Polyglutamic Acid from *Bacillus anthracis* Grown *in vivo*: Structure and Aggressin Activity

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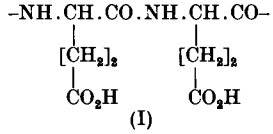
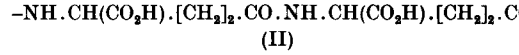
Tomcsik & Szongott (1933) obtained a nitrogenous, polysaccharide-free, capsular material from *Bacillus anthracis* which was identified by Ivanovics & Bruckner (1937*a*, *b*) as poly-D-glutamic acid. A capsule is formed by virulent strains of *B. anthracis* and appears to be connected with the aggressive aspect of virulence, that is, with the ability of the pathogen to interfere with the defence mechanisms of the host (for discussion, see Smith & Keppie, 1955). In order to study the connexion of polyglutamic acid with virulence, we have used material produced during growth of the pathogen *in vivo* when compounds responsible for virulence are necessarily produced. Capsulated organisms were separated from a mixture of plasma and exudate of guinea pigs dying of anthrax (Smith, Keppie & Stanley, 1953). Crude preparations of polyglutamic acid were then obtained from both the organisms and the body fluids (Smith, Zwartouw & Harris-Smith, 1956; Smith & Gallop, 1956). The purification of this polyglutamic acid is described here together with observations on its aggressive activity and structure. Attempts have been made to correlate its biological activity with some physicochemical or structural property.

The important structural problem posed by polyglutamic acid is whether the glutamic acid residues are linked by their α-carboxyl groups (cf. I) or their γ-carboxyl groups (cf. II) or both.



(I)



(II)

No studies of this problem have been made on a polymer from *B. anthracis* grown *in vivo*, but work on samples from cultures *in vitro* has been reported. Hanby & Rydon (1946) produced evidence that in polyglutamic acid from *B. anthracis* there were mainly α-peptide links, and this idea received some support (Hanby, Waley & Watson, 1948; Haurowitz & Bursa, 1949). More recently, evidence has accumulated that the glutamic acid is linked only by its γ-carboxyl group in preparations from *B. anthracis* and *Bacillus subtilis*. The nature of the products from Curtius and Hoffmann degradations of appropriate derivatives indicated this structure (Kovacs & Bruckner, 1952; Bruckner, Kovacs & Nagy, 1953; Bruckner, Kovacs & Denes, 1953). Waley (1955) showed that the infrared spectra of natural polyglutamic acids resembled that of a γ-linked synthetic polymer. He also identified by paper chromatography γ-glutamylglutamic acid in partial hydrolysates of the natural substances.

MATERIALS

Crude capsular polyglutamic acid from Bacillus anthracis *grown* in vivo. This was the fraction precipitated by 1% barium acetate and 5% (v/v) ethanol from the dialysed intracellular substance of *B. anthracis* as described by Smith *et al.* (1956). It contained about 75% of polyglutamic acid.

Crude extracellular polyglutamic acid from the plasma exudate of guinea pigs dying of anthrax. This was fraction *B* obtained from plasma exudate by precipitation with 2% barium acetate and 5% (v/v) ethanol followed by removal of the immunizing aggressin by precipitation with sodium chloride (0·5%) and ethanol (15%, v/v) as described by Smith & Gallop (1956). It contained about 70% of polyglutamic acid.

Polyglutamic acids from other sources. These are specified in Table 4 and were gifts from Mr L. H. Kent (sample 3), Dr C. B. Thorne (sample 4), Dr L. Bichowsky-Slomnitzky (sample 5) and Dr S. G. Waley (sample 6), to whom we owe our thanks.

Polyacrylic acid. A solution of acrylic acid (10%) and potassium persulphate (0·02%) was heated at 60° for 3 hr. The viscous solution was dialysed, adjusted to pH 7 and freeze-dried. Our thanks are due to Polymer Consultants Ltd. for advice on this process.

