THE CHEMICAL BASIS OF THE VIRULENCE OF *BACILLUS ANTHRACIS*. VIII: FRACTIONATION OF THE INTRACELLULAR MATERIAL OF *BACILLUS ANTHRACIS*

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SMITH, Keppie and Stanley (1953*a*) separated capsulated *Bacillus anthracis* from the body fluids of guinea-pigs dying of anthrax in sufficient quantity and purity for chemical analysis. The organisms were almost completely dissolved by two mild methods (Smith, Keppie and Stanley 1953*b*); the fractionation of this material is now described. It is important to note that the crude material is a mixture of capsular and somatic substances and numerous attempts to remove the capsule preferentially from the organism have so far failed (Smith *et al.*, 1953*b*).

The intracellular material of *B. anthracis* resembles the extracellular substances produced *in vivo* in having anti-phagocytic and virulence-enhancing activities (Keppie, Smith and Harris-Smith, 1953), but it differs from them in two important respects. It does not actively immunise animals against infection (Keppie *et al.*, 1953), and it does not contain the anthrax toxin (Smith, Keppie and Stanley, 1955). Any toxic and immunising activities were not destroyed by the method of extraction used because similar treatment of the extracellular products did not affect these activities (Keppie *et al.*, 1953; Smith *et al.*, 1955). Thus, in the fractionation of the mixture of somatic and capsular material of *B. anthracis* grown *in vivo* we are concerned solely with aggressive action, *i.e.*, with ability to interfere with various host defence mechanisms such as phagocytosis.

All previous chemical work on *B. anthracis* has been carried out with organisms grown *in vitro* and has been concerned only with the isolation of one or other of the two established constituents of the organism, *i.e.*, poly-D-glutamic acid or the polysaccharide (Ivanovics and Bruckner, 1937; Ivanovics, 1940). The methods of isolation were relatively drastic. Here the intracellular material has been separated by a mild treatment into its main constituents for subsequent biological assay.

MATERIALS AND METHODS

Ammonium carbonate extract of B. anthracis (*N.P. strain*) *grown* in vivo.—Prepared as described by Smith *et al.* (1953*a*, *b*). The ammonium carbonate, which represented approx. 50 per cent of the total solids in solution, was lost during freeze-drying and constituted only approx. 2 per cent of the freeze-dried solid.

"*Ballotini*" *extract of* B. anthracis (*N.P. strain*) *grown* in vivo.—Prepared as described by Smith *et al.* (1953*a*, *b*). This extract and the ammonium carbonate extract contained the salts of the Locke's solution which were left in the bacterial sludge after the final washing of the latter.

"*Ballotini*" *extracts of* B. anthracis (*strains N.P. and* "*Sterne*") *grown* in vitro.—*B. anthracis* was grown in tryptic meat broth shaken at 37° for 16 hr. The organisms (0·4 g. from

250 ml. culture) were collected by centrifugation, washed with ice-cold Locke's solution and extracted by shaking with " ballotini " as described by Smith *et al.* (1953*a*).

Assays for immunising, anti-phagocytic and virulence-enhancing activity.—See Smith and Gallop (1956). Activities are quoted as the lowest concentration of a material having the stipulated effect.

Anti-complementary activity.—R.B.C. from defibrinated sheep blood (6 per cent v/v in saline) were sensitised at 37° for 10 min. with excess haemolytic antibody (5 M.H.D.). Serial dilutions in saline of the material to be tested (8 drops) were incubated at 37° for 1 hr. with a quantity of guinea-pig complement which was just sufficient to produce complete lysis in control tubes. The suspension of sensitised cells (5 drops) was added and the mixture incubated at 37° for 1 hr. and left at 0° overnight. Activities of samples are quoted as the lowest concentration which inhibited the lysis of at least half of the added R.B.C. This assay detects 2-fold differences in anti-complementary activity.

Analytical methods

Methods for nitrogen, P, carbohydrate, hexosamine, absorption of ultraviolet light and glutamic acid were as used by Smith and Gallop (1956).

Amino N.—The colourimetric method of Frame, Russel and Wilhelmi (1943).

