THE CHEMICAL BASIS OF THE VIRULENCE OF *BACILLUS ANTHRACIS* VII. TWO COMPONENTS OF THE ANTHRAX TOXIN: THEIR RELATIONSHIP TO KNOWN IMMUNISING AGGRESSINS

H. SMITH, D. W. TEMPEST, J. L. STANLEY, PATRICIA W. HARRIS-SMITH AND R. C. GALLOP

From the Microbiological Research Department, Porton, Wilts.

Received for publication March 1, 1956

SMITH, Keppie and Stanley (1955) demonstrated that *Bacillus anthracis* growing *in vivo* forms a specific oedema-producing toxin which is responsible for death of the host. This toxin, present in the plasma of guinea-pigs dying of anthrax, has not been produced by culture *in vitro*. It is unrelated to the capsule of the organism but closely connected with two preparations from *B. anthracis* which actively immunised animals against infection. These purified preparations were extracellular products of the organism, one isolated from an *in vitro* culture (Strange, and Belton, 1954) and the other separated from exudates of infected guinea-pigs. The latter was described in a previous paper (Smith and Gallop, 1956) and was called Fraction Y. Both preparations, when injected into a suitable host, produced antisera which neutralised the effects of the anthrax toxin, but possessed no toxic or oedema-producing activity. Both were antiphagocytic but had no virulence-enhancing activity. They were different in their chemical properties and in their behaviour in the ultra-centrifuge.

In the previous work (Smith and Gallop, 1956), the immunising aggressin Fraction Y was effectively separated by preparative ultracentrifugation after preliminary fractionation of plasma/exudate with salts and ethanol. This preliminary fractionation destroyed the already small toxic, oedema-producing and virulence-enhancing activities of the original plasma/exudate and consequently Fraction Y was devoid of such activities. When attention turned to toxic plasma which contained a much higher level of toxin than the plasma/exudate, the detrimental effect of such preliminary fractionation was known (Smith *et al.*, 1955) and was avoided. Direct ultracentrifugation of the toxic plasma led to the demonstration of two components of the anthrax toxin and to the recognition of an interesting relationship between them and Fraction Y and the immunising aggressin prepared *in vitro*. This work is described here together with a brief account of unsuccessful attempts to produce the anthrax toxin *in vitro*, which emphasise the importance of the *in vivo* environment in relation to toxin formation.

MATERIALS AND METHODS

Toxic plasma—was collected from guinea-pigs dying of anthrax, filtered sterile and freeze-dried as described by Smith *et al.* (1955).

Skin test for oedema-producing activity—as described by Smith *et al.* (1955). In some tests, the sizes of the lesions were noted and are reported here in the nomenclature used by Smith *et al.* (1955). In later experiments, biological assay of fractionation samples was carried out

by injecting serial descending dilutions of the materials under test and noting the null point, *i.e.*, the first dilution not giving a positive reaction.

Test for lethality to mice—as described by Smith *et al.* (1955).

Assay for immunising antiphagocytic and virulence-enhancing activites—as described by Smith and Gallop (1956).

Immunising aggressin produced in vitro—our colleague R. E. Strange supplied us with adequate quantities of a purified preparation. In this paper it is described as the *in vitro* antigen.

Immunising aggressin isolated from in vivo *sources*—this was Fraction Y from plasma/exudate (Smith and Gallop, 1956).

Ultracentrifugation—was carried out in the preparative angle rotor of the Spinco ultracentrifuge at 100,000 × g (40,000 r.p.m.) and at 0°–5°. In the text only the duration of ultracentrifugation is quoted.

Anthrax antiserum—hyperimmune serum prepared in the horse by injection of spores of the " Sterne " uncapsulated strain of *B. anthracis*.

EXPERIMENTAL AND RESULTS

Ultracentrifugation of Toxic Plasma

In the fractionation of plasma/exudate Fraction Y was deposited from a 1·0–1·5 per cent solution of protein (Fraction A described by Smith and Gallop (1956)) in 0·2μ phosphate buffer (pH 8) by ultracentrifuging for 4–6 hr. When the relatively viscous toxic plasma (protein content approx. 6 per cent) was treated similarly the time of ultracentrifugation had to be increased to remove the bulk of the toxic activity from the supernatant.