Polyaspartic acid. The polymer insoluble in hot water, prepared by heating L-aspartic acid at 200° for 48 hr. (Frankel & Berger, 1949) was purified by dissolving it in dilute sodium hydroxide, adjusting the pH to 7 and precipitating the barium salt. After conversion into the sodium salt, dialysing the solution and freeze-drying, it had N, 9·8; sulphated ash, 44·1%. $(C_4H_4O_3NNa)_n$ requires N, 10·2; sulphated ash, 51·8%.

Polymannuronic acid. Two samples of this material were used: sodium alginate (B.D.H. Ltd.) and Mannucol (Alginate Industries Ltd.).

Anthrax antiserum. The antiserum used for precipitation tests with polyglutamic acid was that prepared by Institute Sieroterapico Milanese Serafino Belfanti for the Ascoli thermoprecipitin test.

METHODS

Nitrogen. Determined by a micro-Kjeldahl method employing the catalyst recommended by Chibnall, Rees & Williams (1943).

Total amino nitrogen (nitrous acid). This was determined by the method of Van Slyke (1912), with a reaction time of 4–4·5 min. at 20°.

Total amino nitrogen (colorimetric). The method of Frame, Russel & Wilhelmi (1943) was used for the analysis of hydrolysates and for the estimation of mol.wt. by an end-group assay.

α-Carboxyl amino nitrogen (ninhydrin). This was determined at pH 2·5 by the method of Van Slyke, Dillon, MacFadyen & Hamilton (1941).

Glutamic acid. The paper-chromatographic method of Housewright & Thorne (1950) was used.

Assay for antiphagocytic activity. Keppie, Smith & Harris-Smith (1953) showed that the action of aggressins on the phagocytosis of *B. anthracis* (spores incubated in tryptic-meat broth for 20 min. at 37°) by the polymorphs of normal guinea-pig blood could be measured in two ways. The organisms inside the phagocytes of control and experimental samples could be counted on stained films, or the organisms not phagocytosed could be counted by a viable count; there was no significant extracellular bactericidal action. The second of these methods was more convenient for routine operation and has been used in this work. Serial dilutions of the various test materials were examined in the system described by Keppie *et al.* (1953). In the control samples, the phagocytic action of the guinea-pig blood reduced the viable count to 10–40% of the original count. Addition of aggressins increased the survival rate. The activity of a sample is expressed as the lowest concentration in the test mixture which resulted in a percentage survival 10–15 higher than that in the controls. Statistical analysis showed that, in 90% of the tests, this difference had a significance level of 95% or more; the significance level was never lower than 91·5%. The figures given in the tables below were obtained by averaging three to eight similar results. The assay could detect twofold differences in activity.

EXPERIMENTAL AND RESULTS

Purification of polyglutamic acid from Bacillus anthracis *grown* in vivo

Barium acetate (2 g.) was added to a solution of crude capsular or extracellular polyglutamic acid (1 g.) in water (100 ml.) at pH 7 and 20°. After standing overnight a precipitated impurity was removed from the solution and ethanol added at 0° to a concentration of 4% (v/v). After 16 hr. at 0°, the sticky precipitate of barium polyglutamate (yield about 70%) was collected by centrifuging. It was dissolved in water (70 ml.) and stirred with sufficient of the sodium form of Zeocarb 215 (approx. 2 × 5 g. of the moist granules; The Permutit Co. Ltd.) to remove completely Ba^{2+}. The resulting solution of sodium polyglutamate was shaken with 3·5 g. of washed fuller's earth powder (B.D.H. Ltd., 'For adsorption purposes') for 16 hr. at 0° to remove final traces of protein impurity. After centrifuging, the solution was dialysed against glass-distilled water, adjusted to pH 7·5 with NaOH, and freeze-dried. Yield of sodium polyglutamate, approx. 50%.

Criteria of purity

Our colleagues Drs B. R. Record and R. G. Wallis have shown that the purified samples of extracellular and capsular polyglutamic acid were homogeneous in electrophoresis and sedimentation at pH 8 and 4·4 (see later paper).