Pentose.—By the method of Markham and Smith (1949).

" *Free lipid* ".—The material extracted from the dry solid by ether.

" *Combined lipid*".—Determined as described by Smith and Gallop (1956) under " lipid ".

Polyglutamic acid.—The sample (2 mg.) was placed on strips of filter paper and separated by the paper electrophoretic method of Flynn and de Mayo (1951) using a potential gradient of approx. 6 volts per cm. at 20° for 5 hr. Polyglutamic acid which migrated rapidly towards the anode was detected by the method of Strange and Harkness (1953). The coloured area in test samples was compared visually with that of similarly treated standard samples of purified polyglutamic acid.

Paper chromatography.—By the methods of Partridge (1948 ; 1949) for sugars and of Consden, Gordon and Martin (1944) for amino acids.

EXPERIMENTAL AND RESULTS

Fractionation of the Ammonium Carbonate Extract of B. Anthracis *Grown* in vivo.

The ammonium carbonate and " ballotini " extracts of the organisms from infected guinea-pigs had similar anti-phagocytic (0·5 per cent), anti-complementary (0·25 per cent) and virulence-enhancing (5 per cent) activity. The ammonium carbonate extract was the easier to prepare and was therefore used in the preparation of the following materials. All operations were carried out at 0° and pH 7.

(1) *Diffusate.*—Bacterial extract (1 g.) in water (20 ml.) was dialysed in a Cellophane sac against three changes of water (200 ml.). The combined diffusates were freeze-dried.

(2) *Protein A.*—The dialysate from (1) was diluted to 32 ml. and barium acetate (0·32 g.) added. After 1 hr., the precipitate was collected by centrifugation, and washed with 1 per cent barium acetate solution (5 ml.). It was suspended in water (7 ml.) and the suspension shaken gently with the sodium form of Zeocarb 215 (The Permutit Co. Ltd., approx. 1 g. of moist granules) for 30 min. After standing overnight, the Ba^{++}-free solution was pipetted off from the ion-exchange resin and dialysed against distilled water. Because this fraction was rendered partially insoluble by freeze-drying, it was retained in solution for biological testing and further fractionation. A small amount was freeze-dried for chemical analysis.

(3) *Crude polyglutamic acid.*—Ethanol was added to the supernatant solution from (2) to a concentration of 5 per cent (v/v). After 16 hr., the sticky precipitate was collected and mixed with water (5 ml.) and the sodium form of Zeocarb 215 (approx. 2 g. moist granules). The mixture was shaken for 30 min. and then the Ba^{++}-free solution was separated from the resin, dialysed and freeze-dried.

(4) *Protein B.*—Ethanol was added to the supernatant solution from (3) to a final concentration of 33 per cent (v/v). After 16 hr. the precipitate was collected, washed with a mixture of 1 per cent barium acetate solution (2 vol.) and ethanol (1 vol.). The precipitate was then mixed with water (10 ml.) and the sodium form of Zeocarb 215 (approx. 1 g. of moist granules) and the mixture shaken for 30 min. After removing the resin the Ba^{++}-free solution was dialysed and freeze-dried.

(5) *Crude polysaccharide.*—The supernatant from (4) was dialysed and freeze-dried.

TABLE I.—*Yields, Analysis and Aggressin Activity of Fractions from the Intracellular Material of* B. anthracis

(All values per cent)

Fraction.	Yield.	N.	Carbohydrate (as galactose).	Glutamic acid.	Polyglutamic acid.	Anti-phagocytic activity (lowest active conc.).	Anti-complementary activity (lowest active conc.).
Diffusate . .	45	4·4	8·5	1	—	2·0	Inactive at 1·0
Protein A . .	8	13·7	2·0	19	1	0·1–0·05	0·16
Crude polyglutamic acid . .	8	10·0	2·0	82	75	0·3	0·4
Protein B . .	14	15·1	3·6	20	2	0·1–0·05	0·24
Crude polysaccharide . .	9	7·6	30·0	4	—	Inactive at 2·0	Inactive at 1·0

Table I shows the yields, analyses and aggressive activity of the fractions described above ; all figures are the average of a number of similar experiments. The success of the fractionation method in separating the polyglutamic acid, the polysaccharide and the proteins is indicated by the analyses.

