The Structure of Bacterial Polyglutamic Acid.

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Poly-(γ-L-glutamyl)-L-glutamic acid has been synthesised. Condensation of *N*-benzyloxycarbonyl-γ-L-glutamyl azide with γ-benzyl L-glutamate gave γ-benzyl *N*-(benzyloxycarbonyl-γ-L-glutamyl)-L-glutamate, which was esterified and then hydrogenated to α-methyl *N*-(α-methyl γ-L-glutamyl)-L-glutamate (III). Polymerisation with tetraethyl pyrophosphite (Anderson, Blodinger, and Welcher, *J. Amer. Chem. Soc.*, 1952, **74**, 5309), followed by hydrolysis gave the γ-linked poly-L-glutamic acid. Comparison of its properties with those of the poly-D-glutamic acids from *B. anthracis* (Hanby and Rydon, *Biochem. J.*, 1946, **40**, 297) and from *B. licheniformis* (Bovarnick, Eisenberg, O'Connell, Victor, and Owades, *J. Biol. Chem.*, 1954, **207**, 593) showed that the naturally occurring compound was also γ-linked.

AMONG the many known natural products built up from amino-acid residues there is one that is structurally unique, in that its molecular weight is as large as that of a protein though its structure much simpler. This is poly-D-glutamic acid, formed by certain bacteria of the genus *Bacillus*, such as *B. anthracis* and *B. licheniformis*. The polypeptide is readily purified and on hydrolysis furnishes only D-glutamic acid (Hanby and Rydon, *Biochem. J.*, 1946, **40**, 297; Pongor, *Experientia*, 1950, **6**, 421; see also Bricas and Fromageot, *Adv. Protein Chem.*, 1953, **8**, 64, for a general review of the literature on poly-D-glutamic acid). So the main structural interest centres on whether the glutamyl residues are α-linked (I) or γ-linked (II), or whether both types of linkage are present; this is also, of course, a general problem in protein chemistry whenever glutamyl or aspartyl residues

are encountered (Quastel, Stewart, and Tunnicliffe, *Biochem. J.*, 1923, **17**, 591; Kandel, Kandel, Kovács, and Bruckner, *Naturwiss.*, 1954, **41**, 281). Bovarnick (*J. Biol. Chem.*, 1942, **145**, 415) suggested that the polypeptide from *B. licheniformis* was γ-linked (II) on

(I) (II)

the grounds that it does not give the biuret test and was not racemised by alkali. Hanby and Rydon (*loc. cit.*) inferred the presence of γ-peptide links in the polypeptide from *B. anthracis* from the increase of apparent amino-nitrogen content with the time of reaction with nitrous acid; Sachs and Brand (*J. Amer. Chem. Soc.*, 1954, **76**, 3601) have recently shown that γ-glutamyl peptides with free α-amino- and α-carboxyl groups liberate nearly all their nitrogen in the Van Slyke reaction, with formation of the lactone of α-hydroxyglutaric acid; the relatively slow and incomplete evolution of nitrogen from the polypeptide may result from the low concentration of end groups, or from side reactions in the stepwise degradation. This complicated reaction gives no information on the number of γ-peptide links present. Hanby and Rydon interpreted titration curves as indicating that the polypeptide was predominantly α-linked (I); it now seems likely that they were misled by choosing too low a value for the pK of the α-carboxyl group of a glutamyl residue. Bruckner, Kovács, and their co-workers, on the other hand, produced evidence that the polypeptide was entirely γ-linked (II) (*J.*, 1952, 4255; 1953, 145, 149, 1512; *Nature*, 1953, **172**, 508); they converted methyl poly-D-glutamate into the amide (or hydrazide) and submitted it to Hofmann (or Curtius) degradation: β-formylpropionic acid was the only product isolated. An α-linked polyglutamic acid (I) had already been synthesised (Hanby, Waley, and Watson, *J.*, 1950, 3239) and several of its properties found to differ from those of the natural product; the γ-linked isomer (II) has now been prepared; a preliminary account of this work has been given (Waley, *Chem. and Ind.*, 1954, 1149).