Freeze-dried toxic plasma was re-constituted to its original volume (15 ml.) with ice-cold water. Some insoluble material was removed by centrifugation

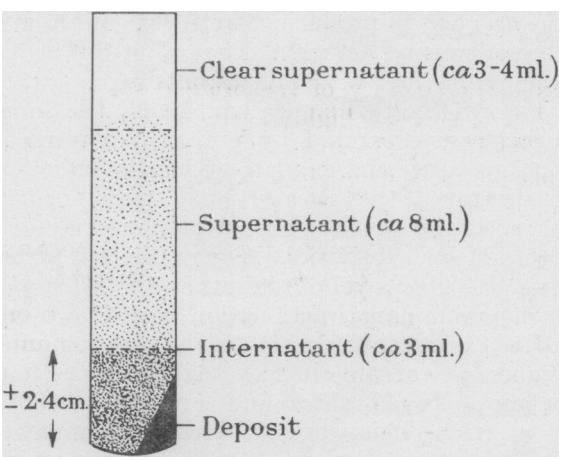

FIG. 1.—Diagram of the contents of a tube after ultracentrifuging the toxic plasma for 16 hr.

(10,000 r.p.m., 20 min.) at 0°, suspended in saline (3 ml.) and reserved. The supernatant (13–14 ml., sufficient to fill a tube of the rotor) was ultracentrifuged for 16 hr. When the tube was removed from the rotor the appearance of its contents was as shown diagrammatically in Fig. 1. *Clear Supernatant* and *Supernatant* were removed carefully with Pasteur pipettes into separate bottles for biological testing. Ice-cold saline (pH 7·4) was added to fill the tube which was then inverted

several times to disperse the internatant in the saline but not to dissolve the deposit; the material was then ultracentrifuged for 16 hr. After this second ultracentrifugation, the whole of the liquid was removed carefully from the deposit and concentrated on collodion ultrafilters to approx. 3 ml.; this constituted the *Internatant.* The deposit was dissolved in a quantity of ice-cold saline (pH 7·4; sufficient to fill the tube) and then ultracentrifuged for 6 hr. The supernatant was concentrated on collodion ultrafilters to approx. 1·5 ml. and constituted the *Washings.* The *Washed Deposit* was dissolved in ice-cold saline (1·5 ml., pH 7·4), centrifuged to remove a little insoluble matter, and reserved for biological testing. Table I summarises the yields and the results of tests for toxicity in 4–6

TABLE I.—*Toxicity Tests on Ultracentrifugal Fractions of Toxic Plasma*

Fraction.	Vol.* (ml.).	N (approx.) per cent.	Skin test (size of lesion) (ml.). 0·4.	0·2.	0·1.	0·05.	Mouse lethality test (ml.). 2.	1.	0·5.	0·25.
Soluble, re-constituted toxic plasma	13–14	1·1	—	8 : 38	7 : 32	6 : 30	—	10/10	—	—
Residue insoluble in water	3	1·4	—	3 : 18	*tr.*	—	—	0/2†	—	—
Clear supernatant	3–4	0·16	—	4 : 16	*tr.*	—	—	0/10	0/10	—
Supernatant (II)	7–8	0·7	6 : 30	5 : 22	*tr.*	—	6/25	1/25	0/25	—
Internatant	3	1·7	—	5 : 30	4 : 24	—	—	1/20	—	—
Washings	1·5	0·6	—	6 : 30	5 : 26	—	—	0/5	—	—
Washed deposit (I)	1·5	0·6	—	7 : 30	6 : 28	5 : 24	—	0/5	0/5	0/5
In vitro antigen	—	0·015‡	—	*Nil*	*Nil*	*Nil*	1/10	0/10§	0/10	—

* The yields from a single ultracentrifuge tube are quoted here. † Injected intraperitoneally. ‡ Equivalent to 0·1 per cent w/v solution. § Unaltered by dissolving the *in vitro* antigen in normal guinea-pig plasma.

similar experiments. The toxicity of the original toxin was largely lost in the fractions from it. Even the most highly active fraction, *i.e.*, the *Washed Deposit* was less active than the original toxin although eight times more concentrated than in the toxic plasma from which it was obtained.