The diffusate had P, 1·3 ; hexosamine, *nil*, and ash 63 per cent. Paper chromatography after hydrolysis with 0·5 N-HCl at 100° for 1 hr. showed the presence of glucose and ribose ; after hydrolysis with 3 N-HCl at 100° for 16 hr. glycine, alanine, aspartic acid and glutamic acid were detected. The ultra-violet absorption spectrum of a solution had a maximum at 260 mμ. The anti-phagocytic activity of this fraction was non-specific and was caused by the hypertonicity of solutions. Identical anti-phagocytic activity was shown by solutions of NaCl which had a similar hypertonicity. The diffusate had no virulence-enhancing activity at 2 per cent.

Polyglutamic acid.—The crude polyglutamic acid had appreciable anti-phagocytic activity. The purification of polyglutamic acid from this fraction, its criteria of purity and its biological and chemical properties are given in detail by Zwartouw and Smith (1956). Only relevant facts are reported here. Removal of active protein impurities reduced the anti-phagocytic activity, and the final purified product, which contained less than 0·2 per cent of foreign amino acids, was active at 0·8 per cent. It was active in the virulence-enhancing test at 2 per cent and had no anti-complementary activity at 1 per cent.

The polysaccharide was purified (see Smith and Zwartouw, 1956) and had no anti-phagocytic, virulence-enhancing or anti-complementary activity at 1 per cent.

The Protein Fractions of B. anthracis *Grown* in vivo.

Proteins A and B had the highest aggressive activity of the fractions from *B. anthracis*. The remainder of this paper is concerned with their chemistry and with the relevance of their biological activity to the aggressive action of intact *B. anthracis in vivo*. Both proteins were inactive as immunising antigens at 0·7 per cent and showed no marked activity in the virulence-enhancing test at 1 per cent. Relatively high concentrations of some batches of Protein A had a lethal effect on *B. anthracis* (strain N.P.). This peculiar effect was destroyed by incubating a sterile solution of the protein at 37° overnight, a procedure which did not alter the anti-phagocytic or anti-complementary activity.

The analysis of proteins A and B is given in Table II. Paper chromatography of their acid hydrolysates showed that the following amino acids were present in both fractions: glycine, alanine, phenylalanine, valine and/or methionine, leucine and/or iso-leucine, threonine, serine, cysteine, proline, aspartic acid, glutamic acid, lysine, histidine and arginine. Their ultracentrifugal and electrophoretic behaviour at pH 8 showed them to be heterogeneous.

TABLE II.—*Analyses of Proteins A and B from* B. anthracis *Grown* " in vivo "

	Protein A (%).	Protein B (%).
Nitrogen	13·7	15·1
Phosphorus	0·25	0·81
Carbohydrate (as galactose)	2·0	3·6
(as ribose)	1	2
Pentose	1	2
Glutamic acid	19	20
Polyglutamic acid	1	2
Free lipid	*Nil*	*Nil*
Combined lipid	5·7	1·6
Combined lipid phosphorus	0·02	*Mil*
Absorption of ultraviolet light	No max. at 260 mμ	Max. at 260 mμ

Exhaustive attempts were made to purify the aggressin in proteins A and B without success.

Attempts at subfractionation.—Fractionation with ammonium sulphate, with ethanol at 0°, with barium acetate (1 per cent) and ethanol at 0°, and by electrophoresis in the Tiselius apparatus and on paper gave subfractions which all had similar activity. The active material was not preferentially adsorbed on ion exchange resins, Fuller's earth powder and precipitates of calcium phosphate or benzoic acid. Paper chromatography with 20 per cent (w/v) NaCl as solvent did not separate the active material.

Removal of protein by the Sevag method.—Solutions of proteins A and protein B (1 per cent) containing NaCl (6 per cent) were shaken with a half volume of chloroform-butanol mixture (4 : 1). The precipitated gel was removed and the process repeated until no further gel formed. The deproteinised solutions (0·1 per cent) which contained most of the original P and pentose, were inactive in the anti-phagocytic test.

