THE CHEMICAL BASIS OF THE VIRULENCE OF *BACILLUS ANTHRACIS*. II: SOME BIOLOGICAL PROPERTIES OF BACTERIAL PRODUCTS.

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IN a previous paper a method is described for collecting and separating large quantities of *Bacillus anthracis* and its extracellular products (plasma/exudate) from infected guinea-pigs. Two sterile extracts of the intracellular material (ammonium carbonate extract and ballotini extract) were prepared by dissolving the organisms almost completely under mild conditions (Smith, Keppie and Stanley, 1953*a*, *b*). These crude preparations have been examined for biological properties connected with virulence as a preliminary to chemical fractionation for the compounds responsible for such activity. This paper describes the behaviour of these preparations in tests for toxicity, tissue-damaging activity, aggressins, and protective antigen. Similar studies on the extracellular products have already been reported (Treffers, 1947), but this is not so for the intracellular products of *B. anthracis* grown *in vivo*. The behaviour of the latter in the biological tests is therefore of special interest.

The results are discussed in relation to anthrax in the guinea-pig.

METHODS AND RESULTS.

In these experiments the strain of *B. anthracis* (N.P.) and the breed of guinea-pigs used were those employed in the infection from which the bacterial products were obtained (Smith *et al.*, 1953*a*, *b*,). Abbreviations used to describe the latter are P.E. (plasma exudate,) A.C.E. (ammonium carbonate extract) and B.E. (ballotini extract).

Non-toxicity of B. anthracis *Products*

Eurich and Hewlett (1930) and Sobernheim (1931) doubted the toxicity of anthrax products, and this was supported by the work of King and Stein (1950). They found that intra- and extracellular products of a moderately virulent strain of *B. anthracis* grown under various conditions *in vitro* were non-toxic to mice in the doses given. The use of extracts of infected mouse spleens gave equally negative results.

In addition to using highly virulent *B. anthracis* harvested from infected guinea-pigs, a more quantitative approach to this question of toxicity was made in the experiments reported below. The aim was to treat guinea-pigs (500 g.) with sterile bacterial extract equivalent to more bacteria than there are in a guinea-pig when it dies of anthrax, and with a volume of P.E. greater than 100 ml. as this is approximately the volume of extracellular fluid in a guinea-pig (500 g.).

The number of bacteria l chains (4–8 bacteria) in guinea-pigs dead from anthrax was estimated as accurately as po ssible. Six guinea-pigs were examined immediately after

death, and haemocytometer counts were carried out on samples of the blood, thoracic and peritoneal exudates, liver, spleen, lung and kidney in two cases. The latter tissues were weighed after being removed from the animal, ground with water and sand in a mortar to form a fine suspension, and then suitable dilutions of these suspensions were taken for counting. Table I gives the number of anthrax bacilli estimated to be present in the whole guinea-pig and in the main viscera. The total counts for each animal in the series were similar.

TABLE I.—*An Estimate of the Number and Location of Anthrax Bacilli in Infected Guinea-Pigs at Death.*

Guinea-pig (500 g.).	Blood (estimated vol. 50 ml.).	Spleen (1·25–4 g.).	Liver (14–23 g.).	Lungs (3·5–4 g.).	Kidneys (5–8 g.).	Peritoneal and thoracic exudate.	Total.
1	2·75	0·7	0·44	1·08	—	—	4·97
2	1·54	0·92	0·40	0·27	—	—	3·13
3	1·89	0·70	0·30	0·18	0·20	0·66 (15 ml.)	3·93
4	2·75	0·93	0·52	0·40	—	—	4·60
5	3·08	0·64	0·64	0·43	—	—	4·79
6	2·73	0·88	0·56	0·12	0·11	0·38 (15 ml.)	4·78

Figures are the total number ($\times 10^{10}$) of bacterial chains (4–8 bacilli) in each tissue as determined by haemocytometer counts.

Since it was intended to inject P.E. in volumes of 100 ml. or more, the tolerance of guinea-pigs (500 g.) to the intraperitoneal injection of large volumes of normal guinea-pig plasma was investigated. Injection of these large volumes was carried out high in the flank to avoid subsequent leakage. Citrated plasma was toxic in the required volumes, but heparinised plasma (or plasma from defibrinated blood) was not toxic up to a limit of 200 ml. (see Table III).

