THE CHEMICAL BASIS OF THE VIRULENCE OF *BACILLUS ANTHRACIS*. I: PROPERTIES OF BACTERIA GROWN *IN VIVO* AND PREPARATION OF EXTRACTS.

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STUDIES on the purification and chemical identification of bacterial products have been related mostly to their immunising action and their serological behaviour. Less attention has been given to the part played by these products in the disease process and in causing the death of the host. This is particularly true of those organisms capable of producing septicaemic conditions in which no potent extra-cellular toxins seem to play a part. The research now undertaken is directed towards chemical identification of substances and processes connected with the virulence of organisms producing such septicaemic diseases.

The virulence of a bacterium is best defined as its capacity to infect the host and establish disease. To do so the organism must overcome the defence mechanisms of the host. The course of the disease is influenced by the strength of the latter, which may affect the metabolism of the organism. There is abundant evidence that the complex metabolic activity of a pathogen in establishing disease is different from its metabolism *in vitro*. The particular nutritional conditions of the host tissues undoubtedly play their part, but the possibility that the organism reacts in some way to the challenge of the host's defence mechanisms must be borne in mind.

Our limited knowledge of bacterial nutrition makes it impossible at the moment to reproduce *in vitro* the conditions of growth *in vivo*. It seemed therefore that the best way of studying compounds and reactions responsible for virulence was to use organisms and their products isolated from infected animals in spite of the technical difficulties involved. This does not necessarily mean, as Bail and Weil suggested (1911), that there is an absolute difference between "animal organisms " and those grown *in vitro*. If the compounds and reactions responsible for virulence in the host could be identified, it might lead to the production in defined media of some of the metabolic processes which occur *in vivo*. The recent preparation of the immunising antigen of *Bacillus anthracis* by artificial culture (Gladstone, 1946, 1948) is encouraging in this respect.

It was therefore decided to carry out work on the chemical basis of virulence, using as starting materials the bacteria and their products from infected animals. *B. anthracis* was chosen as the pathogen to be studied for two main reasons. In the first place anthrax is characterised by the presence in the host of large numbers of organisms, and this made it probable that the yield of bacteria would be satisfactory. Secondly, the extracts from the literature given below show that the precise chemical basis of invasiveness, killing power and immunity to the disease is unknown. However, sufficient biological knowledge has been gained to indi-

indicate the general lines of attack on these problems and the probable value of materials harvested from growth *in vivo*.

Reviews of the literature on the pathology and immunochemistry of *B. anthracis* and its infections are available (Sobernheim, 1913, 1931 ; Cromartie, Bloom and Watson, 1947 ; Treffers, 1947). Only the main outlines of present knowledge of the disease are mentioned here, and it will be seen that attention has been focussed on three main topics.

Immunity to anthrax has received much attention. For nearly half a century following the use of live attenuated cultures by Pasteur (1881), and sterile oedema fluid from infected animals by Bail (1904), for successful vaccination against the disease, no further progress was made. Dead bacteria and various bacterial extracts (Gladstone, 1946) were ineffective. Gladstone (1946, 1948) however using *ad hoc* methods produced an extracellular immunising antigen by *in vitro* culture, a fact which has been confirmed (Heckly and Goldwasser, 1949 ; Wright, Hedberg and Feinberg, 1951). This protective antigen has not yet been connected by experimental evidence with any of the usual serological reactions (Watson, Cromartie, Bloom, Kegeles and Heckly, 1947), or with any aspect of virulence, although Bail (1904) suggested its identity with the aggressin. Its chemical nature is unknown and it does not appear to be connected with either of the two known antigens of *B. anthracis* (Staub and Graber, 1943 ; Staub, 1949), the D-polyglutamic acid of Ivanovics and Bruckner (1937) or the somatic polysaccharide of Ivanovics (1940). Watson, Cromartie, Bloom, Kegeles and Heckly (1947) found that immunising activity was associated with the globulin fraction of rabbit oedema fluid.

With regard to the ability of *B. anthracis* to grow in the host tissues, Bail and Weil (1911) found that anthrax lesions contain substances which interfere with host resistance. These substances were termed aggressins, and together with similar substances from other organisms formed the basis of the much discussed "aggressin theory" (see Wilson and Miles, 1948). Gruber and Futaki (1907) and Preiss (1909) suggested that these substances were associated with the formation of the capsule, because crude capsular material protected the organism from phagocytosis and the bactericidal action of certain body fluids. Bail and Weil (1911) did not think that the aggressin and the capsule were connected. No chemical study has progressed far enough to correlate these aggressins with one or more definite chemical compounds, but Watson, Cromartie, Bloom, Heckly, McGhee and Weissman (1947) showed that their crude "inflammatory factor" inhibited completely the anthracidal substance from leucocytes and other tissue cells.