Enzymic degradation.—Trypsin at pH 8 and ficin at pH 7 produced considerable hydrolysis of both proteins but no significant loss of anti-phagocytic activity.

Some concentration of active material was effected by dialysis after treatment with trypsin.

A 1 per cent solution of mixed protein A and B containing a little $CHCl_3$ was treated with crystalline trypsin (0·01 per cent) and maintained at pH 8 and 20° for 24 hr. A further quantity (0·01 per cent) of trypsin was added and after a second 24 hr. the mixture was divided into two parts. One was ultrafiltered through a collodion sac almost to dryness and the ultraconcentrate and ultrafiltrate reserved. The other part was dialysed against water, the dialysate reserved and the diffusate recovered by freeze-drying. Table III summarises the results of biological assays on these fractions. It will be noted that although the anti-phagocytic activity was not affected by treatment with trypsin the anti-complementary activity was destroyed.

TABLE III.—*Action of Trypsin on a Mixture of Proteins A and B*

Sample.	Amino N as per cent total N.	Yield per cent.	Anti-phagocytic activity (lowest active conc. per cent).	Anti-complementary activity (lowest active conc. per cent).
(1) Untreated protein	11	—	0·1	0·1
(2) Trypsin control*	—	—	Inactive	Inactive
(3) Protein control*	17	—	0·1	0·2
(4) Protein + trypsin*	40	—	0·1	Inactive at 1·0
Fractions from (4)				
Ultraconcentrate	23	43	0·05	Inactive at 1·0
Ultrafiltrate	51	48	Inactive at 0·1	Inactive at 1·0
Dialysate	16	39	0·05	—
Diffusate	52	47	Inactive at 0·1	—

* The samples were kept for 48 hr. at pH 8 and 20°.

An Increase of Phagocytosis Resistance in B. anthracis *after Treatment with Proteins A and B.*

In view of the failure of all attempts to fractionate the anti-phagocytic material, attention was given to the behaviour of proteins A and B in the anti-phagocytic test. It was possible that these mixtures of cellular constituents were not true aggressins which directly interfered with phagocytosis but merely appeared to have aggressive activity by virtue of a nutritional effect on the growing test organism (*B. anthracis* strain N.P.). The nutritional effect might operate in two ways, by stimulating growth so that more organisms were present to be phagocytosed or by inducing the production by the test organism of anti-phagocytic substances or mechanisms. Such explanations could account for the failure to fractionate the protein mixtures (obtained from *B. anthracis* strain N.P.) because a number of different constituents might have so acted. Therefore, an analysis was made of the mode of action of proteins A and B in the routine assay for anti-phagocytic activity. Details of this test, which is based on the viable count of organisms surviving phagocytosis, are given by Keppie *et al.* (1953); the numbers of organisms counted in the tests were such that statistical analysis has shown that a difference of 10–15 in the percentage of organisms

surviving phagocytosis in experimental and control samples was significant (Smith and Gallop, 1956).

Effect on growth

Proteins A and B did not cause the numbers of the test organisms to increase in the routine anti-phagocytic assay. The test was made without the phagocytes, *i.e.*, using plasma (0·2 ml.) instead of blood (0·4 ml.). After 1½ hr. at 37° the bacterial count in tubes containing a mixture of proteins A and B (0·5 per cent) or their hydrolysate (3 N-HCl, 100°, 16 hr.) was the same as that in control tubes.

Increased resistance to phagocytosis by treatment with proteins A and B at 37°.

Anthrax bacilli were tested for susceptibility to phagocytosis after the following treatment. The organisms were held for ¾ hr. in a system comparable to the usual test but containing plasma (0·2 ml.) instead of blood ; the experimental sample contained a mixture of proteins A and B (0·5 per cent). The mixtures were then centrifuged and the supernatants removed. Half the deposit of organisms was reserved and the other half was washed twice at 0° with gelatin-Locke solution (2 ml.). The unwashed and washed pre-treated organisms were then used in the phagocytosis test but no further aggressin was added and the mixtures were only incubated ¾ hr. In twelve experiments of this kind it was found that the pre-treated organisms had acquired resistance to phagocytosis. Thus, the average percentage of organisms remaining unphagocytosed among the washed and unwashed bacilli was respectively 14 and 19 in the controls but 30 and 44 after pre-treatment with bacterial proteins.