In the collection of *B. anthracis* and its products (Smith *et al.*, 1953*a*) for these toxicity experiments, heparin (10 I.U./ml.) and not citrate was used as the anticoagulant.

Non-toxicity of bacterial extracts.

The bacteria were used immediately after the differential centrifugation procedure and removal of the final P.E. The washing with Locke's solution was omitted to avoid the danger of removing or destroying toxic principles by this treatment. Bacterial counts of this sludge showed that the total quantity of bacteria was appoximately 10 times the number estimated to be present in one guinea-pig (500 g.) when it dies of anthrax. The material was divided into 5 parts, and after the treatments described below, each part was injected into a single guinea pig (500 g.) This meant that each guinea-pig received bacterial extract equivalent to approximately twice the number of anthrax bacilli estimated to be present in a guinea-pig killed by anthrax It is unlikely that the error in the latter estimate is more than twofold.

The total bacterial sludge was homogenised in distilled water (800 ml.) and divided into 5 parts which were treated as follows.

(*a*) *Filtered A.C.E.*—Prepared as described previously (Smith *et al.*, 1953*b*). After filtering, the solution (160 ml. + 40 ml. water used to wash the filter) was adjusted to pH 7·2 with acetic acid and made isotonic by adding sodium chloride (1 g.).

(*b*) *Filtered B.E.*—Prepared as described previously. After filtering, the solution (160 ml. + 40 ml. water used to wash the filter) was made isotonic by adding sodium chloride (1·4g.)

(*c*) *Penicillin-treated A.C.E.*—Prepared as described for (*a*), but instead of being filtered sterile, the solution (160 ml.) was adjusted to pH 7·2, rendered isotonic and incubated at 37° for 2 hr. with penicillin (1000 units/ml.).

(*d*) *Penicillin-treated B.E.*—Prepared as described for (*b*) and treated with penicillin as described for (*c*).

(*e*) *Heat- and penicillin-treated bacterial suspension.*—The suspension (160 ml.) was heated to 60° for 30 min., adjusted to pH 7·2, rendered isotonic and treated with penicillin as described for (*c*).

To determine whether the bacterial extracts after any of the above treatments were toxic, each preparation was injected intraperitoneally into a single guinea-pig (500 g.), which was kept under observation for 7 days. Table II (Exp. 1 and 2) summarises the results of two experiments, and it is evident that no toxin was demonstrable under these conditions. Preparations (*c*), (*d*) and (*e*) were made to rule out a possible loss of toxin during filtration of (*a*) and (*b*), although this was unlikely since the filters were of sintered glass. As (*c*), (*d*) and (*e*) were not wholly free from *B. anthracis* and other organisms, the appropriate guinea-pigs were given daily injections of oily penicillin (30,000 I.U.) to try to prevent infection ; this dose of penicillin was innocuous in control animals. Although this was inadequate in a few instances, it was clear that the unfiltered extracts were as non-toxic as those filtered.

For comparison with these toxicity experiments, guinea-pigs (500 g.) were injected with the untreated live bacteria from infected guinea-pigs in quantities 1/100 and 1/10,000 of those made into the preparations described above. These animals died very rapidly of anthrax infection ; the times for death to occur are included in Table II.

TABLE II.—*Non-Toxicity of Bacterial Extracts when Injected Alone and Together with P.E.*