The synthesis of a γ-linked polyglutamic acid presents several problems: direct polymerisation of α-methyl glutamate is out of the question as preferential cyclisation to methyl 5-oxopyrrolidine-2-carboxylate would occur. So it is necessary to prepare a γ-linked dipeptide with both the α-carboxyl groups suitably protected (*e.g.*, III); further this must be formed from a precursor in which the protective groups on the amino- and the γ-carboxyl groups can be removed preferentially (*e.g.*, IV; R = Me). Now the γ-carboxyl group of

(III)

(IV)

glutamic acid can be preferentially esterified and this is the basis of the scheme chosen: γ-methyl L-glutamate was converted into benzyloxycarbonyl-γ-L-glutamylhydrazide (by a somewhat simplified method) and the azide condensed with γ-benzyl L-glutamate by the method of Boothe *et al.* (*J. Amer. Chem. Soc.*, 1949, **71**, 2304) to give a satisfactory yield of γ-benzyl *N*-(benzyloxycarbonyl-γ-L-glutamyl)-L-glutamate (IV; R = H). Under some conditions benzyloxycarbonyl-γ-L-glutamyl azide gives a mixture of α- and γ-linked peptides (Sachs and Brand, *J. Amer. Chem. Soc.*, 1954, **76**, 1815), but only the γ-linked product was obtained in this case, as shown by hydrogenation to γ-L-glutamyl-L-glutamic acid, free from the α-isomer. Esterification with diazomethane gave the dimethyl ester (IV; R = Me), from which hydrogenation removed the benzyl ester and benzyloxycarbonyl groups to give the dipeptide (III). An earlier attempt to obtain the ester (IV; R = Me) required α-benzyl α-methyl L-glutamate: no pure product could be isolated after treatment of crude γ-benzyl α-methyl-*N*-formyl-L-glutamate with hydrogen chloride in benzyl alcohol (cf. Waley, *Chem. and Ind.*, 1953, 107), and the formyl derivative itself was not obtained pure.

Several ways of polymerising peptides have been examined (Frankel, Liwschitz, and

Zilkha, *Experientia*, 1953, **9**, 179; Wieland and Bernhard, *Annalen*, 1953, **582**, 218; Noguchi and Hayakawa, *J. Amer. Chem. Soc.*, 1954, **76**, 2846) but in general only very low degrees of polymerisation were achieved. Although some polymeric material was obtained by the action of phosphorus trichloride in pyridine on the dipeptide (III) (cf. Goldschmidt and Lautenschlager, *Annalen*, 1953, **580**, 68) the polypeptide was contaminated with a phosphorus-containing polymer; γ-linked poly-L-glutamic acid was obtained pure, however, by heating the dipeptide (III) with tetraethyl pyrophosphite in diethyl phosphite (Anderson, Blodinger, and Welcher, *J. Amer. Chem. Soc.*, 1952, **74**, 5309), followed by brief hydrolysis with cold, dilute alkali; the ready hydrolysis of esters of the naturally occurring poly-D-glutamic acid was already known (Fraenkel-Conrat and Olcott, *J. Biol. Chem.*, 1945, **161**, 259). Detailed comparison of this synthetic poly-L-glutamic acid with the poly-D-glutamic acids (from *B. anthracis* and *B. licheniformis*) showed that the latter were, indeed, γ-linked.

The infrared spectra of the synthetic γ-linked and α-linked polypeptides differed: the most prominent of the peaks not shared by both isomers were at 8·25, 8·88, and 9·80 μ in the γ-linked one, and at 7·12, 7·90, 8·58, and 12·70 μ in the α-linked one. Most of these peaks are in the skeletal region, but the peak at 8·25 μ (in the γ-linked isomer) is present in acylamino-acids and is assigned to the carboxyl group; the environment of this group is closer to that in an acylamino-acid in (II) than it is in (I). Clayton and Kenner (*Chem. and Ind.*, 1953, 1205) noted a shift in the carbonyl-stretching band of the carboxyl group (5·84 to 4·77 μ) in the conversion of an α-glutamyl peptide into a γ-glutamyl one. For polyglutamic acids, however, the peak in this region (5·82 μ) is common to both isomers. The poly-D-glutamic acid from *B. licheniformis* had an infrared absorption curve very similar to the synthetic γ-linked polypeptide and, although the curve of the product from *B. anthracis* was poorly resolved, it clearly belonged to the γ-linked type.