A Synergic Mixture of the Deposit (Factor I) and Supernatant (Factor II) from the Toxic Plasma

Skin test and mouse lethality tests were carried out on mixtures of the *Supernatant* and the solution of the *Washed Deposit*, the amounts being equal to the smallest of those referred to in Table I. The average results of a number of similar experiments on these mixtures are shown in the first line of Table II. The toxicity of the mixture in skin and mouse lethality tests was greater than that shown separately by each component at four times their strength in the mixture (see Table I). *Washed Deposit* and *Supernatant* therefore contained two distinct components (Factors I and II respectively) of the anthrax toxin which formed a synergic mixture.

A number of materials were tested for ability to replace Factor II in the above tests. In skin tests, the lesion (5 : 24) produced by injection of the solution (0·05 ml.) of Factor I was not significantly increased by admixture with 0·1 ml. of sodium polyglutamate (0·5 per cent), normal guinea-pig plasma and its ultrafiltrate,

TABLE II.—*Synergic Mixtures of Washed Deposit (Factor I) with Supernatant (Factor II) and of Each Factor with the* in vitro *Antigen*

Mixture.†	Vol. (ml.).	Skin tests. Alone.	With* normal serum.	With* anti-serum.	Vol. (ml.).	Mouse lethality tests. Alone.	With* normal serum.	With* anti-serum.
Washed Deposit (Factor I) + Supernatant (Factor II)	0·05 + 0·1	7 : 34	7 : 35	*Nil*	0·25 + 0·5	5/5	—	—
Washed Deposit (Factor I) + *In vitro* antigen (0·1 per cent)	0·05 + 0·1	7 : 33	7 : 35	*Nil*	0·25 + 0·5	5/5	9/9	0/9
Supernatant (Factor II) + *In vitro* antigen (0·1 per cent)	0·1 + 0·1	5 : 22	5 : 28	*Nil*	0·5, 1·0‡ + 0·5, 1·0‡	2/5, 8/10	5/5, 5/5	0/5, 0/10

* 1/5 of the volume of the mixture. † Details of the preparation and strength of the components and their toxicity when injected alone see text and Table I. ‡ A 0·1 per cent solution of *in vitro* antigen in Supernatant—1 ml. injected.

ultrafiltrate from Factor II, a solution of $NaHCO_3$ (0·1, 0·02 and 0·004 per cent), "ballotini" and ammonium carbonate extracts of *B. anthracis* grown *in vivo* (Smith, Keppie and Stanley, 1953*a*, *b*), filtrates of cultures of *B. anthracis* (strain N.P.) in normal guinea-pig plasma or whole blood, A.T.P. (0·1 per cent) and 0·02 M solutions of $FeCl_3$, $FeSO_4$, $MgSO_4$, $CaCl_2$ and KCl. In mouse lethality tests no animals died when batches of 4 were injected with mixtures of a solution (0·25 ml.) of Factor I with 0·5 ml. of sodium polyglutamate (0·5 per cent) in saline, normal guinea-pig plasma and the ultrafiltrate from Factor II.

In view of the synergic action between the two factors the remaining fractions from ultracentrifugation were tested for the presence of Factors I and II by combining them in skin tests with the solutions of Factors II and I respectively. The residue insoluble in water and *Clear Supernatant* contained no significant amounts of either factor. As would be expected, the *Internatant* and *Washings* were mixtures of Factors I and II, the proportions of which varied somewhat from batch to batch. They were mixed together and treated by a similar process to that used on the original toxin to recover more of Factors I and II.