A comparison of hydrolysates (3N-HCl, 100°, 16 hr.) of the polyglutamic acids with those of small amounts of bacterial protein (Smith *et al.* 1956) and plasma protein on paper chromatograms indicated that less than 0·2% of protein impurity was present.

Analysis and properties

Freeze-dried sodium polyglutamate was a crisp white solid which rapidly deliquesced. In contrast to the polyglutamic acid from *B. subtilis* and the synthetic α-linked polyglutamic acid (Waley, 1955), acidification of a solution of the sodium salt did not precipitate the free acid. The results of analysis and biological tests on a number of different samples of the capsular and extracellular sodium polyglutamate were similar; average values are shown in Table 1. It is apparent that these polymers of D-glutamic acid have aggressive activity but no immunizing or toxic properties.

Structural investigation

Several methods have been used to investigate whether glutamic acid was linked by the α- or γ-carboxyl group in the polymer. This work was mainly carried out with the extracellular polyglutamic acid, and the most significant results were confirmed with the capsular material.

End-group analysis after partial hydrolysis. Sodium polyglutamate (0·5% in 0·2N-HCl) was heated at 100° and after various time intervals the hydrolysate was analysed for glutamic acid, α-carboxyl amino nitrogen (ninhydrin), total amino nitrogen (colorimetric) and total amino nitrogen (nitrous acid). The results obtained are shown in Table 2. The apparent incompleteness of the hydrolysis in 0·2N-HCl was probably due to the partial conversion of glutamic acid or its peptides into pyrrolidone carboxylic acids (cf. Hanby & Rydon, 1946); a shorter treatment (16 hr.) with stronger acid (3N-HCl) produced complete hydrolysis and prevented the formation of pyrrolidone carboxylic acid.

A consideration of the results in Table 2 provides the following evidence for the presence of γ-linkages in the polymer.

(i) Comparison of the glutamic acid nitrogen values with the higher amino nitrogen (ninhydrin)

values shows that α-carboxyl amino groups were present on glutamic acid residues still combined in peptides, presumably by their γ-carboxyl groups.

(ii) The ninhydrin α-carboxyl amino nitrogen values were the same as the amino nitrogen values obtained by the colorimetric method. Since the latter method detects NH_2 groups both with and without α-carboxyl groups (Frame *et al.* 1943) no $NH_2.CH(CO.R).CH_2.CH_2.CO_2H$ groups (i.e. α-linkages) were present. In contrast, a sample of partially hydrolysed synthetic α-linked polyglutamic acid contained amino nitrogen (ninhydrin) 20 % of total nitrogen, and amino nitrogen (colorimetric) 53 % of total nitrogen.

(iii) The peptide nitrogen of γ-linked but not of α-linked glutamyl peptides reacts with nitrous acid (Sachs & Brand, 1953, 1954). A consideration of the analytical values in Table 2 shows that some of the peptide nitrogen in the partial hydrolysates had reacted with nitrous acid. Let us consider, for example, the sample after hydrolysis for 5 hr. If the nitrous acid value (78 %) represented only free NH_2 groups then 22 % of the linkages remained intact. Hence, at least (i.e. assuming all unhydrolysed material is present as dipeptide) 56 % (78–22 %) of the NH_2 groups should be present as free glutamic acid. The measured glutamic acid value (and the α-carboxylamino nitrogen value) was much lower than this and therefore the nitrous acid amino nitrogen value does not represent only free amino groups but includes peptide nitrogen.

Table 1. *Properties of sodium poly-*D*-glutamate obtained from* B. anthracis *grown* in vivo