The partial hydrolysis of poly-D-glutamic acid had not previously been studied; it was found that hydrolysis in constant-boiling hydrobromic acid at 100° was rapid; after ¼ hr. the hydrolysate gave a series of ninhydrin-positive spots on a paper chromatogram. The two of these with the highest R_F values were identified as glutamic acid and γ-glutamylglutamic acid; the other spots were assigned to higher members of the homologous series of peptides, such as are formed by the action of hydrobromic acid on poly-L-lysine (Waley and Watson, *Biochem. J.*, 1953, **55**, 328). This assignment is supported by the linear plot of $\ln[(1/R_F) - 1]$ against the assumed number of glutamic acid residues in the peptide (Pardee, *J. Biol. Chem.*, 1951, **190**, 757; Waley and Watson, *J.*, 1953, 475); the values of the constants A and B in Pardee's equation were −510 cal./mole and −100 cal./mole respectively. Similar results were obtained on partial hydrolysis of the synthetic γ-linked polypeptide; it can be concluded from these results, and from the infrared spectra, that there is no evidence for any α-glutamyl linkages in the naturally occurring poly-D-glutamic acids.

The γ-linked polypeptide also resembled the natural products (and differed from the synthetic α-linked polypeptide) in its ready solubility in water and the value of its ionisation constant. In the titration of a weak acid with a strong base, the value of the ionisation constant (K) at any stage of the titration is accurately given by

$$K = [H^+](b + [H^+] - [OH^-])/(a - b - [H^+] + [OH^-])$$

where b is the concentration of added base, and a the total concentration of acid; in the pH region (2·5 — 5) with which we are concerned, the term $[OH^-]$ may be neglected, giving

$$K = [H^+](b + [H^+])/[a - b - [H^+]) \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

The titrations of these polypeptides were carried out in N-potassium chloride, so as to minimise the interaction between the charged groups; if there is no such interaction, the titration curve of a polymeric acid may be represented by one titration constant (Simms, *J. Amer. Chem. Soc.*, 1926, **48**, 1239; von Muralt, *ibid.*, 1930, **52**, 3518). The value of K [found by means of equation (1)] decreased, however, with increasing degree of ionisation, showing that even at this ionic strength interaction occurs. The value of pK of the synthetic γ-linked and natural polyglutamic acids was 3·6 ± 0·1 at 50% ionisation, in N-potassium chloride at 25°.

The positive ninhydrin reaction given by γ-linked peptides of glutamic acid has been mentioned. It was found that a 0·25% solution of sodium poly-D-glutamate approximately matched a 0·005% solution of glutamic acid in the intensity of colour produced on heating with ninhydrin in acetate buffer: if the only groups reacting were the terminal amino-groups of the polymer, its degree of polymerisation would be about 50. In fact, the degree of polymerisation of this sample was of the order of a thousand, so that much of the amide-nitrogen must enter into the reaction with ninhydrin, which thus resembles the reaction with nitrous acid.

The molecular weights of these polypeptides were measured by arylation of their amino-groups with 1-fluoro-2 : 4-dinitrobenzene, with a slight modification of Levy's method (*Nature*, 1954, **174**, 126), followed by hydrolysis with perchloric acid in formic acid (Hanes, Hird, and Isherwood, *Biochem. J.*, 1952, **51**, 25); the amount of 2 : 4-dinitrophenylglutamic acid thus liberated was compared with the amount obtained after reaction of the glutamic acid with 1-fluoro-2 : 4-dinitrobenzene. The molecular weight of the synthetic polypeptide was about 9000; that of the polypeptide from *B. licheniformis* about 90,000, and of that from *B. anthracis* of the order of 100,000 [Dr. L. H. Kent found a value of 180,000 for the polypeptide from *B. anthracis* (personal communication)]. During this work, *N*-2 : 4-dinitrophenylglutamic acid was obtained crystalline for the first time.