Synergic Mixtures of the In Vitro *Antigen with Factors I and II*

The solution of Factor I was mixed with the *in vitro* antigen, the amounts being equal to the smallest referred to in Table I. The average results of a number of similar skin tests and mouse lethality tests on these mixtures are shown in the second line of Table II. As with the mixture of Factors I and II, the toxicity of the mixture in both tests was greater than that shown separately by each component of the mixture at four times their combined strengths. The toxic action of the synergic mixture was specifically inhibited by anthrax antiserum.

Although the *in vitro* antigen will replace Factor II in skin and mouse lethality tests, the two materials are not identical. This was first shown by the fact that mixtures of the *in vitro* antigen with Factor II acted synergically and not additively in mouse lethality tests. Thus, in amounts equal to the two lowest of the three referred to in Table I, they formed mixtures significantly more lethal to mice (line 3, Table II) than the top concentration of each component (see Table I) when they were injected separately. The lethal action of the mixture was specifically inhibited by antiserum. These mixtures of Factor II with the *in vitro* antigen did not show a synergic action in the skin test.

Synergism of Mixtures of Factors I and II in Null Point Skin Assays; Inactivity of the In Vitro *Antigen*

For the further fractionation of Factors I and II a null point assay in the skin of guinea-pigs (see Methods) has been used. The following null points (average from 5–6 similar experiments) were obtained when the initial materials (0·2 ml.) were injected separately: original toxic plasma 1/128; solution of Factor I 1/64; and solution of Factor II (separated from the *Internatant* at a level a few mm. higher than in the previous experiments) 1/2.

Factor I (0·1 ml. of a 1/64 dilution) was mixed with Factor II (0·1 ml. of a 1/2 dilution). When injected, the mixture produced a positive skin reaction and could be diluted 1/8 before the null point was reached. If Factor II was replaced by the *in vitro* antigen (0·1 per cent) no skin reaction was produced. Null point assays were carried out in which one factor in the mixture of the two was held constant at half the strength of its null point when injected separately, while the other factor was serially diluted. In these, as in the first experiments, the mixtures of Factors I and II produced a synergic effect but mixtures with the *in vitro* antigen did not. Thus, a 1/64 dilution of Factor I (0·1 ml.) produced no skin reaction when injected alone or with a 0·1 per cent solution of the *in vitro* antigen (0·1 ml.) but on admixture with a solution of Factor II (0·1 ml.) the latter had to be diluted 1/16 before a skin reaction was not evoked. Similarly the solution of Factor II (0·1 ml.) produced no skin reaction when injected alone or with a 0·1 per cent solution of the *in vitro* antigen (0·1 ml.) but on admixture with a solution of Factor I (0·1 ml.) the latter had to be diluted 1/1024 before the null point was reached.

There does not seem to be a large excess of Factor I or II in the original toxin since addition of solutions of either Factor I (0·1 ml. of 1/64) or Factor II (0·1 ml. of 1/2) to the toxin (0·1 ml. of 1/128) formed a mixture which failed to produce a skin reaction on injection.

Stability of Factors I and II

Factors I and II retained both their inherent activity and that shown on recombination with a fixed and separately unreactive amount of the other, after freeze-drying and for at least 28 days in the dry state (*cf.* original toxin, Smith *et al.*, 1955).

In solution at 0°, both factors gradually lost activity especially if the solutions contained any slight bacterial contamination. For this reason, the solutions were filtered through "Millipore Filters" as soon as they were collected and freeze dried unless they were to be used immediately.