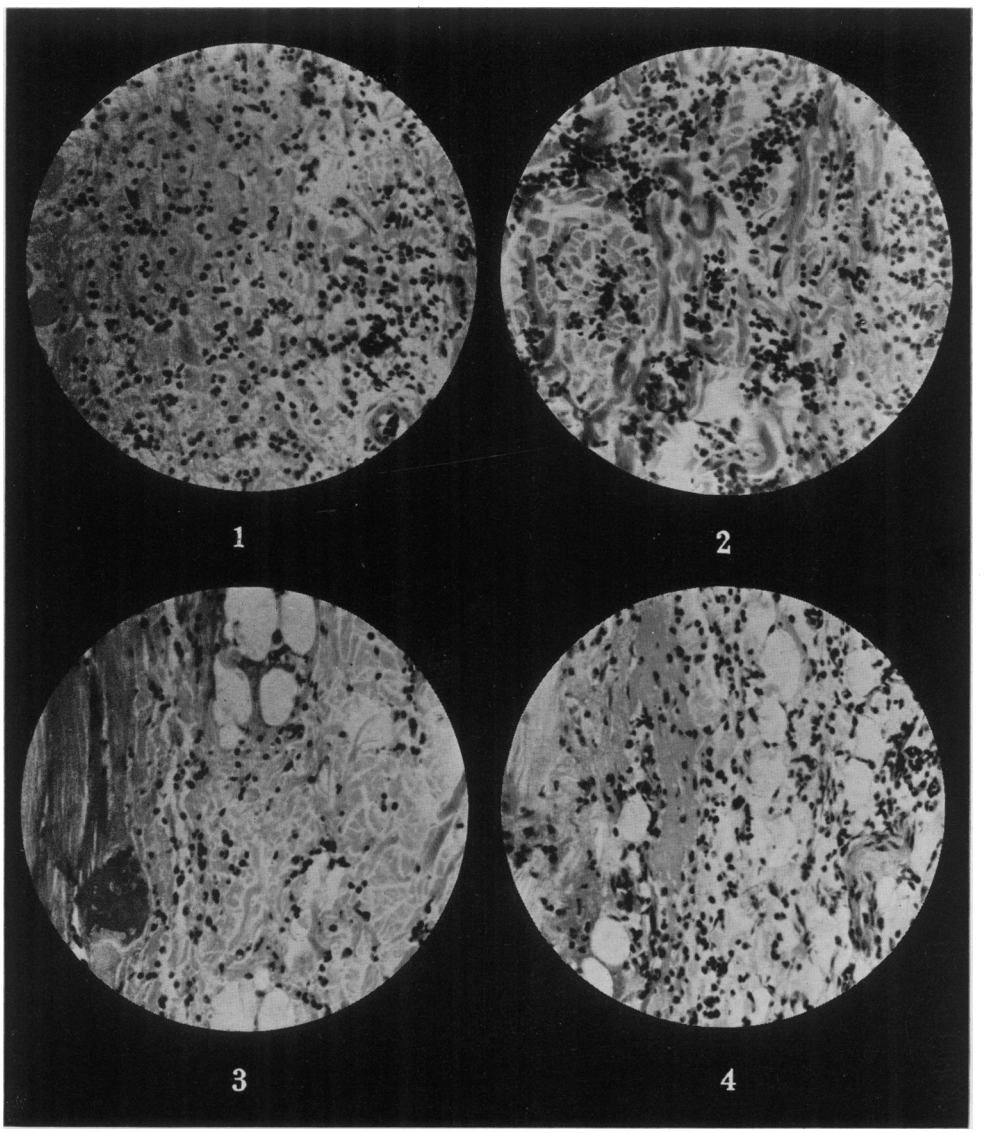
Nature of material injected i/p (160–200 ml.).	Bacterial extracts alone. Exp. 1 (each extract equiv. to $7 \cdot 5 \times 10^{10}$ bacterial chains). No. of guinea-pigs.	Result.	Exp. 2 (each extract equiv. to $10 \cdot 4 \times 10^{10}$ bacterial chains). No. of guinea-pigs.	Result.	Bacterial extracts with solids from P.E. (100 ml.). (each extract equiv. to $9 \cdot 9 \times 10^{10}$ bacterial chains). No. of guinea-pigs.	Result.
Filtered A.C.E. (sterile)	1	Alive	1	Alive	1	Alive
Filtered B.E. (sterile)	2*	,,	1	,,	1	,,
Penicillin-treated A.C.E. (non-sterile)	1	,,	1	Died of anthrax 6th day	1	Died of anthrax in 40 hr.
Penicillin-treated B.E. (non-sterile)	1	,,	1	Died of anthrax in 48 hr.	1	Alive
Heat- and penicillin-treated extract (non-sterile)	1	,,	1	Alive	1	Died of streptococcal infection in 52 hr.
Live bacteria 1/100 quantity in each extract	2	Died in 15 and 16 hr.	3	Died in 10, 11 and 12 hr.	3	Died in 11, 12 and 13 hr.
Live bacteria 1/10,000 quantity in each extract	1	Died in 38 hr.	4	Died in 17, 18, 19 and 19 hr.	4	Died in 18, 19, 20 and 21 hr.