Apparently poly-D-glutamic acid from *B. anthracis* has one of the longest peptide chains known; it is found both inside and outside the cells of *B. anthracis* grown *in vivo* (Smith and Zwartouv, *Biochem. J.*, 1954, **56**, viii; Smith, Zwartouv and Gallop, *ibid.*, p. ix), and is concentrated in the capsules surrounding the cells. It has been reported (Ivanovics and St. Horváth, *Acta Physiol. Sci. Hung.*, 1953, **4**, 401) that the carboxyl groups of the polypeptide in the capsules are not free, and the polypeptide may play an important part in the spatial organisation of the capsule.

Experimental

Benzyloxycarbonyl-γ-L-glutamylhydrazide.—Hydrogen chloride was passed into a cooled suspension of L-glutamic acid (125 g.) in methanol (2500 ml.). After 4½ hr., the solution was neutralised with aqueous ammonia (*d* 0·88; 65 ml.) and kept in the refrigerator overnight. γ-Methyl L-glutamate (99 g.) was collected, and acylated by stirring it with water (700 ml.) containing potassium hydrogen carbonate (137 g.) and benzyl chloroformate (120 ml.) at room temperature for 6 hr. The solution was extracted with ether and then acidified: the γ-methyl benzyloxycarbonyl L-glutamate was isolated with ethyl acetate [the ethyl acetate solution being washed with water containing potassium hydrogen carbonate (5 g.)], dissolved in ethanol (250 ml.), and treated with 90% hydrazine hydrate (100 ml.). After 48 hr., the benzyloxycarbonyl-γ-L-glutamylhydrazide was isolated as described by Le Quesne and Young (*J.*, 1950, 1959): the yield was 120 g. (48% overall from glutamic acid), and the m. p. 173—174° (pure enough for the next stage).

γ-Benzyl N-(*Benzyloxycarbonyl-γ-L-glutamyl*)-L-*glutamate.*—Sodium nitrite (10·7 g.) in water (107 ml.) was added during 12 min. to a cooled, stirred solution of benzyloxycarbonyl-γ-L-glutamylhydrazide (32 g.) in water (256 ml.) containing 6N-hydrochloric acid (109 ml.) covered with ether (528 ml.). After separation, the aqueous layer was extracted with cooled ether, and the combined ethereal layers were washed thrice with cooled water and promptly added to γ-benzyl L-glutamate (25 g.) (Hanby, Waley, and Watson, *J.*, 1950, 3239; average yield 30%) and potassium hydrogen carbonate (32 g.) in water (320 ml.). The mixture was stirred at room temperature for 6 hr. The next day, the filtered solution was covered with ethyl acetate and acidified (caution: hydrazoic acid evolved). The ethyl acetate extracts were washed, dried, concentrated to 120 ml., and diluted with ether (220 ml.); *γ-benzyl* (*benzyloxycarbonyl-γ-L-glutamyl*)-L-*glutamate* (31·6 g., 58%; m. p. 154—156°) separated. Recrystallisation by dissolution in acetone–ethyl acetate, concentration, and dilution with ether raised the m. p. to 159·5—160°, $[\alpha]_D^{21}$ +11·5° (*c*, 1·92 in acetic acid) (Found: C, 59·7; H, 5·9; N, 5·8. $C_{25}H_{28}O_9N_2$ requires C, 60·0; H, 5·6; N, 5·6%).

γ-Benzyl α-Methyl N-(*α-Methylbenzyloxycarbonyl-γ-L-glutamyl*)-L-*glutamate.*—Excess of ethereal diazomethane was added to γ-benzyl (benzyloxycarbonyl-γ-L-glutamyl)-L-glutamate (17 g.) in methanol (300 ml.), and the solvents were removed. The residual *γ-benzyl α-methyl* (*α-methylbenzyloxycarbonyl-γ-L-glutamyl*)-L-*glutamate* (12·6 g., 70%), recrystallised from ethyl

acetate–light petroleum (b. p. 80—100°), had m. p. 117—118°, $[\alpha]_D^{21}$ +6° (*c*, 1·88 in acetic acid) (Found: C, 61·7; H, 5·8; N, 5·15. $C_{27}H_{32}O_9N_2$ requires C, 51·4; H, 6·1; N, 5·3%).