Finally, the cause of the death of the host has received some attention, but no definite conclusions have been reached. The main property of the crude "inflammatory factor" of the above workers was its ability to produce the oedematous tissue damage characteristic of the disease, but it was not grossly toxic. This property and its anticoagulant activity showed the difference between this preparation, which contained a high proportion of glutamic acid, and the D-polyglutamic acid of *Bacillus subtilis*. Eurich and Hewlett (1930) and Sobernheim (1931) regarded the presence of a definite toxin as unproven, and some recent work by King and Stein (1950) supports this view. Several different workers agree that a marked hyperglycaemia occurs in anthrax infection (Cromartie *et al.*, 1947 ; Bloom, McGhee, Cromartie and Watson, 1947). An interference with

calcium metabolism is suggested as a possible cause of death by the work of Bloom *et al.* (1947). The symptoms of the disease were compared with those of calcium deficiency, and the protective effect of certain calcium salts was noted. Deoxygenation of the blood and mechanical capillary obstruction have also been put forward as possible explanations, but have not yet been substantiated (Cromartie *et al.*, 1947 ; Bloom *et al.*, 1947). De Moulin (1936) noted lesions in the nervous system in anthrax, and attributed the symptoms of the disease and the death of the host to damage of this system.

In a previous paper (Smith, Keppie and Stanley, 1953) a method is described for the collection and separation of *B. anthracis* and body fluids containing its extracellular products from infected guinea-pigs in quantities of 1·5–2·0 g. and 1·5–2·51 respectively, from 100 animals. The bacteria were almost free from blood cells (not more than 0·03 per cent), and the quantity of these products was sufficient for a chemical approach to the question of virulence of *B. anthracis* in guinea-pigs.

The sterile extracellular products, although grossly contaminated with plasma constituents, could be examined directly for biological properties connected with virulence, and undergo chemical extraction for the substances responsible for these biological properties. Before the same procedure could be adopted for the bacterial substance two questions remain to be answered. First, were these bacteria appreciably different from those obtained by growing the same strain under a variety of conditions *in vitro* ? Secondly, could sterile extracts containing nearly 100 per cent of the original bacterial substance be prepared by mild methods ? The work described in this paper gives an affirmative answer to both questions.

METHODS AND RESULTS.

In these experiments the strain of *B. anthracis* (N.P.) and the breed of guinea-pig used were those employed in the infection from which the bacterial products were obtained (Smith *et al.*, 1953).

The Difference between B. anthracis *from Infected Guinea-pigs and those Cultivated* in vitro.

The organisms from infected guinea-pigs were shown to be different from those cultivated *in vitro* by a combination of three criteria. Two of these, morphology and susceptibility to phagocytosis, are well known. The behaviour of the organisms in a dilute solution of ammonium carbonate however has not been previously described.

For this comparison *B. anthracis* was grown *in vitro* in four different media—tryptic meat broth (T.M.B.), a modified medium of Brewer, McCullough, Mills, Roessler and Herbst (1946) (B.M.), sheep serum (S.S.), and citrated guinea-pig plasma (G.P.P.). Using turbidity measurements as a guide, samples were taken at four different times—at the beginning, the middle and the end of the phase of rapid growth and at 24 hr. Table I gives the details of the preparation of these cultures. The bacteria were collected by centrifugation (12,000 g) at 0°, and washed with Locke's solution as described for the organisms obtained from infected guinea-pigs.

Morphology and susceptibility to phagocytosis.

Bacteria isolated from infected guinea-pigs had well-developed capsules, and were completely resistant to the action of guinea-pig phagocytes in the system

TABLE I.—*Turbidity Measurements during the Growth of* B. anthracis *in Different Media.*

Medium.*	Time shaken at 37° (hr.).												
	0.	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	12.	24.
T.M.B.	0·04	0·05	0·17	0·44	0·95	1·06	1·14	1·18	—	—	—	—	1·06
B.M.	0·14	—	0·10	—	0·12	0·26		0·48	0·75	0·93	0·95	0·88	0·71
S.S.	0·07	—	—	—	0·08	0·22	0·35	0·53	0·72	0·83	0·82	0·81	0·86
G.P.P.†	0·12	0·25	0·33	0·45	0·60	0·79	0·99	—	1·4	—	—	—	2·0

Initial inoculum 3×10^7 spores per ml. of medium. Figures are turbidity readings (—log percentage transmission at wave length 610 mμ.) compared with a blank of the original medium.