The guinea-pigs used weighed 500 g. and were observed for 7 days.
* The result of a single preliminary experiment with filtered B.E. is included here.

DESCRIPTION OF PLATE.

Tissue changes in guinea-pigs 24 hr. after intradermal injection (0·2 ml.) of solutions (5 per cent w/v) of sterile anthrax products.

FIG. 1.—P.E., adjacent to cutaneous muscle.
FIG. 2.—P.E., a more superficial field.
FIG. 3.—A.C.E.
FIG. 4.—B.E.



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Non-toxicity of P.E.

Quantities equivalent to 75, 100, 150 and 200 ml. of undiluted P.E. were injected into guinea-pigs (500 g.). As described previously (Smith *et al.*, 1953*a*), the undiluted P.E. collected had to be mixed with an equal volume of diluent to ensure satisfactory filtration through " Millipore " filters. Since the maximum volume tolerated by a guinea-pig (500 g.) was 200 ml., the 150 ml. and 200 ml. quantities of undiluted P.E. were obtained in isotonic solution by diluting with water before filtration, freeze-drying and reconstituting. Any possible adverse effects of this treatment were ruled out for the 75 ml. and 100 ml. quantities by diluting with Locke's solution and injecting 150 ml. and 200 ml. respectively after filtration.

Table III summarises the results obtained in 3 experiments with different batches of P.E. It includes the results of controls given normal heparinised guinea-pig plasma. No toxic extracellular substances were detected in the P.E. under these conditions.

TABLE III.—*Non-Toxicity of P.E. when Injected Alone.*

	Control (normal heparinised plasma).		P.E. (combined results on three different batches).	
Volume of material injected (i.p., ml.).	No. of guinea-pigs.	Result.*	No. of guinea-pigs.	Result.*
200	5	3 alive, 2 died (1st and 6th day)	4	3 alive, 1 died (6th day).
150	10	Alive	5	Alive
100	3	2 alive, 1 died (7th day)	6	,,
75	—	—	2	,,

*The animals were observed for 7 days.

Non-toxicity of bacterial extracts and P.E. when injected together.

The experiment which showed the non-toxicity of bacterial extracts when injected alone was repeated but instead of adding sodium chloride to make the preparations isotonic, the freeze-dried solids from 100 ml. undiluted P.E. were dissolved in solutions (*a*), (*b*), (*c*), (*d*) and (*e*) described above. The results of this experiment are included in Table II. The mixtures of products were non-toxic under these conditions.

Tissue-damaging Activity of B. anthracis *Products.*

Watson, Cromartie, Bloom, Heckly, McGhee and Weissman (1947) showed that crude oedema fluid, and a fraction of it, produced a lesion if injected intradermally into rabbits. The histopathology of this lesion was similar to that produced by *B. anthracis* in a similar site. Some tissue damage was produced by our products which, however, was not extensive or of long duration.

Intradermal injections of 5 per cent w/v solutions (0·2 ml.) of A.C.E., B.E. and P.E. (dialysed) were made in guinea-pigs.

Macroscopically A.C.E. and B.E. produced only a trace of oedema, which quick-

ly faded. P.E. produced a small oedematous plaque within 24 hr., but the oedema and congestion were short-lived and were diminishing by 48 hr.

The tissue changes are shown in Fig. 1, 2, 3, and 4. The tissue reactions produced by P.E. and the two bacterial extracts were similar, and differed only in degree, the P.E. reaction being the more widespread. The whole depth of the corium was involved, the changes being greatest in the deeper layers adjacent to the cutaneous muscle. No selective damage to any particular tissue was found.

The site of the injection showed a loss of the superficial layers of the epidermis, leaving an accumulation of disintegrating polymorphs with a fibrinous coagulum. The deeper layers of the epidermis showed little change although only a thin layer of the epithelium remained. The papillary connective tissue in a small area under the bleb showed hyaline necrosis with a few polymorphs at the margin. Downwards through the corium there was some oedema which pushed the collagen fibres apart. Polymorphs were widely scattered in all the spaces, but were accumulated in the clefts between fibres in the mid-zone of the corium (Fig. 2), where a localised abscess may be formed. There was no evidence of destruction or fragmentation of collagen (Watson *et al.*, 1947), but some fibres showed hyaline change and stained deeply with eosin (Fig. 2). Oedema was greater towards the muscle, and there was some fibrinous coagulum in the tissue spaces. Scattered polymorphs were present in the oedematous tissue and some red cells were free in the tissue spaces (Fig. 1). Deep to the muscle there was oedema and fibrinous accumulation, and there the red cells were present in greater numbers and there were haemorrhages from damaged capillaries.