α-Methyl N-(*α-Methyl-γ-*L*-glutamyl*)-L*-glutamate.*—*γ*-Benzyl α-methyl (α-methylbenzyloxycarbonyl-*γ*-L-glutamyl)-L-glutamate (12·6 g.) in methanol (270 ml.), acetic acid (10 ml.), and water (20 ml.) was reduced in hydrogen in the presence of palladium black (2 g.) for 2½ hr. The filtered mixture was evaporated, and the residue recrystallised from 95% *iso*propanol; *α-methyl* N-(*α-methyl-γ-*L*-glutamyl*)-L*-glutamate* (5·4 g., 70%) had m. p. 123—124°, $[\alpha]_D^{21}$ +23° (*c*, 2·03 in acetic acid) (Found: C, 44·6; H, 6·95; N, 9·15. $C_{12}H_{20}O_7N_2,H_2O$ requires C, 44·7; H, 6·8; N, 8·7. Found, on material dried at 100°: C, 48·0; H, 6·5. $C_{12}H_{20}O_7N_2$ requires C, 47·3; H, 6·6%).

*Poly-(γ-*L*-glutamyl)-*L*-glutamic Acid.*—α-Methyl *N*-(α-methyl-*γ*-L-glutamyl)-L-glutamate hydrate (0·6 g.), diethyl phosphite (2·8 ml.), and tetraethyl pyrophosphite (1·6 ml.) were heated at 100° for 10 min., the solvent was distilled off, and the residue was triturated under ether. The sticky polymethyl ester was hydrolysed with N-sodium hydroxide (8 ml.) for 10 min., and the solution then dialysed against running tap-water for 40 hr. and finally against distilled water for 4 hr. Freeze-drying gave *sodium poly-(γ-*L*-glutamyl)-*L*-glutamate* (0·12 g., 31%) as a dihydrate, which, however, still contained phosphorus [Found: C, 32·4; H, 5·6; N, 7·6; P, 1·8; degree of polymerisation (see below), 55. $(C_5H_6O_3NNa),2H_2O$ requires C, 32·1; H, 5·35; N, 7·5%). For further purification, the aqueous solution of this salt was treated with aqueous copper sulphate, the precipitated copper salt was washed thoroughly and dissolved in 0·25N-hydrochloric acid, and the solution was dialysed against 0·005N-hydrochloric acid for 24 hr.; freeze-drying gave *poly-(γ-*L*-glutamyl)-*L*-glutamic acid* (Found: C, 40·9; H, 5·9; N, 9·55; P, 0·6. $C_5H_7O_3N,H_2O$ requires C, 40·8; H, 6·1; N, 9·5%).

*γ-*L*-Glutamyl-*L*-glutamic Acid.*—*γ*-Benzyl (benzyloxycarbonyl-*γ*-L-glutamyl)-L-glutamate was hydrogenated in aqueous acetic acid, and *γ*-L-glutamyl-L-glutamic acid isolated as described by Le Quesne and Young (*loc. cit.*); it had $[\alpha]_D^{20}$ +3·4° (*c*, 1·0 in 0·5N-hydrochloric acid), and on hydrolysis furnished optically pure L-glutamic acid (Sachs and Brand, *J. Amer. Chem. Soc.*, 1953, **75**, 4608, give $[\alpha]_D^{24}$ +3·8° in 0·5N-hydrochloric acid); paper chromatography showed it to be free from the α-dipeptide.

*Isolation of Poly-*D*-glutamic acid from* B. licheniformis.—The organism was kindly supplied by Dr. Bovarnick, and was grown as described by Bovarnick, Eisenberg, O'Connell, Victor, and Owades (*J. Biol. Chem.*, 1954, **207**, 593); the copper salt was precipitated from the medium (600 ml.), washed with water acidified to pH 3, and decomposed with 0·5N-hydrochloric acid (10 ml.). The filtered solution was dialysed against 0·005N-hydrochloric acid at 2° for 70 hr., filtered again, and freeze-dried, giving 74 mg. of a colourless solid (Found: C, 41·7; H, 5·75; N, 9·25. Calc. for $C_5H_7O_3N,H_2O$: C, 40·8; H, 6·1; N, 9·5%).

