of synthetic α-poly-D-glutamic acid decreases quickly owing to racemisation (to be published later), whereas under the same conditions that of the native polypeptide remains unchanged (Bovarnick, *loc. cit.*). Although it may seem relatively less important that synthetic α-poly-D-glutamic acid gives no serological reaction (Hanby, Waley, and Watson, *loc. cit.*)—since in the case of the native substance these reactions may possibly depend on the presence of intermittent γ-peptide-bonded chains—this fact remains nevertheless remarkable in view of the very distinct serological reaction of native poly-D-glutamic acid of low molecular weight (*e.g.*, 6400—7000), which, according to Hanby and Rydon, would contain only α-glutamyl bondings.

Primarily, our present work was designed to decide, at least qualitatively, whether in native poly-D-glutamic acid the α- or the γ-glutamyl bonds predominate, especially as lack of a reliable method for distinction between the different types of peptide bonds seems to have handicapped structural investigation of proteins (*Ann. Reports*, 1949, **46**, 218).



(I; R = CO_2R') (II; R = CO·NH·NH_2)
(I*a*; R = CO_2H) (III; R = NH_2)



(IV; R = CO_2R') (IV*a*; R = CO_2H) (V; R = CO·NH·NH_2) (VI; R = NH_2)

After conversion of a polyglutamic acid (I*a*) into its methyl ester (I; R′ = Me) and thence into the corresponding polyhydrazide (II), the last would be expected to give on Curtius degradation a monotone polypeptide (III). A glutamic acid of partial structure (IV*a*) would, on the other hand, give ultimately, *via* a polyhydrazide (V), a substance of structure (VI). Now, on hydrolysis a polypeptide (III) would give αγ-diaminobutyric acid, whereas a polypeptide (VI) would give under mild conditions β-formylpropionic acid. If both (I*a*) and (IV*a*) were present in the polypeptide, the hydrolysate would contain both products in a ratio corresponding to that of the α- and γ-glutamyl bonds originally present in the polypeptide.

We have applied this method to native poly-D-glutamic acid prepared by Iványovics and Bruckner's method (*loc. cit.*) from a suitable *B. subtilis* strain. The molecular weight, calculated from van Slyke amino-nitrogen values, was 6400. On Hanby and Rydon's argument—if this should apply also to the *B. subtilis* polypeptide—α-glutamyl bonds would be expected to predominate. We find, however, that on degradation of 83 mg. of the polyhydrazide no αγ-diaminobutyric acid could be detected in the hydrochloric acid hydrolysate, even as its very insoluble diflavianate, whereas 20 mg. of β-formylpropionic acid could be easily separated from the solution in the form of its *p*-nitrophenylhydrazone. Further, according to Hanby and Rydon's arguments, 83 mg. of a polyhydrazide prepared from polypeptide of molecular weight 6400 should produce no more than 1·0 mg. of β-formylpropionic acid, whereas αγ-diaminobutyric acid should be produced in such quantity that its presence could not have escaped detection, not only as diflavianate but even as the dipicrate.

It is true that in our experiments only 14·5% of the theoretical quantity of β-formylpropionic acid *p*-nitrophenylhydrazone was isolated which would be expected from polyglutamic acid of structure (IV*a*), but the yield would be decreased by three factors: (*a*) the Curtius degradation of the polyhydrazide is not quantitative; (*b*) β-formylpropionic

acid is rather unstable under the conditions of the acid hydrolysis; and (*c*) the separation of the *p*-nitrophenylhydrazone of this acid is not quantitative. With these limitations, we conclude that in native poly-D-glutamic acid of *B. subtilis* the γ- rather than the α-glutamyl bonding predominates.

We are extending our investigations to native poly-D-glutamic acid of higher molecular weight, prepared from *B. anthracis*, and are refining our method for a reliable assay on the α : γ ratio of the glutamyl bonds by the quantitative determination of ammonia present in the hydrolysate obtained after degradation, 2 equivalents of ammonia being expected to result for each γ-glutamyl bond. Further, experiments to be reported shortly, in which we converted the polymethyl ester into the polyamide, instead of the polyhydrazide, and degraded this amide by Hofmann's method, agree with those now reported.

Experimental

Polymethyl Ester of Native Poly-D-glutamic Acid.—(*a*) *By the action of diazomethane on the polypeptide* (*M*, 6400) *prepared by the original method from* B. subtilis. To the polypeptide, in dilute methanol, ethereal diazomethane was added. From the resulting product a water-insoluble fraction was isolated [Found : OMe, 20·2. Calc., for a polymethyl ester $(C_6H_9O_3N)_n$ corresponding to a polyglutamic acid $(C_5H_7O_3N)_n$ of neutralisation equiv. 129·1 : OMe, 21·7%]. Since our polyacid has a neutralisation equiv. of only 140 (Bruckner, Iványovics, and Kovács Oskolás, *loc. cit.*), an OMe value of 20·1% is theoretically attainable.