Increased resistance to phagocytosis by treatment with proteins A and B at 0°.

Experiments were made to test whether the increased resistance to phagocytosis of the test organism was the result of metabolic uptake by the bacteria or due to surface adsorption of the proteins.

A suspension (0·2 ml.) of the test organism (see Smith and Gallop, 1956) was mixed with plasma (0·2 ml.) and gelatin-Locke solution (0·2 ml.), and incubated for ¾ hr. at 37°. The cultures were cooled to 0° and maintained at this temperature for ½ hr. before a solution (0·2 ml.) of one or other of the proteins was added to the test samples and Locke solution (0·2 ml.) to the controls. After standing for a further ¾ hr. at 0° the organisms were collected by centrifugation at 0° and half the organisms washed as described in the previous section. Table IV summarises the results of numerous experiments in which the susceptibility to phagocytosis of the treated organisms (washed and unwashed) was compared with that of control organisms. It was necessary to compare the results obtained with unwashed organisms with those from additional control samples for the following reason. Approximately 1/15th of the original protein mixture remained around the unwashed organisms of the experimental sample and was therefore present during their subsequent phagocytosis. This quantity of material, which would not have affected a normal anti-phagocytic test using recently germinated spores (see Smith and Gallop, 1956), inhibited to some extent the phagocytosis of the organisms described above which had become partially resistant by growing for ¾ hr. in plasma. Furthermore, the degree to which the phagocytosis of these organisms reacted to the residual protein was related to the sample of guinea-

pig plasma in which they were grown. To provide a correct basis for comparison with experimental samples a quantity of protein equal to that trapped around the centrifuged deposit of treated organisms was added to a normal control mixture of blood- and plasma-grown organisms; the phagocytosis of organisms from these samples is included in Table IV as " corrected controls ". The results show that *B. anthracis* which has grown in plasma for $\frac{3}{4}$ hr. can adsorb from proteins A or B a material which confers on them an increased resistance to phagocytosis. Protein B may also contain a little of this material. The adsorption depends to some extent on the preliminary growth of the organisms in plasma since organisms grown in tryptic meat broth for $\frac{3}{4}$ hr. did not show the phenomenon.

TABLE IV.—*Treatment of Phagocytosis-sensitive Organisms with Proteins A and B at* 0°

Sample.	Conc. (per cent) in contact with organisms at 0°.	Number of experiments from which the average result obtained.	Average per cent of organisms surviving phagocytosis. Washed organisms. (*a*) Control.	(*b*) Expt.	(*b*–*a*).	Unwashed organisms. (*a*) Control.	(*a*) Control (corrected)*	(*b*) Expt.	(*b*–*a*).
Mixture of proteins A and B	0·25	3	11	30	*19*	15	25	41	*16*
Protein A	0·125	11	17	30	*13*	17	24	40	*16*
„ B	0·5	7	32	36	*4*	29	39	44	*5*
	0·25	7	20	28	*8*	21	31	40	*9*

For details of the treatment of organisms see text.
* See text for details of correction.

Protein Fractions from in vitro*-grown Phagocytosis-sensitive Organisms.*

To assess the importance of the protein fractions described above in the resistance to phagocytosis of *B. anthracis in vivo*, phagocytosis-sensitive organisms grown *in vitro* were extracted to see if they contained protein fractions with similar biological activity to those obtained from the resistant organisms. Two strains of *B. anthracis* were grown *in vitro*, the virulent N.P. strain and the avirulent non-capsulated " Sterne " strain. When grown in tryptic meat broth, both strains were readily phagocytosed but they differed in degree of sensitivity. The virulent N.P. strain was 10–20 per cent less susceptible to phagocytosis than the " Sterne " strain when examined in a standard phagocytosis test (Smith and Gallop, 1956); also, relatively higher concentrations of an aggressin were needed to interfere with the phagocytosis of the "Sterne " strain of organisms.