Partial Hydrolysis of Polyglutamic Acid.—Sodium poly-D-glutamate (10 mg.) in constant-boiling hydrobromic acid (0·1 ml.) was kept at 100°; samples (0·02 ml.) were periodically withdrawn and added to 50% pyridine (0·2 ml.), and this solution analysed by paper chromatography, using as solvent *n*-butanol–acetic acid–water–pyridine (30 : 6 : 24 : 20). The relative intensities (evaluated visually) of the spots are shown in the Table below.

Of the products, glutamic acid and *γ*-glutamylglutamic acid were identified by comparison with authentic samples; the assignment of the higher peptides has already been discussed. There were no ninhydrin-positive spots at the beginning of the reaction with either specimen of poly-D-glutamic acid; the synthetic polypeptide, however, showed some spots near the starting line which disappeared during the course of the reaction; this difference may be attributed to the higher molecular weights of the natural polypeptides.

Product	Time of hydrolysis (hr.). 0·25	0·5	0·75	1·25
Glutamic acid	+	++	+++	++++
γ-Glutamylglutamic acid	++	++	+	±
Assumed tripeptide	+	+	±	—
Assumed tetrapeptide	+	±	—	—
Assumed pentapeptide	+	—	—	—
Assumed hexapeptide	+	—	—	—

End-group Estimation.—Sodium poly-(*γ*-L-glutamyl)-L-glutamate (7 mg.) in 0·025M-borax (0·2 ml.) and 1-fluoro-2 : 4-dinitrobenzene (1 mg.) were stirred at 40° in the dark for 80 min. The cooled solution was extracted with ether, acidified, extracted with ether again, dialysed against distilled water for 6 hr., and evaporated to dryness *in vacuo*. The residue was transferred with a 10% solution of 60% perchloric acid in 98% formic acid (0·3 ml.) to a test-tube, the end

of which was then drawn out into a capillary (as considerable pressure is generated in the subsequent heating). After being kept for 4 hr. at 100° in the dark, the solution was diluted with water (3 ml.) and extracted thrice with ether (solution A). The aqueous solution was extracted thrice with water-saturated ethyl methyl ketone (to remove formic acid) and once with ether. The combined ethyl methyl ketone extracts were washed with a little water, which was added to the aqueous solution. This was then brought to pH 9 with N-sodium hydroxide (0·6 ml.), borax (90 mg.) and 1-fluoro-2 : 4-dinitrobenzene (16 mg.) added, and the mixture stirred at 40° in the dark for 80 min. The cooled solution was extracted with ether, acidified, and extracted thrice with ether, and this ethereal solution (B) diluted to a volume of 20 ml. One-third of solution A and 0·5 ml. of solution B were run on a paper chromatogram, using as solvent 1·5M-phosphate buffer (pH 6), and the amounts of dinitrophenylglutamic acid were estimated as described by Levy (*Nature*, 1954, **174**, 126); after correction for the paper blanks, the optical density of the solution corresponding to solution A was 0·175, and of that corresponding to solution B, 0·71. Hence the number-average degree of polymerisation was 55. The end-group estimation on other polymers was carried out in the same way.

N-(2 : 4-*Dinitrophenyl*)-L-*glutamic Acid*.—L-Glutamic acid (209 mg.) was treated with 1-fluoro-2 : 4-dinitrobenzene (350 mg.) as described by Levy (*loc. cit.*). The crude product was washed first with benzene, and then with ether–benzene (1 : 19), giving crystalline 2 : 4-dinitrophenyl-L-glutamic acid (248 mg., 58%), m. p. 93·5—94·5° (Found : C, 41·9; H, 3·7; N, 13·3. Calc. for $C_{11}H_{11}O_8N_3$: C, 42·2; H, 3·5; N, 13·4%).

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