The peak of the reaction had occurred by 24 hr. and by 48 hr. resolution had begun. On the 7th day healing was almost complete.

The Aggressin Activity of B. anthracis *Products.*

A.C.E., B.E. and P.E. have been tested for aggressin, or the capacity to interfere with the host defence mechanisms (Bail, 1904 ; Bail and Weil, 1911). Tests for such activities as the promotion of infection by sub-lethal doses of organisms and interference with the phagocytic and bactericidal power of blood will be described. The two latter tests have been developed into biological assays which can be used for detecting aggressin during the chemical purification of crude products. The activity of crude products in the tests will be described here, but the results of the chemical fractionation and a statistical examination of the assays will be given elsewhere.

The promotion of B. anthracis *infection in guinea pigs by* B. anthracis *products.*

Preliminary experiments showed that 50 *B. anthracis* spores in gelatin-Locke solution (20 ml.) were non-lethal for guinea-pigs (300–350 g.) by the intraperitoneal route ; 1×10^4 spores by this route are needed to kill all animals. In order to test solutions of P.E. (2 per cent w/v), A.C.E. (1·25 per cent w/v) and B.E. (1·25 per cent w/v) for their capacity to promote infection, 50 spores were injected with 20 ml. of each of these solutions using groups of 5 guinea-pigs. All of these animals died, whereas no deaths occurred in 30 control animals.

Aggressin activity was thus demonstrated in the intracellular products as well as in the extracellular products. The large amount of material necessary for this *in vivo* test makes it unsuitable as a routine assay for chemical fractionation.

The antiphagocytic activity of B. anthracis *products.*

The A.C.E., B.E., and P.E. were examined for antiphagocytic activity in the following *in vitro* phagocytosis test. Citrate has a definite antiphagocytic action in this test (Allgöwer, 1947): therefore the samples of P.E. were dialysed against distilled water before use.

To fresh defibrinated guinea-pig blood (0·4 ml.) contained in a waxed bottle (5 ml.) gelatin-Locke solution (0·4 ml.) containing the test material was added, and the mixture was kept at 37° for 30 min. A broth suspension (0·2 ml.) of *B. anthracis* was then added. This suspension contained ten times as many organisms in 0·2 ml. as there were polymorphs in 0·4 ml. of the blood. It was prepared by adding the correct number of spores to broth and keeping the mixture at 37° for 30 min. The spores had then formed small stainable diploid organisms which were easy to count when inside the phagocytes. The final mixture was rotated at 37° for 30 min., cooled in ice and centrifuged at 500 r.p.m. (10 min.) and then at 2000 r.p.m. (10 min). The well-defined white cell layer was filmed and stained with Leishman's stain. Organisms in 100 phagocytes were counted, and the mean phagocytic index for the experimental sample was compared with that of the control. Several concentrations of the materials under test were examined, and Table IV summarises the results of one of a number of tests carried out on each sample. The antiphagocytic activity of A.C.E., B.E. as well as P.E. is well marked. Absence of citrate ($\leqslant$ 0·3 per cent) in the freeze-dried products was confirmed by the chemical method of Hargreaves, Abrahams and Vickery (1951).

TABLE IV.—*Antiphagocytic and Antibactericidal Activity of* B. anthracis *Products.*

Nature of test material.	Concentration per cent.	Antiphagocytic test. Mean phagocytic index from count of 100 phagocytes.	Antibactericidal test. (Figures are the viable counts in equivalent samples of the mixtures.) Initial	Final.
	Nil (control)	6·13	104	23
A.C.E.	5	0·8	103	48
	2·5	4·29	100	55
	1·25	5·89	96	45
	0·62	—	94	42
	Nil (control)	6·13	85	28
B.E.	5	1·32	81	65
	2·5	5·08	89	50
	1·25	4·78	80	41
	0·62	—	88	39
	Nil (control)	9·21	91	25
P.E. (Batch 1)	5	7·62	91	57
	2·5	9·59*	95	43
	1·25	—	92	43
	0·62	—	100	33
	0·31	—	90	29
	Nil (control)	10·7	91	25
(P.E. Batch 2)	5	5·32	90	90
	2·5	7·40	87	67
	1·25	10·2*	85	52
	0·62	9·93*	91	48
	0·31	—	96	41

Whole defibrinated guinea-pig blood was used. Details of the tests are given in the text.
* Figure not significantly lower than the control.