Further Purification of Factor I : Its Immunising and Aggressin Activity

The *Washed Deposit* (*ca.* 60 mg.) obtained from one ultracentrifuge tube of toxic plasma (13–14 ml.) was dissolved in ice-cold phosphate buffer (pH 8, 0·2 μ, 13–14 ml.). The solution was ultracentrifuged for 6 hr. and the process repeated 4 times. The final deposit (*ca.* 6 mg.) was dissolved in phosphate buffer (1 ml.) for biological testing. Analytical ultracentrifuge diagrams of the final deposit are shown in Fig. 2. The main component (S = approx. 15) in this diagram corresponds

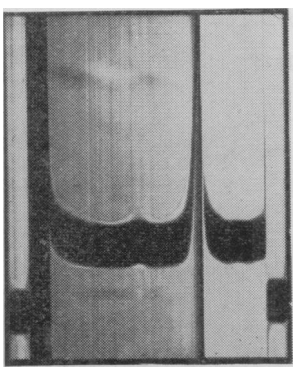

FIG. 2.—Analytical ultracentrifuge diagram of purified Factor I of the anthrax toxin. The conditions were the same as those described in Fig. 2 of Smith and Gallop (1956); the protein concentration was 1·2 per cent.

with Fraction Y (see Fig. 2(*e*) of Smith and Gallop, 1956); the small fast-moving component is present in normal guinea-pig plasma.

The toxic, immunising, and aggressin activity of the original *Washed Deposit* and the final deposit after 5 washings with phosphate buffer are given in Table III, which also includes a description of the behaviour of the Fraction Y in the biological tests. The final deposit retained an ability to form a synergic mixture with Factor II. Furthermore Factor I in this deposit was free from much impurity. Its anti-phagocytic and immunising activities were similar to those of Fraction Y, but in

TABLE III.—*Purification of Factor I and a Comparison with Fraction Y.*

Fraction.	Toxic action. Conc. (per cent protein).	Skin reaction (null point) dilution. Inherent activity.	Skin reaction (null point) dilution. Combined with† Factor II.	Mouse lethality test.‡ No. dead / No. injected.	Immunising* activity (lowest conc. giving 50 per cent protection).	Anti-phagocytic* activity (lowest active conc. per cent).	Virulence* enhancing activity (lowest active conc. per cent)
Factor I							
(*a*) Original Washed Deposit	4	1/64	1/1024	5/5	0·020 (0·007–0·058)	0·15	0·5
(*b*) Deposit—after washing five times in the ultra-centrifuge	0·6	1/32	1/512	7/9	0·004 (0·0013–0·012)	0·03	0·08
Fraction Y	0·5	Undiluted	Undiluted	0/9	0·006 (0·003–0·012)	0·02	Inactive at 0·5

* For details of these tests and methods of expressing activities see Smith and Gallop (1956). † Factor II held constant at 0·1 ml. of a 1/2 dilution, *i.e.*, half the strength of its null point. ‡ Solution (0·5 ml.) mixed with solution of Factor II (0·5 ml.).

contrast to the latter, which had no ability to form a toxic mixture with Factor II, it had marked virulence-enhancing properties. Further tests showed that this purified Factor I formed a toxic mixture with the *in vitro* antigen but Fraction Y did not.

The supernatant from the ultracentrifuging of the toxic plasma retained an immunising activity but a detailed investigation of the immunising and aggressin activity of Factor II cannot be made until some purification of it has been attained.

Attempts to Produce the Anthrax Toxin In Vitro

The following experiments were carried out in an attempt to produce the anthrax toxin *in vitro*. In contrast to the attempts of previous workers (Eurich and Hewlett, 1930 ; Sobernheim, 1931 ; King and Stein, 1950) we were aided by the knowledge that a small sublethal concentration of the toxin could be detected by a skin reaction ; it had been established that oedema-producing activity in the skin of a guinea-pig and mouse lethality were due to the same toxin (Smith *et al.*, 1955). Filtrates of numerous cultures were examined for toxin using this skin test. None of the preparations which are described below contained even the small amount of toxin detectable by this test. The cultures described represent a progressive approach towards the conditions occurring *in vivo*.

Growth in laboratory media

B. anthracis (strain N.P.) was grown at 37° for 16 hr. in tryptic meat broth and in the medium used by Belton and Strange (1954) to produce the *in vitro* antigen. No toxin was produced.