(*b*) *By the action of dry methanol and catalytic amounts of acetyl chloride on the polypeptide specified above* (Fraenkel-Conrat, Cooper, and Olcott, *J. Amer. Chem. Soc.*, 1945, **67**, 950; Fraenkel-Conrat and Olcott, *J. Biol. Chem.*, 1945, **161**, 259). The polypeptide (289 mg.) was finely powdered and dried at 100°/1 mm. over phosphoric oxide until of constant weight (45 hours) (Found : Ash, 2·5; amino-N, 0·3%; corresponding to *M*, 4670). To 250 mg. of this substance 4 ml. of dry methanol were added, and the suspension mixed with 0·03 ml. of acetyl chloride and shaken. In 2 hours almost complete dissolution occurred. After 24 hours, the mixture was filtered from a trace of insoluble matter, and to the filtrate 30 ml. of dry ether were added. The viscous gummy precipitate was separated on the centrifuge, redissolved in dry methanol, and reprecipitated with dry ether, then dried in a vacuum-desiccator over phosphoric oxide and paraffin; it was powdered and dried at 100°/1 mm. (P_2O_5). On addition of 15 ml. of water, the product slowly dissolved. The solution was evaporated in the frozen state, and the remaining solid foam was dried at 100°/1 mm. (P_2O_5), giving an almost colourless amorphous powder (247 mg.) (Found : OMe, 19·7%).

(*c*) *By the action of methanolic hydrogen chloride on the sodium salt of the polypeptide.* From cultures of *B. subtilis* the copper salt of the polypetide was isolated by the usual method, and dialysed against citrate buffer solution (0·5M, pH 5·0), and then against distilled water (see Bovarnick, *loc. cit.*). The solution was finally evaporated *in vacuo* and the dry powdered product was dried to constant weight (200 hours) at 100°/1 mm. (P_2O_5) (Found : Ash, 18%). To 500 mg. of this substance 15 ml. of absolute methanol and then 0·4 ml. of concentrated hydrochloric acid were added, and the mixture shaken for 5 hours, complete dissolution occurring. By next morning a gel was formed. This was mixed with 45 ml. of absolute methanol and then centrifuged. After decantation, the residue was mixed with 30 ml. of absolute methanol and again centrifuged. The combined methanolic solutions were concentrated *in vacuo* to 3 ml., and mixed with absolute ether until no more precipitate formed. The precipitate was then separated on the centrifuge, redissolved in methanol, and reprecipitated with ether. After being separated on the centrifuge, the substance was washed with ether, dried in a vacuum-desiccator, powdered, and dried to constant weight (16 hours) at 100°/1 mm. over phosphoric oxide. It formed an almost colourless, water-soluble, amorphous powder (Found : Ash, 4; OMe, 20·1; total N, 9·3; amino-N, 0·31%, corresponding to *M*, 4500).

Polyhydrazide of Native Poly-D-glutamic Acid.—(i) A solution of the polymethyl ester (96 mg.) in anhydrous hydrazine (3 ml.) was kept for 3 days and then completely evaporated at 0·001 mm., liquid air being used for cooling. The powdered residue was dried in a vacuum-desiccator for 8 days and then at 78°/1 mm. over phosphoric oxide for 6 hours. It formed a nearly colourless powder (95 mg.) insoluble in water and completely soluble in dilute hydrochloric acid (Found : OMe, 0·0; total N, 26·6. Calc. from OMe content of starting material : total N, 29·9%).

(ii) The polymethyl ester (240 mg.) was converted into polyhydrazide, as described in (i) except that, after evaporation of the hydrazine solution, the residue was ground with 6 ml. of water, and dried in the frozen state and then at 100°/1 mm. over phosphoric oxide (48 hours).

The properties of the substance (240 mg.) were similar to those described under (i) (Found : total N, 26·7%). In a repetition of this procedure 280 mg. of polyester afforded 278 mg. of polyhydrazide (Found : total N, 28·1%).

Degradation of the Polyhydrazide of Native Poly-D-glutamic Acid.—(i) To a solution of 83 mg. of the polyhydrazide, obtained as in (i), above, water (1 ml.) and N-hydrochloric acid (1·2 ml.), sodium nitrite solution (1·974% ; 1·86 ml.) was slowly added (10 minutes) with ice-cooling and shaking. Fine gummy particles of the polyhydrazide separated. When the mixture was warmed over a micro-burner with N-sodium hydroxide solution (0·7 ml.) for 1—2 minutes, foaming occurred and the substance was deposited on the walls of the flask. The liquid (*A*) was removed by decantation, the remaining solid dissolved in concentrated hydrochloric acid (4·5 ml.), and the solution heated under gentle reflux for 2 hours.

1 Ml. of this solution was evaporated *in vacuo*, and the residue (12 mg.) dissolved in two drops of water and mixed with 3 drops of saturated aqueous picric acid ; no crystallisation occurred after long storage, or even after seeding with crystals of αγ-diaminobutyric acid dipicrate.