Extracts of the organisms from 16 hr. shaken cultures in tryptic meat broth (see Methods) were separated into five fractions by the method described already for the ammonium carbonate extract of the organisms grown *in vivo*. The extracts were prepared by shaking the organisms with " ballotini " because when grown *in vitro* they do not lyse in ammonium carbonate solution as do those grown *in vivo* (Smith *et al.*, 1953*a*, *b*). Hence a ballotini extract of the latter was prepared and fractionated to permit a strictly comparative study. The yields, analyses and biological activity of the four high M.W. fractions from these extracts

are shown in Table V; corresponding figures for fractions from the ammonium carbonate extract of organisms grown *in vivo* are shown in Table I. It is apparent from Table V that the virulent N.P. organisms formed small amounts of polyglutamic acid when growing *in vitro* in tryptic meat broth but the avirulent uncapsulated " Sterne " strain produced none of this substance.

TABLE V.—*Properties of Fractions from " Ballotini " Extracts of* B. anthracis *Grown* in vivo *and* in vitro

Organism from which extract prepared.	Fraction. Per cent ethanol in barium-acetate ethanol fractionation.	Yield per cent.	Polyglutamic acid per cent.	Anti-phagocytic activity (lowest active conc. per cent).	Anti-complemenary activity (lowest active conc. per cent).
In vivo, N.P.	0 (protein A)	11*	<2	0·07	0·25
	5	6	10–20†	0·5	0·2
	33 (protein B)	14	7	0·16	0·12
	Sol.	9	—	Inactive 0·5	Inactive 2
In vitro, N.P.	0 (protein A)	9*	<2	0·07	0·4
	5	6	7	0·5	0·6
	33 (protein B)	24	3	Inactive 0·5	1·0
	Sol.	4	—	,, 0·5	Inactive 2
In vitro, " Sterne "	0 (protein A)	9*	<2	0·07	1
	5	5	<2	0·5	1·5
	33 (protein B)	19	<2	Inactive 0·5	2
	Sol.	4	—	,, 0·5	Inactive 2

* Proteins A did not go into solution when treated with Zeocarb 215 as easily as did the corresponding fraction from the ammonium carbonate extract of *in vivo*-grown organisms.

† Paper chromatography on hydrolysates showed 15–20 per cent more glutamic acid than in bacterial protein. The paper electrophoretic method indicated approx. 10 per cent polyglutamic acid but some polyglutamic acid appeared to remain with the protein at the origin.

Extracts of the organisms grown *in vitro* contained substances which were active in the anti-phagocytic test but they were present in smaller quantity than in the extracts of organisms grown *in vivo*. Thus, the yield and activity of protein A fractions from organisms grown *in vitro* were similar to those obtained from the organisms grown *in vivo*, but the protein B fractions had negligible activity. In the anti-complementary test, the fractions from N.P. strain grown *in vitro* were less active than those from the *in vivo*-grown organisms while those from the " Sterne " strain were almost inactive.

The different protein fractions were tested for their ability to confer phagocytosis resistance on *B. anthracis* when it was treated with them at 0°. The experiments were made as described in the previous section for proteins A and B from the ammonium carbonate extracts of the organisms grown *in vivo*; the results are summarised in Table VI. Proteins from the virulent organisms grown *in vitro* conferred resistance to phagocytosis but to a smaller extent than those obtained from material grown *in vivo*. On the other hand, treatment with the proteins from the avirulent " Sterne " strain left the test organism phagocytosis-sensitive. Furthermore, in five experiments, organisms of the " Sterne " strain grown in plasma for ¾ hr. were not made more resistant to phagocytosis either by

treatment at 0° with the proteins from N.P. organisms or when these materials were included during the preliminary growth in plasma.