	Capsular	Extracellular	Calc. for $(C_5H_6O_3N\text{-}Na)_n$ or reported for other polyglutamic acids and D-glutamic acid
Nitrogen %	9·0	9·0	9·3 (calc.)
Sulphated ash, %	46·2	45·2	47·0 (calc.)
$[\alpha]_D^{20}$ in water (C, 4)	−16·9	−14·5	—
$[\alpha]_D^{20}$ in 3N-HCl (C, 4)	+25·2	+22·7	+23·5 (*a*)
$[\alpha]_D^{20}$ in 3N-HCl (C, 3) of glutamic acid resulting from hydrolysis (100°, 16 hr.)	−26·9	−28·5	−30·0 (*b*) −24·2 (*c*)
Molecular weight			
(1) From sedimentation and diffusion data*	88 000	253 000	—
(2) From end-group (NH_2) analysis	33 000	71 000	14–53 000 (*c*); 100 000 (*d*)
Dilution precipitating with antiserum	$1:6·4 \times 10^5$	$1:6·4 \times 10^5$	$1:8 \times 10^5$ (*e*)
Biological tests† (lowest active concn., %)			
(1) Antiphagocytic	0·8	0·8	—
(2) Virulence-enhancing	1	2	—
(3) Anticomplementary	Inactive at 1	Inactive at 1	—
(4) Tissue-damaging‡	Inactive at 1	Inactive at 1	—
(5) Immunizing	—	Inactive at 2	—

(*a*) Ivanovics & Bruckner (1937*b*); (*b*) Schmidt (1945); (*c*) Hanby & Rydon (1946); (*d*) Waley (1955); (*e*) Ivanovics & Bruckner (1937*a*).

* See later paper.
† For details of these tests see Smith & Gallop (1956), Smith, Keppie & Stanley (1955) and Smith *et al.* (1956).
‡ In addition, the materials were non-toxic when injected intravenously (Smith *et al.* 1955).

Table 2. *Analysis of partial hydrolysates of sodium polyglutamate*

Sodium polyglutamate (0·5 %) was heated at 100° in 0·2N-HCl, and at various time intervals the hydrolysate was analysed as shown below. Results have been calculated as % of total nitrogen.

Time of hydrolysis (hr.)	Glutamic acid N	α-Carboxyl amino N (ninhydrin)	Total amino N (colorimetric)	N liberated by nitrous acid
1	1	9·6	9·9	40·4
2·5	5	22·4	20·8	64·7
5	13	35·3	36·2	77·9
9·5	24	52·8	55·8	76·2
22	59	67·3	69·9	81·3
69	68	70·8	70·7	70·3

Table 3. *Peptides from a partial hydrolysate of polyglutamic acid*

Sodium polyglutamate (0·5%) was partially hydrolysed in 0·2N-HCl (5 hr., 100°), and the products of hydrolysis were separated on a column of Dowex 50. Five fractions were eluted with 0·1N-HCl. They were examined on paper chromatograms developed in either (*A*) butanol–acetic acid–water (2:1:1, v/v) or (*B*) phenol saturated with water (in the presence of NH_3 and HCN vapours) and compared with a glutamic acid marker. Fraction 1 was a mixture of large peptides and fraction 5 was glutamic acid; fractions 2–4 produced only single spots and these fractions were analysed as shown below. For comparison, calculated and reported values for various glutamyl peptides are included.

Fractions from column			*R* (glutamic acid)		N analysis	
					α-Carboxyl amino N	N liberated by HNO_2
No.	Eluate vol. (ml.)	Yield (%)	*A*	*B*	Total N	Total N
2	146–226	10	0·60	0·09	0·24	0·85
3	289–410	13	0·71	0·22	0·31	0·89
4	571–740	15	0·87	0·43	0·48	1·0
Glutamyl peptides						
Tetra-γ-			—	—	0·25 (calc.)	—
Tri-γ-			—	—	0·33 (calc.)	0·9*
Di-γ-			0·9*†	0·45†	0·5*	1·0*
Di-α-			1·1–1·2*†	0·45†	Nil*	0·52*

* Sachs & Brand (1953, 1954). † Williams & Thorne (1954).

Identification of small peptides after partial hydrolysis. Preliminary examination of partial hydrolysates of sodium polyglutamate on two-dimensional paper chromatograms indicated the presence of γ- but not α-glutamylglutamic acid. Larger quantities of dipeptides and higher peptides were isolated by the following method, which was based on that of Williams & Thorne (1954).