The time (hr.) at which the four samples of cultures were taken for comparison with organisms grown *in vivo* by the methods described in the text were as follows: T.M.B. 3, 3½, 5, 24; B.M. 6½, 7½, 10, 24; S.S. 6½, 8, 10, 24; G.P.P. 3, 5, 8, 24.

* For definition of media see text.

† Spores previously germinated in tryptic meat broth.

described in the following paper (Keppie, Smith and Harris-Smith, 1953). Young organisms from T.M.B. cultures were almost devoid of capsule and had no resistance to phagocytosis. Young organisms from the other three media had capsules which varied in width according to the medium, but were smaller than those of organisms from animals. They also resisted phagocytosis except for a small minority of cells which appeared to be degenerate and had lost their capsule.

It is true therefore for the strain N.P. as for other virulent strains of *B. anthracis* that the organisms from an infected host have better-developed capsules and are more completely resistant to phagocytosis than are most organisms grown *in vitro*.

The action of 0·16 *per cent w/v ammonium carbonate solution.*

B. anthracis isolated from guinea-pigs dissolve readily in ammonium carbonate solution. This property has been used to distinguish these organisms from the 16 samples which were grown *in vitro* and collected as described above.

The washed organisms from 20 ml. of culture were immediately homogenised with ice-cold distilled water (20–22 ml.). This suspension (9·6 ml.) was placed in two spectrophotometer tubes. To one tube 4 per cent w/v ammonium carbonate solution (0·4 ml.) was added, and to the control the same amount of water. The tubes were kept at 0–3°, and at various time intervals turbidity readings (— log per cent transmission of light of wave length 610 mμ.) were taken using a distilled water blank. Any lysis of the organisms in 0·16 per cent w/v ammonium carbonate solution was thus detected.

It was important that the amount of organisms treated with ammonium carbonate should be of the same order in all samples. Although the amounts of organisms in 20 ml. of the cultures described above were not identical; they all produced a turbidity reading within the range 0·4–0·8 before the addition of ammonium carbonate. The amounts of bacteria isolated from guinea-pigs and used in five comparison experiments were selected so that their initial turbidity readings extended over this range. Variation of initial turbidity within this range did not significantly affect the degree of lysis. The figures quoted in Table II are the averages of the five experiments.

Table II summarises the results of these experiments. In most of them there was a slight change in the turbidity reading of the control as the experiment proceeded. For the sake of brevity in presenting a large number of comparisons of experiment with control, the figures quoted are the differences between the reading of the control, and that of the experiment expressed as a percentage of the former, *e.g.*, control 0·67, experiment 0·46, figure quoted 31; control 0·65,

TABLE II.—*The Action of* 0·16 *per cent Ammonium Carbonate Solution at* 0° *on* B. anthracis *grown* in vivo *and* in vitro.

Sample of *B. anthracis*.*		Time of readings (hr.).								
		1.	2.	3.	4.	5.	6.	7.	8.	24.
Organisms isolated from guinea-pigs		13	24	36	44	51	61	67	72	86
T.M.B.	(1)	1	3	3	5	8	16	25	35	85
	(2)	0	1	3	4	5	7	11	15	73
	(3)	0	0	0	0	0	0	2	4	33
	(4)	0	2	5	0	1	4	4	4	18
B.M.	(1)	0	0	0	2	2	2	2	2	11
	(2)	0	0	0	0	0	0	0	0	13
	(3)	0	2	0	0	0	0	0	0	3
	(4)	2	1	2	2	1	2	2	3	0
S.S.	(1)	0	2	4	5	5	5	5	5	22
	(2)	0	0	3	3	0	0	0	0	24
	(3)	0	0	0	0	0	0	0	0	5
	(4)	0	0	0	0	0	0	0	0	5
G.P.P.	(1)	1	4	4	5	5	6	7	8	14
	(2)	2	2	2	4	4	5	5	6	21
	(3)	2	4	5	8	8	8	8	8	12
	(4)	0	2	6	6	6	9	8	8	8

The figures are a measure of the lysis of organisms in ammonium carbonate solution, 0 indicating no lysis ; they are the difference between the turbidity readings (– log percentage transmission at wave-length 610 mμ.) of the control and those of the experiment, expressed as a percentage of the former.

* For details of preparation see Table I.

experiment 0·17, figure quoted 74. All figures are the average of two or more experiments, and are a measure of the lysis of the organisms in the dilute ammonium carbonate solution.