Another 1 ml. of the above hydrolysate was evaporated, and the residue mixed with 2 drops of a freshly prepared and filtered solution of *p*-nitrophenylhydrazine in N-hydrochloric acid. Instant precipitation of golden-yellow flocks occurred. These were filtered off, washed with 1 ml. of cold water, and dried first in a vacuum-desiccator, then at 100°/1 mm. over phosphoric oxide ; 3·4 mg. of yellow needles were obtained ; they melted at 178° alone or mixed with an authentic specimen of β-formylpropionic acid *p*-nitrophenylhydrazone, in agreement with the literature (Harries, *Ber.*, 1912, **45**, 2585).

The turbid solution (*A*), above, was mixed with an equal volume of concentrated hydrochloric acid, refluxed for 2 hours, set aside for 4 days, and finally evaporated *in vacuo* to dryness. The residue was redissolved in 1 ml. of a freshly prepared solution of *p*-nitrophenylhydrazine in N-hydrochloric acid ; needles of the *p*-nitrophenylhydrazone instantly separated. These were filtered off, washed repeatedly with cold water, and dried (P_2O_5), the product (5 mg.) melting at 174° undepressed by authentic β-formylpropionic acid *p*-nitrophenylhydrazone. The total yield of the latter is therefore $4·5 \times 3·4 + 5·0 \simeq 20$ mg. from 83 mg. of the polyhydrazide, corresponding to a 14·5% yield from 137·8 mg. of pure γ-polypeptide starting material.

(ii) A solution of the polyhydrazide (50 mg.) in 0·1N-hydrochloric acid (3·2 ml.) was treated with 1·76% sodium nitrite solution as described under (i). The mixture containing natant polyazide precipitate was mixed with concentrated hydrochloric acid (5 ml.) and warmed until the solution became clear. This was evaporated *in vacuo* to dryness, and the residue redissolved in concentrated hydrochloric acid (5 ml.), then refluxed for 90 minutes, and again freed from water *in vacuo*. This procedure was repeated, and the residue then mixed with 7 ml. of a freshly prepared solution of *p*-nitrophenylhydrazine in N-hydrochloric acid. The yellow crystals were filtered off, washed with 1·5 ml. of cold water, and dried at 100° ; the yield was 11 mg., or 13·2% of the quantity calculated as above and the m. p. 175°. After one recrystallisation (giving 6·6 mg.) from 4 ml. of water the m. p. rose to 180°, undepressed by admixture of an authentic specimen (Found : C, 50·9 ; H, 4·6. Calc. for $C_{10}H_{11}O_4N_3$: C, 50·6 ; H, 4·7%).

(iii) A solution of polyhydrazide (195 mg.) in 0·2N-hydrochloric acid (5 ml.) was converted into a suspension of the polyazide as described above under (i), N-sodium hydroxide solution (1·6 ml.) was added, and the mixture warmed for 15 minutes on the steam-bath ; it was then acidified with concentrated hydrochloric acid (11 ml.) and refluxed for 2 hours. The pale yellow solution was evaporated *in vacuo*, the residue redissolved in water (5 ml.), and the solution again evaporated. This procedure was repeated, then the residue was dissolved in 20 ml. of water, and the solution halved.

One half was concentrated to 2 ml., and a solution of phosphotungstic acid (2 g.) in water (3 ml.) was added. Instantly, copious precipitation occurred, and the precipitate was separated, and treated in an open flask with gently boiling barium hydroxide solution. The mixture was filtered, barium ions precipitated with dilute sulphuric acid, and the filtrate concentrated *in vacuo* to a small volume, and mixed with 2 ml. of a concentrated solution of flavianic acid. No precipitation occurred on long storage, and the solution remained clear after being seeded with crystals of αγ-diaminobutyric acid diflavianate.

The other half of the above solution was evaporated, and the *p*-nitrophenylhydrazone of β-formylpropionic acid was precipitated as usual.

Control Experiments on the Precipitation of β-Formylpropionic Acid as its p-Nitrophenylhydrazone.—Small amounts of crystalline β-formylpropionic acid, m. p. 147°, were investigated as to their precipitability as *p*-nitrophenylhydrazone, and the influence of the acid hydrolysis upon this procedure. (i) β-Formylpropionic acid (7 mg.) was dissolved in 1 drop of N-hydro-

chloric acid and 4 drops of the reagent; on gentle warming, the solution solidified to a mass of crystals, which were filtered off and washed with 5 ml. of cold water; the dry product weighed 11·2 mg. (68·9%).

(ii) A solution of β-formylpropionic acid (9 mg.) and sodium chloride (11 mg.) in water (3 ml.) mixed with concentrated hydrochloric acid (3 ml.) was evaporated *in vacuo*, and the residue was redissolved in 7 drops of the reagent. The product, after collection, washing, and drying, weighed 10 mg. (47·8%).

(iii) A solution of β-formylpropionic acid (7 mg.) and sodium chloride (7 mg.) in concentrated hydrochloric acid (2 ml.) was refluxed for 2 hours, set aside for 2 hours, and then evaporated *in vacuo* to dryness. The residue was redissolved in a small amount of water, which was distilled off *in vacuo*. On dissolution of the residue in 4 ml. of the reagent, crystallisation occurred after seeding. The product, isolated as usual, weighed 2 mg. (12·3%).

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