Growth in guinea-pig plasma

Cultures of *B. anthracis* in heparinised (10 i.u. per ml.) guinea-pig plasma were grown for 24 hr. under the following conditions : static, 37°, aerobic ; static, 37°, anaerobic ; static, 20°, aerobic ; static, 20°, anaerobic ; shaken, 37°, air containing 5 per cent CO_2 ; all were without activity. Since the plasma A.T.P., glutamine and $NaHCO_3$ levels were reduced by the growth of *B. anthracis* during the final bacteraemia of anthrax in the guinea-pig, A.T.P. (0·1 per cent), glutamine (0·1 per cent) and $NaHCO_3$ (1 per cent) were added separately to static 24 hr. cultures of *B. anthracis* in guinea-pig plasma but failed to stimulate toxin synthesis. Seven-hour cultures (37°) of *B. anthracis* in guinea-pig plasma alone and with the addition of yeast ribonucleic acid (0·1 per cent) or its alkaline digest (0·1 per cent) did not contain toxin.

To test whether related strains of organisms could produce the anthrax toxin some of the experiments described above were carried out using *B. anthracis* (Sterne), *B. anthracis* (Stamatin and Stamatin, 1936) and *Bacillus cereus*. Only cultures of the latter produced a skin reaction which was, however, not inhibited by anthrax antiserum.

Growth in infected blood

The blood of a guinea-pig infected with *B. anthracis* and approximately 8–10 hr. from death was removed by heart puncture. The bacterial count in the blood was approx. 1×10^6 chains per ml. (see Keppie, Smith and Harris-Smith, 1955) and only a small amount of toxin was detectable in the plasma. If the blood had not been removed until the animal died, the bacterial count would have been approx. $0{\cdot}5–1 \times 10^9$ chains per ml. (Keppie *et al.*, 1955) and approximately 30 × more anthrax toxin would have accumulated. The infected blood, or the plasma after removal of the red cells by slow centrifugation, was shaken in a Warburg apparatus for 8–10 hr. with continuous gassing using air containing 5 per cent CO_2. Growth of the bacteria occurred almost at the same rate as the growth *in vivo* but no significant amounts of toxin were produced. Addition of $NaHCO_3$ (0·1 per cent), of tissue slices (spleen, kidney or liver), of A.T.P. (1, 5 and 25 mg. per 100 ml.) and glutamine (0·1 per cent) to this system were without effect in producing toxin. Neither increasing the rate of shaking

and surface area of the liquid in the Warburg flask nor the use of static culture resulted in toxin production. In some of these experiments, however, samples taken at different times in the growth of the culture showed some evidence of a slight rise and then a destruction of toxin.

DISCUSSION

The work described shows that the specific toxin of *B. anthracis* present in the plasma of guinea-pigs dying of anthrax has at least two components (Factors I and II). They have a marked synergic action on each other in both the skin test and in the mouse lethality test. There does not seem to be a large excess of one or other of the two components in the plasma since addition of the separate components to the toxic plasma does not increase its activity.

Factor I of the toxin was quickly deposited by ultracentrifugation under conditions described for the separation of the immunising aggressin (Fraction Y) in a previous paper (Smith and Gallop, 1956). When purified by repeated ultracentrifugation, its ultracentrifuge diagram was almost identical with that of the latter preparation. The two preparations also had similar immunising and antiphagocytic properties but they differed in two important respects. First, Factor I was toxic when recombined with Factor II but Fraction Y was not. Second, Factor I had virulence-enhancing activity, as had the original toxin (and plasma/exudate) but Fraction Y had not. Fraction Y is however, closely connected with the toxin since a hyperimmune serum prepared against it neutralised the toxin. It seems highly probable therefore, that Fraction Y is a " toxoided " form of Factor I. In fact, when purified Factor I was treated with barium acetate and ethanol (as was Fraction Y), its power of recombining with Factor II to form a toxic mixture was largely destroyed (*cf.* the action of barium acetate and ethanol on the original toxin (Smith *et al.*, 1955)). The fact that at least one component of the anthrax toxin is an active aggressin means that the aggressive action of *B. anthracis* in the early stages of anthrax is to some extent due to the toxin which later in the disease acts to kill the host.