The antibactericidal activity of B. anthracis *products.*

This test was carried out as described for the anti-phagocytic test, except that the number of germinated *B. anthracis* spores added was one-tenth of the number of polymorphs in the

blood, and rotation of the final mixture was continued for 1½ hr. Immediately after adding the organisms and before rotation at 37°, a sample was taken from each waxed bottle. After appropriate dilution in gelatin-Locke, a measured volume was spread on 4 nutrient agar plates for a viable count. The same procedure was repeated after rotation in the hot room, but 6 plates were used for each sample The bactericidal action of defibrinated guinea-pig blood, which reduced the viable count of the control to a low figure, was inhibited by A.C.E., B.E. and the two citrate-free samples of P.E.; the greater the aggressin activity of the test material, the higher was the final viable count.

Table IV includes typical results which demonstrate anti-bactericidal activity in A.C.E. and B.E. as well as in P.E.

The nature of the bactericidal action of defibrinated guinea-pig blood.

It could be shown that normal guinea-pig serum had no bactericidal action on broth cultures of *B. anthracis*. The presence of an anthracidal substance in leucocytes (Bloom, Watson, Cromartie and Freed, 1947) suggested the possibility that secretion of such a substance by the white cells might occur after the bacilli were added (see also Bail and Weil, 1911). This was disproved by determining the fate of all the organisms used in phagocytosis tests. These were carried out without the addition of any experimental material. After 30 min. rotation at 37°, samples were taken for viable and phagocytic counts as described before. Rotation was continued and further samples were taken at 60 min. and 90 min. Table V gives the results obtained in several experiments. These show that the sum of the organisms remaining viable and the number phagocytosed equalled the number of organisms initially added. This means that under these conditions there is no extracellular bactericidal activity.

TABLE V.—*Nature of the Action of Defibrinated Guinea-Pig Blood on* B. anthracis *from Broth Culture.*

		Distribution of bacteria after incubation for the time interval stated.								
		30 min.			60 min.			90 min.		
Exp.	Number of organisms added to the system.	Viable	Phago-cytosed	Total	Viable	Phago-cytosed	Total	Viable	Phago-cytosed	Total
1	6·0	—	—	—	3·7	2·5	6·2	3·8	2·5	6·3
2	9·2	4·9	3·8	8·7	3·6	5·7	9·3	3·3	6·1	9·4
3	9·9	7·0	2·9	9·9	4·9	5·0	9·9	3·5	5·6	9·1
4	2·3	1·3	0·7	2·0	—	—	—	1·1	0·9	2·0

Figures are the total count ($\times 10^6$) in the mixture (1 ml.).
The ratio of organisms to phagocytes in this mixture is approximately 10 : 1.

It is possible that the viable counts after the action of the blood were reduced by the number of polymorphs present, since these may each have contained one viable organism which gave rise to a colony in the viable count. Application of this correction does not affect the main conclusion, that there is no extensive extra-cellular bactericidal activity.

These results were confirmed in a smaller number of experiments using the conditions of the bactericidal test.

The Immunising Activity of B. anthracis *Products.*

The literature already reviewed (Smith *et al.*, 1953*b*) clearly shows that extracellular products of *B. anthracis* whether produced *in vivo* or by special conditions *in vitro* contain a protective antigen. Attempts to detect such an antigen in extracts of bacteria grown *in vitro* have been unsuccessful, but preparations from bacteria grown *in vivo* have not so far been examined. The results of active immunisation experiments on A.C.E. and B.E. are therefore of special interest.