TABLE VI.—*The Treatment of Phagocytosis-sensitive Organisms at* 0° *with Proteins A and B from Extracts of* B. anthracis *Grown* in vivo *and* in vitro

Sample.	Number of experiments from which the average result obtained.	Conc. per cent in contact with organisms at 0°.	Average per cent of organisms surviving phagocytosis. Washed organisms, (a) Control	(b) Expt.	(b–a).	Unwashed organisms. (a) Control	(a) Control (corrected).	(b) Expt.	(b–a).
N.P. *in vivo*									
Protein A	3	0·125	23	38	*15*	24	37	50	*13*
„ B	6	0·5	29	36	*7*	31	40	46	*6*
	6	0·25	25	30	*5*	25	25	30	*5*
N.P. *in vitro*									
Protein A	6	0·25	24	30	*6*	17	20	37	*17*
„ B	2	1·0	30	28	*−2*	32	41	43	*2*
"Sterne" *in vitro*									
Protein A	4	0·5	26	26	*0*	31	33	30	*−3*
	10	0·25	28	25	*−3*	26	31	33	*2*
„ B	2	1·0	30	26	*−4*	30	44	44	*0*

DISCUSSION

It is apparent from recent work (Smith and Gallop, 1956; Smith, Tempest, Stanley, Harris-Smith and Gallop, 1956; this paper) that the overall aggressive action of *B. anthracis* growing *in vivo* is not due to a single substance but to an armoury of different products, some produced extracellularly and some forming integral parts of the organism. It seemed improbable that each one would satisfy all tests for aggressive activity that we have used, namely those for anti-phagocytic, virulence-enhancing, anti-complementary and anti-anthracidal activity, and this has proved to be so.

The two most important aggressins which seem to assist *B. anthracis* to establish itself in the tissues of a host are the extracellular toxin (Smith *et al.*, 1955), which later in the disease acts specifically in killing the host, and capsular poly-glutamic acid. The toxin is clearly an aggressin because at least one purified component of it has anti-phagocytic, virulence-enhancing, and anti-anthracidal properties (Smith *et al.*, 1956). The following fact emphasises the fundamental importance of the toxin as an aggressin. It and its components are antigens which actively protect animals against the disease, and it is in the very early stages of the latter that the respective antibodies abort the infection (Cromartie, Bloom and Watson, 1947; Cromartie, Watson, Bloom and Heckly, 1947). The production of oedema by the toxin may be responsible for part of its aggressive action (*cf.* Dubos, 1954). Isolated polyglutamic acid which has no toxic or immunising activity is anti-phagocytic and is present in the capsule surrounding *B. anthracis*. Since *in vivo* this material is concentrated at the place where it can be most effective, *i.e.*, between the organism and the phagocyte, its anti-

phagocytic action (Zwartouw and Smith, 1956) probably plays a large part in preventing the ingestion of the capsulated organism. Polyglutamic acid is also found free in the plasma of the host and here it may help to combat any bacteriolytic powers which might be possessed by the latter.

The work described here shows that a further non-toxic factor may contribute to the anti-phagocytic action of virulent *B. anthracis*. It was present in an impure lipoprotein fraction which had a high anti-complementary activity. There is some evidence that this substance, which is of high molecular weight and apparently not connected with the anti-complementary activity, could act at the surface of the organism; it renders phagocytosis-sensitive organisms more resistant to ingestion when they are treated with it at 0°. It was not present in an avirulent uncapsulated strain of *B. anthracis*. Recognition of the possible existence of such a surface material was difficult for two reasons. First, extracts of organisms used in this work were a mixture of somatic and capsular material; it was thus impossible to say whether or not any active fraction was a surface component. Second, protein from virulent capsulated organisms had anti-phagocytic activity yet the bulk of it must have been within the bacillus where it is doubtful that it played any aggressive rôle. Thus the somatic protein from uncapsulated phagocytosis-sensitive organisms was similarly active in the simple anti-phagocytosis test, although it was unable to induce resistance in test organisms after pre-treatment.

The possible addition of a protein fraction to polyglutamic acid as a further anti-phagocytic substance operating in the capsule of *B. anthracis* recalls the relationship between the M protein and hyaluronic acid of capsulated virulent streptococci. Not only is the capsular hyaluronic acid responsible for virulent and anti-phagocytic properties but the M protein, which can be isolated from whole extracts of the organisms, plays its part and some of it is present on the surface of the capsulated organisms (Rothbard, 1948; Morris and Seastone, 1955).