Sodium polyglutamate (0·3 g.) was hydrolysed in 0·2N-HCl (60 ml.) for 5 hr. at 100° and the mixture evaporated *in vacuo*. The residue, in water (2 ml.), was applied to a column (1·36 cm. × 45 cm.) of Dowex 50 (Dow Chemical Co. Inc., U.S.A.) in the hydrogen cycle. Elution was with 0·1N-HCl, and five well-separated fractions were detected in the eluate by the ninhydrin-colour reaction. Paper chromatography revealed that fraction 1 was a mixture of higher peptides and fraction 5 was glutamic acid. Fractions 2–4 produced single spots on paper chromatograms and they were analysed for total nitrogen, α-carboxyl amino nitrogen, and amino nitrogen (nitrous acid). The analytical and chromatographic results on these fractions are shown in Table 3 and are compared with reported and calculated values for various glutamyl peptides. Fractions 2–4 correspond with the tetra-, tri- and di-γ-linked glutamyl peptides. Fraction 4 had $[\alpha]_D^{20}$ (C, 3) = 0 (± 3)°; this agrees with the values for γ- but not α-glutamyl dipeptides (Sachs & Brand, 1953, 1954; Williams & Thorne, 1954).

Attempts to correlate antiphagocytic activity with some physicochemical property of polyglutamic acid

Non-reversal of the activity by Ca^{2+}. Calcium ions appear to be concerned in phagocytosis (Lambin & Desvignes, 1953), which suggests the possibility that the antiphagocytic action of polyglutamic acid may be due to its binding Ca^{2+}. This has been excluded by the observation that the addition of Ca^{2+} equivalent to the free carboxyl groups of polyglutamic acid does not diminish its antiphagocytic activity.

Similar activity of different polyglutamic acids. Table 4 shows that polyglutamic acids from widely different sources have similar antiphagocytic activity. Hence this activity does not depend on whether the polyglutamic acid has been produced as a capsule or extracellularly, by avirulent or virulent organisms, growing *in vivo* or *in vitro*. Neither does it depend on the presence of D- or L-glutamic acid residues nor on α- or γ-peptide linkages.

Independence of activity and viscosity. The results in Table 4 show that the viscosity of equally active solutions varies widely, and that some inactive solutions are more viscous than other active solutions.

Antiphagocytic activity of other polyacids. Table 5 shows that various organic polyacids have antiphagocytic activity. Unpolymerized glutamic acid is inactive at 0·8%.

DISCUSSION

Poly-D-glutamic acid has been obtained from the capsule of *B. anthracis* grown in infected guinea pigs and from the body fluids of these animals. The method of purification, via the barium salt followed by treatment with fuller's earth powder, has provided material which showed no evidence of heterogeneity when examined by physical and chemical methods.

Table 4. *Different polyglutamic acids with similar antiphagocytic activity*

Samples of polyglutamic acid from various sources and with different properties were examined for their ability to interfere with the phagocytosis of *B. anthracis* in a standard test (see Methods).

Sample no.	Source of polyglutamic acid: Organism	Growth conditions	Capsular or extracellular	Method of preparation	Configuration of glutamic acid residues	Linkage	η (37°) of solution relative to water: 2%*	1%†	Antiphagocytic test (lowest active concn. %)
1	*B. anthracis* (N.P.) virulent	*In vivo*	Capsular	This work	D	γ	4·73	—	0·8
2	*B. anthracis* (N.P.) virulent	*In vivo*	Extracellular	This work	D	γ	30·4	9·94	0·8
3	*B. anthracis* (H.M.) avirulent	*In vitro*	Capsular	Hanby & Rydon (1946)	D	γ	34·0	10·43	0·8
4	*B. subtilis*	*In vitro*	Extracellular	Thorne, Gomez, Noyes & Housewright (1954)	D and L	γ	4·75	2·61	0·8
5	*B. subtilis*	*In vitro*	Extracellular	—	D and L	γ	3·08	—	0·8
6	←	Synthetic	→	Hanby *et al.* (1948)	L	α	1·93	—	0·6

* This solution (0·4 ml.) was active when diluted to 0·8% in the antiphagocytic-test system (1 ml.).
† This solution was inactive at 0·4% when diluted similarly.