P.E., A.C.E. and B.E. were examined for their power to produce active immunity in the following test. Guinea-pigs (300–350 g.) were given 3 subcutaneous injections (1 ml.) of the test material at 7-day intervals. One week after the last injection the animals were challenged with 1000 L.D. of *B. anthracis* spores. Deaths were recorded up to 10 days. This test is now used as a biological assay of the protective antigen in the chemical fractionation of P.E. Full details of this fractionation and of the biological assay, especially the statistical treatment of data, will be published elsewhere.

Activity of P.E.

Several batches of freeze-dried P.E. have been tested for their immunising power in guinea-pigs with similar results. The combined results show that with concentrations (per cent) of 4, 1, 0·5 and 0·025 the number of survivors was 10/10 35/38, 11/15 and 3/15 respectively. It must be noted that a 4 per cent solution of freeze-dried P.E. is approximately equivalent to undiluted P.E. collected from guinea-pigs ; approximately 1/3rd of the dry solids are the sodium citrate and salts of the Locke's solution added during harvesting.

Further experiments have shown that P.E. does not lose appreciable immunising activity on freeze-drying and does not confer passive immunity ; it also protects rabbits against a *B. anthracis* infection. When examined separately, both the blood plasma and the cavity exudate from infected guinea-pigs have immunising activity.

Inactivity of A.C.E. and B.E.

The following solutions, 3 per cent w/v A.C.E., 2·5 per cent w/v A.C.E. (different batch) and 3 per cent w/v B.E. gave no protection to 5, 4 and 4 guinea-pigs respectively in the test for protective antigen. The quantity of bacterial extract used for one guinea-pig in these experiments was derived from the bacteria removed from approximately 100 ml. of P.E. in the harvesting procedure (Smith *et al.*, 1953*a*). This quantity of P.E. by virtue of its content of extracellular antigen would immunise 150 guinea-pigs. Tests have shown that treatment with ammonium carbonate or shaking with ballotini does not affect the protective activity of P.E.

The results obtained with these bacterial extracts show that the intracellular products of *B. anthracis* grown *in vivo* are as devoid of immunising activity as are similar *in vitro* preparations. This further confirms the essentially extracellular nature of the immunising antigen of *B. anthracis*.

There was a possibility that the immunising antigen was in high concentration at the surface of the cell, and was removed by the Locke's solution when the bacteria were washed. This was excluded by examination of the activity of the

three individual wash solutions. Activity was only apparent in the first washing, and this was no more than that accounted for by the presence of P.E.

Connection between the protective antigen and the aggressin of P.E.

The fractions of P.E. resulting from purification studies have been tested in parallel for protective antigen and for aggressin by means of the tests described earlier. In samples from three different types of fractionation, preliminary results have shown an interesting correlation worth mentioning here between protective antigen and aggressin. The results are summarised in Table VI.

TABLE VI.—*Relative Activity of P.E. Fractions in Tests for Protective Antigen and Aggressins.*

Type of fractionation.	Fraction.	Lowest active concentration* (per cent)		
		Immunising test.	Antiphagocytic test.	Antibactericidal test.
Cohn/Edsall plasma fractionation	1	0·2	0·17	0·17
	2	0·2	0·5	0·5
	3	Inactive at 1·6	Inactive at 1·5	Inactive at 1·5
Ammonium sulphate	1	0·2	0·17	0·5
	2	0·8	0·5	1·5
	3	Inactive at 1·6	Inactive at 4·5	Inactive at 4·5
A combination of barium acetate and ethanol	1	0·2	No test	0·26
	2	0·05	,,	0·06
	3	1·6	,,	1·6

* The volume of solutions used and other details of these tests are as described in the text ; figures in the table are concentrations of solutions used as test material.

The samples tested were not pure materials, and therefore the possibility remains that two different substances are responsible for each activity. However, the correlation up to the present is noteworthy, and is supported by the finding that a sample of purified protective antigen produced in artificial cultures (Strange and Belton, 1953 ; personal communication) had aggressin activity in both tests.

DISCUSSION.

The evidence given in the first part of this paper strongly indicates the absence of any well-defined endo- or exotoxin in the materials from a *B. anthracis* infection in guinea-pigs. This supports earlier work (Eurich and Hewlett, 1930 ; Sobernheim, 1931 ; King and Stein, 1950) using materials prepared by culture *in vitro*. Gross toxicity is almost certainly ruled out by our experiments. The quantities of materials injected into a single guinea-pig were unusually large, and the methods of preparing the extracts were very mild. An absence of gross toxicity might be expected in view of the large numbers of bacilli present in the guinea-pig at death, which suggests that the toxicity per organism cannot be large, or the host would probably die before so many could be produced.