The existence of a possible third component in the aggressive mechanism of *B. anthracis* can only be proved by an examination of the constitution of the capsule of *B. anthracis* if this can be removed preferentially from the somatic material. It is however relevant to point out here that Tomcsik (1956) has suggested that the ability of capsulated *B. anthracis* organisms to produce polyglutamic acid antisera may possibly be due to a connection with protein in the capsule. It should also be remembered that Smith and Gallop (1956) showed that the body fluids of infected guinea-pigs contained a protein aggressin resembling the bacterial protein, in addition to polyglutamic acid from the capsule of the organism.

SUMMARY

A mixture of the capsular and somatic material of *B. anthracis* grown *in vivo* has been extracted and substances with aggressive action isolated.

In addition to capsular polyglutamic acid, which has aggressive activity, some evidence has been obtained that a second aggressin, present in protein fractions, may have an anti-phagocytic action at the surface of the capsulated organism.

The overall aggressive activity of *B. anthracis* is discussed It is due mainly to the action of the extracellular toxin (see previous papers in this series) which later in the disease kills the host, and to capsular polyglutamic acid. The protein

aggressin must be shown to occur in the capsule of *B. anthracis* before its importance in virulence can be assessed.

REFERENCES

CONSDEN, R., GORDON, A. H. AND MARTIN, A. J. P.—(1944) *Biochem. J.*, **38**, 224.

CROMARTIE, W. J., BLOOM, W. L. AND WATSON, D. W.—(1947) *J. infect. Dis.*, **80**, 1.

Idem, WATSON, D. W., BLOOM, W. L. AND HECKLY, R. J.—(1947) *Ibid.*, **80**, 14.

DUBOS, R. J.—(1954) ' Biochemical Determinants of Microbial Diseases.' Cambridge, Mass. (Harvard University Press), p. 35.

FLYNN, F. V. AND DE MAYO, P.—(1951) *Lancet*, ii, 235.

FRAME, E. G., RUSSEL, J. A. AND WILHELMI, A. E.—(1943) *J. biol. Chem.*, **149**, 255.

IVANOVICS, G.—(1940) *Z. ImmunForsch.*, **97**, 402.

Idem AND BRUCKNER, V.—(1937) *Ibid.*, **90**, 304.

KEPPIE, J., SMITH, H. AND HARRIS-SMITH, PATRICIA W.—(1953) *Brit. J. exp. Path.*, **34**, 486.

MARKHAM, R. AND SMITH, K. M.—(1949) *Parasitology*, **39**, 330.

MORRIS, M. AND SEASTONE, C. V.—(1955) *J. Bact.*, **69**, 195.

PARTRIDGE, S. M.—(1948) *Biochem. J.*, **42**, 238.—(1949) *Nature, Lond.*, **164**, 443.

ROTHBARD, S.—(1948) *J. exp. Med.*, **88**, 325.

SMITH, H. AND GALLOP, R. C.—(1956) *Brit. J. exp. Path.*, **37**, 144.

Idem, KEPPIE, J. AND STANLEY, J. L.—(1953*a*) *Ibid.*, **34**, 471.—(1953*b*) *Ibid.*, **34**, 476.—(1955) *Ibid.*, **36**, 460.

Idem, TEMPEST, D. W., STANLEY, J. L., HARRIS SMITH, PATRICIA W. AND GALLOP, R. C.—(1956) *Ibid.*, **37**, 263.

Idem AND ZWARTOUW, H. T.—(1956) *Biochem. J.*, **63**, 447.

STRANGE, R. E. AND HARKNESS, N.—(1953) *Nature, Lond.*, **171**, 77.

TOMCSIK, J.—(1956) *Symp. Soc. gen. Microbiol.* " Bacterial Anatomy." Cambridge (University Press), p. 46.

ZWARTOUW, H. T. AND SMITH, H.—(1956) *Biochem. J.*, **63**, 437.