The rapid solution of the *in vivo*-grown organisms is not shown by the 16 samples of organisms grown *in vitro* even by those obtained from media containing plasma constituents.

Preparation of Sterile Bacterial Extracts by Mild Methods.

In these experiments the washed *B. anthracis* from infected guinea-pigs were used.

Attempts to remove the capsule from B. anthracis.

The evidence that the capsule of *B. anthracis* is composed of polyglutamic acid consists solely of the fact that non-capsulated organisms produce none of this material. When a capsule is formed there is no reason to suppose that it consists entirely of polyglutamic acid. Indeed Tomcsik (1951) has put forward some evidence for the presence of polysaccharide in the capsule. A preferential removal of the capsule would have been of great value in these studies in view of the possible connection of the capsule with virulence (Treffers, 1947). Following the publication of the work of Amies (1951) on the removal of the capsule from *Pasteurella pestis* by solution of KCNS, in which it is mentioned that the same method worked for *Bacillus mycoides*, considerable attention was devoted to this problem, but without success.

The classical method of wet India ink preparations was used to indicate the presence of a capsule. The following substances were examined in various concentrations both at room temperature and at 0° to see if they would preferentially remove the capsule :—KCNS, KI, NH_4CNS, sodium salicylate, guanidine hydrochloride, urea, ammoniacal copper sulphate, ammonium acetate, mono-, di- and triethanolamine, tergitol, sodium taurocholate, sodium desoxycholate, formamide, ethylene glycol, diethylene glycol, pyridine, ether water, chloroform water, dilute ammonia solutions, Na_2CO_3, NaOH and acetic acid. Not one had the desired effect.

Although no clear-cut removal of the capsule could be effected, stained films of *B. anthracis* grown *in vivo* show that some capsular material tends to become detached from the organism. This is supported by the fact that chemical fractionation of the plasma/exudate has revealed the presence of polyglutamic acid. There is a distinct possibility that the three washings of the bacteria with Locke's solution (Smith *et al.*, 1953) may have removed some capsular material. These washings have been kept separate for chemical and biological examination.

A bacterial extract prepared by the action of 0·16 *per cent w/v ammonium carbonate.*

It was noticed in the above experiments that 50 per cent aqueous pyridine and 0·1 N ammonia solution dissolved the organisms completely at 0°. This was not solely a pH effect since $NaHCO_3$, Na_2CO_3 and NaOH solutions did not give the same degree of lysis.

Concentrating on the action of the ammonium ion rather than that of pyridine, experiments were designed to find the lowest pH at which this ion would effect complete solution of the organisms. Mixtures of ammonia, ammonium carbonate and ammonium acetate in dilute solution were used. A 0·16 per cent w/v ammonium carbonate solution (pH 8·8) was the most satisfactory, and after the organisms had dissolved the pH of the solution dropped to a minimum of 8·3. Mixtures of ammonium carbonate and ammonium acetate with reactions below pH 8·2 did not dissolve the organisms. Spores were not affected. After lysis the viable count corresponded to the few spores present in the original suspension.

The procedure for preparing the extract is as follows : The sludge (50 ml.) of bacterial chains ($3–5 \times 10^{11}$) from 100 guinea-pigs was transferred in ice-cold distilled water to a bottle (900 ml.) containing glass beads (100 ml.). Ammonium carbonate solution (4 per cent w/v, 32 ml.) was added and the bottle filled with water and shaken at 0–3° overnight. The solution was clarified from a slight turbidity by centrifugation at 0–3° for 15 min. at 10,000 r.p.m. (12,000 g) in a refrigerated centrifuge. After filtering through sintered glass filters and testing for sterility the extract was freeze-dried. The small residue from centrifugation was re-extracted with 0·16 per cent w/v ammonium carbonate solution (80 ml.).

Table III shows the details of five routine preparations of this extract in which the yield of insoluble material is also given. This material, which was autoclaved, freeze-dried and finally dried at 0·1 mm. Hg and at 60° before weighing, consisted of spores and bacterial débris.

A totally unexpected advantage of this method was the sublimation of the ammonium carbonate during freeze-drying. Determination of ammonia as ammonium salts in 7 different freeze-dried preparations corresponded to 1–2 per cent of ammonium carbonate. This small quantity of ammonia is probably present as the ammonium salts of other acids.