The important inference to be drawn from these toxicity experiments is that the killing power of *B. anthracis* is associated mainly with the growth of the organism in the tissues of the host, rather than with any particularly active endo-

or exotoxin. The rapid death produced by the relatively small initial dose of live organisms described in the toxicity experiments shows the importance of this growth. There are two essentials for rapid growth in the host tissues, a satisfactory nutritional medium (Bacon, Burrows and Yates, 1951), and the production by the organisms of substances which combat the normal host defence mechanisms. At the moment, therefore, it is thought that the most important chemical substances to study in connection with the virulence of *B. anthracis* in guinea-pigs are the aggressins. They are present in the intra- and extracellular products of the organism, and work is in progress on the fractionation of these crude materials for the compounds responsible for activity. It is relevant to mention here that Sterne (1937), on the basis of observations on the virulence and immunising power of *B. anthracis* variants, suggested that virulence was perhaps determined by two factors. One was connected with the capsule of the organism, but the other was extracellular and was also responsible for immunising power. The results described above support this theory Both intra- and extracellular aggressins are present and evidence from chemical fractionation connects an extracellular aggressin with the protective antigen.

Once the organisms can grow abundantly, the death of the host could result either from the combined action of a number of relatively weak or slowly acting harmful effects, or through the host's tissues being deprived of essential nutrients by the metabolic activity of the organism.

A number of the effects of infection mentioned in the literature (some of which have been noticed in this work with guinea-pigs) could be harmful. A slight tissue-damaging factor has been demonstrated in this and other work (Sobernheim, 1931 ; Watson *et al.*, 1947). The abnormalities of the blood in the terminal phases such as the slight haemolysis, the increased red cell fragility, the delayed clotting and the agglutination of red cells must result from the action of some as yet undefined harmful product of *B. anthracis*. The possibility that calcium metabolism is interfered with has been suggested by Bloom, McGhee, Cromartie and Watson (1947), and de Moulin (1936) has drawn attention to evidence of damage to the tissues of the nervous system. In this connection it is important to bear in mind that weakly toxic materials—*e.g.*, the lecithinase of McGaughey and Chu (1948)—may be fixed on the host tissues as soon as they are formed, and would not be detected in the products harvested.

It should be feasible in connection with the second of these possibilities to determine whether the bacilli deprive the host of essential materials by studying the rate at which these organisms remove physiological essentials from the fluids of the host.

In connection with anthrax in the guinea-pig, it is important to decide whether the final septicaemia, which appears to coincide with the period of greatest distress in the animal, is essential to cause death. In view of the reports in the literature (Cromartie, Bloom and Watson, 1947 ; Bloom, McGhee, Cromartie and Watson, 1947) that death from anthrax occurs in some species without a marked septicaemia, blood films were examined from approximately 200 guinea-pigs dying from the form of experimental anthrax used in this work. In every case the blood contained large numbers of bacilli. The counts made on the blood of the six animals described earlier in this paper are additional evidence that a marked septicaemia precedes death in the guinea-pig. This is supported by the fact that the total yield of bacteria collected from successive batches of 100 guinea-pigs (Smith *et al.*,

1953*a*) is a remarkably constant amount. Further work is necessary to examine the suggestion that guinea-pigs treated differently from those under discussion may die from anthrax in the absence of a marked septicaemia.

Study of the virulence of *B. anthracis* in guinea-pigs is being continued on the lines indicated in this discussion.

SUMMARY.

As a preliminary to chemical fractionation, products of *B. anthracis* harvested from infected guinea-pigs have been examined in various biological tests connected with virulence.

Large quantities of intra- and extracellular products were non-toxic when injected intraperitoneally.

Intra- and extracellular products contain aggressins as shown by their ability to promote infection by sub-lethal doses of *B. anthracis* and to interfere with phagocytosis.

Extracellular products produced active immunity to anthrax in guinea-pigs but intracellular products did not. Evidence is presented that an extracellular aggressin and the protective antigen may be the same substance.

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