Table 5. *Antiphagocytic activity of polyacids*

Various polyacids were examined for their ability to interfere with the phagocytosis of *B. anthracis* in a standard test (see Methods).

Nature of polyacid	Antiphagocytic activity of soln. (lowest active concn. %)
Polyglutamic	0·8
Polyaspartic	0·3
Polymannuronic (sodium alginate)	0·2
Polymannuronic (Mannucol)	0·2
Polyacrylic	0·2

End-group analysis and identification of peptides in partial hydrolysates of both the samples of polyglutamic acid showed that at least the majority of the glutamyl residues were linked by their γ-carboxyl groups, and produced no evidence of linkage by the α-carboxyl groups. Similar conclusions have been reached for polyglutamic acids from other sources by previous workers who used different methods (see above). Professors V. Bruckner and J. Kovacs (private communication) have examined our material by their methods and have obtained the same result.

The only difference observed between the capsular and extracellular polyglutamic acid was the higher molecular weight of the latter. The lower values for the molecular weights obtained by the 'end-group assay' method (see Table 1) might indicate a structure involving more than one terminal amino group. On the other hand, they may have been due to hydrolysis during the assay procedure or to the influence of molecular association on the physical determination of molecular weight.

The purified polyglutamic acids were aggressins, i.e. compounds which interfere with defence mechanisms of the host, since they inhibited phagocytosis of organisms *in vitro* and had properties which enhanced virulence *in vivo*. They would not actively immunize animals against anthrax. In contrast to the 'inflammatory factor' of Watson *et al.* (1947) which contained polyglutamic acid, our preparations had no tissue-damaging properties when injected intradermally into guinea pigs. A high concentration of polyglutamic acid was necessary to inhibit phagocytosis of organisms *in vitro*. Such a concentration of the capsular polyglutamic acid surrounds *B. anthracis* when growing *in vivo* and almost certainly plays a part in the resistance of the organism to phagocytosis. The extracellular polyglutamic acid, which is present in the body fluids at a relatively low concentration, may play a different role in the aggressive action of the organism. Watson *et al.* (1947) showed that the 'inflammatory factor' and polyglutamic acid from *B. subtilis* inhibited the action of

anthracidal substance from rabbit leucocytes. In preliminary experiments, our colleague Dr J. Keppie has shown that the two polyglutamic acids described here inhibit the anthracidal action of either horse serum or extracts of guinea-pig leucocytes at concentrations of less than 0·1 %.

The antiphagocytic action of polyglutamic acid does not appear to be due to any part of the fine structure of the molecule, since different polymers from various sources had similar activity. In searching for some less specific property which might be responsible for its activity, we have excluded any effect of viscosity or binding of calcium ions. The activity appears to be associated with the multivalent negative charge of the polyglutamate ion. This is indicated by the antiphagocytic activity of other polyacids. It is relevant to point out here that other capsular materials from pathogenic bacteria play a role in their aggressive action and have a polyacidic nature, e.g. hyaluronic acid from streptococci and certain pneumococcal polysaccharides (Wilson & Miles, 1946).

SUMMARY

1. Polyglutamic acid showing no evidence for heterogeneity has been obtained from the capsule of *Bacillus anthracis* grown in infected guinea pigs and from the body fluids of the host. The isolation involved only mild treatment.

2. Studies on partial hydrolysates have shown that the glutamic acid residues in these materials were linked by their γ-carboxyl groups.

3. The polyglutamic acids are associated with the aggressive action of *B. anthracis in vivo*.

4. Their antiphagocytic activity appears to be due to the multivalent negative ion.

We are grateful to Dr J. Keppie and Mrs P. W. Harris-Smith for carrying out aggressin tests, and to Messrs R. E. Strange and A. G. Ness for some chemical analysis. The work described in this paper formed part of a Ph.D. thesis submitted to the University of London by one of us (H.T.Z.).

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