TABLE III.—*The Extraction of* B. anthracis *with Ammonium Carbonate Solution and by Shaking with Ballotini.*

Nature of extract (vol. approx. 800 ml.).	Initial material (from 100 guinea-pigs). Total No. ($\times 10^{11}$) of B.C.	Soluble material: pH of filtered extract.	Soluble material: Wt. (g.) of freeze-dried extract.	Soluble material: *Wt. (g.) of extract corrected for moisture and solids of the Locke's soln. in bacterial sludge.	Insoluble material: Dry wt. (g.).	Insoluble material: Percentage of soluble material.
Ammonium carbonate solution (0·16 per cent w/v).	4·0	8·3	2·2	1·8	0·087	4·8
	3·8	8·5	1·8	1·4	0·062	4·4
	3·8	8·7	1·7	1·3	0·055	4·2
	4·9	8·7	2·1	1·7	0·075	4·4
	4·9	8·7	2·5	2·1	0·080	3·8
Ballotini extract	4·5	7·1	1·7	1·3	0·098	7·5
	5·4	6·9	2·2	1·8	0·075	4·2
	4·1	6·8	1·8	1·4	0·070	5·0
	5·1	6·7	2·0	1·6	0·061	3·8

* Moisture : Loss at 0·1 mm. Hg and 60°. Vol. of sludge is 50 ml. ; the weight of the solids of the Locke's solution in the sludge cannot be more than 0·5 g., and is estimated to be 0·25 g.

A bacterial extract prepared by shaking with ballotini at 0°.

A more conventional method of extraction was that used by Curran and Evans (1942) and King and Alexander (1948). The organisms were shaken with water and "ballotini" Grade 12, *i.e.*, minute glass beads with an average diameter of 0·13 mm. (Messrs. Chance Bros., Smethwick, England). After preliminary experiments to find the best proportions of ballotini, distilled water and bacterial sludge the following procedure was used :

The bacterial sludge from 100 guinea-pigs was transferred with ice-cold water to a bottle (1150 ml.) containing ballotini (350 ml.). The bottle was filled with distilled water and shaken 24 hr. at 0–3°, only a small air bubble being left so that foam (and denaturation) was reduced to a minimum. The extract, the pH of which hardly varied from 7·0, was treated in the same manner as the ammonium carbonate extract. The re-extraction of the residue was done with water (80 ml.) and ballotini (35 ml.).

Table III includes the details of four routine preparations in which the yield of insoluble material (treated as described above) is quoted. This material consisted of spores and bacterial debris.

Results of freezing and thawing experiments.

By freezing the diluted bacterial sludge at —20°, then allowing it to thaw at room temperature and repeating the process 15–20 times, the viable count of the sample dropped to 10^2–10^3/ml. However, the samples after this procedure remained grossly turbid, and there was no hope of sterile filtration yielding a soluble extract containing the same high proportion of bacterial substance as in the previous two methods. This method was therefore abandoned.

DISCUSSION.

The widely held view that organisms from an infected host differ from those obtained from cultures has been substantiated for *B. anthracis* (N.P.) from infected guinea-pigs. A study of morphology and susceptibility to phagocytosis sharply differentiated organisms grown *in vivo* from those grown in broth, but not from those cultivated in the other three media which contained some plasma constituents. However, the rapid lysis of organisms from guinea-pigs in ammonium carbonate solution was not shown by any of the organisms grown *in vitro* including those from media containing plasma constituents. This lysis, the basis of which is unknown, serves to underline the chemical difference between these organisms.

After the failure of attempts to remove the capsule of *B. anthracis*, two methods, the use of ammonium carbonate solution and shaking with ballotini have been used to dissolve the bacterial substance with excellent results. The proportion of material dissolved was over 95 per cent. Of the two methods, extraction with dilute ammonium carbonate solution was easier to carry out, and it is doubtful whether any gross harm was done to constituents by a pH of 8·8–8·3 at 0–3°. However, the second method of extraction at pH 7·0 rules out any possible detrimental effect of these slightly alkaline conditions.

Future work on the chemical basis of virulence of *B. anthracis* in guinea-pigs will follow three main lines. Plasma/exudate (extracellular products) and both bacterial extracts will be examined in biological tests connected with virulence. The crude materials will then be examined chemically to identify the substances responsible for particular biological activity. This involves the extension of the above tests into biological assays for fractionation samples. Finally, observations on the metabolism of *B. anthracis* in body fluids may be attempted together with its repercussions on the metabolism of the host. The following paper describes the first of these investigations.

SUMMARY.

B. anthracis organisms harvested from infected guinea-pigs have been shown to be different from those grown under a variety of conditions *in vitro* mainly by their rapid lysis in ammonium carbonate solution.

Sterile bacterial extracts of *B. anthracis* grown *in vivo* have been prepared by two mild methods and contain over 95 per cent of the bacterial substance.

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