Factor II of the toxin remains in the supernatant after ultracentrifugation. Its synergic action with Factor I cannot be simulated by polyglutamic acid or by its own ultrafiltrate. An antigen produced *in vitro* can replace Factor II in the synergic mixtures tested for mouse lethality and oedema-producing activity provided sufficient of Factor I is present. However, two findings show that *in vitro* antigen is not identical with Factor II. First, addition of the *in vitro* antigen to Factor II forms a synergic not an additive mixture in a mouse lethality test although the skin reaction is unchanged. Second, whereas Factor II acts synergically with I even when the latter is in a non-reacting concentration, the *in vitro* antigen is only capable of increasing the effect of a dose of Factor I which already produces a slight skin reaction independently.

Further purification of the two components of the anthrax toxin is proceeding and the relationship, if any, of Factor I with the S20 component of normal serum is being investigated.

It would have been convenient for chemical fractionation to have been able to produce the anthrax toxin *in vitro* but all attempts to do this failed. The failure of experiments involving the use of ordinary laboratory media was not surprising in view of the unsuccessful attempts in the past (Eurich and Hewlett, 1930 ; Sobernheim, 1931 ; King and Stein, 1950).

It is interesting that the blood of an infected guinea-pig at the early bacteraemic stage (Keppie *et al.*, 1955) which 8–10 hr. later *in vivo* would be toxic, produces no toxin when removed and incubated *in vitro* although the rate of growth and extent of capsulation of the organisms were almost equal to those obtained *in vivo*. Hence the particular factor of the host environment responsible for toxin production remains undetermined.

SUMMARY

The specific toxin of *B. anthracis* in the plasma of guinea-pigs dying of anthrax has at least two components which form a synergic mixture.

One component (Factor I) deposited quickly by ultracentrifugation has been partially purified ; it is an immunising antigen and an aggressin. The immunising aggressin (Fraction Y) separated from plasma/exudate (see Smith and Gallop, 1956) is probably this component which has been " toxoided " by the extraction process. A second component (Factor II) remains in the supernatant after ultracentrifugation. The immunising antigen purified from *in vitro* sources by Strange and Belton (1954) is connected with Factor II of the toxin but is not identical with it.

Polyglutamic acid produced by *B. anthracis in vivo* is not a component of the toxin.

Attempts to prepare the anthrax toxin *in vitro* using conditions approaching those occurring *in vivo* have failed.

Our thanks are due to Mr. R. C. Hale, Mr. G. S. Grace, Mr. J. Hayton, Mr. D. Waite and Mr. A. Annetts for technical assistance.

REFERENCES

Belton, F. C. and Strange, R. E.—(1954) *Brit. J. exp. Path.*, **35**, 144.

Eurich, F. W. and Hewlett, R. T.—(1930) "A System of Bacteriology" 5, Chap. 10, London ; Medical Research Council.

Keppie, J., Smith, H. and Harris-Smith, Patricia W.—(1955) *Brit. J. exp. Path.*, **36**, 315.

King, H. K. and Stein, J. H.—(1950) *J. gen. Microbiol.*, **4**, 48.

Smith, H., Keppie, J. and Stanley, J. L.—(1953*a*) *Brit. J. exp. Path.*, **34**, 471.—(1953*b*) *Ibid.*, **34**, 477.—(1955) *Ibid.*, **36**, 460.

Idem and Gallop, R. C.—(1956) *Brit. J. exp. Path.*, in press.

Sobernheim, G.—(1931) ' Handbuch der Pathogenen Mikroorganismen,' **3**, part 2, p. 1041. Jena (Gustav-Fischer, Urban and Schwarzenburg).

Stamatin, N. and Stamatin, L.—(1936) *C.R. Soc. Biol., Paris*, **122**, 491.

Strange, R. E. and Belton, F. C.—(1954) *Brit. J. exp. Path.*, **35**, 153.