THE CHEMICAL BASIS OF THE VIRULENCE OF *BACILLUS ANTHRACIS*. III: THE RÔLE OF THE TERMINAL BACTERAEMIA IN DEATH OF GUINEA-PIGS FROM ANTHRAX.

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Bacillus anthracis and its products have been obtained from infected guinea-pigs in sufficient quantity for chemical extraction (Smith, Keppie and Stanley, 1953*a*). It has been suggested that the examination of this type of *in vivo*-produced material might be the best way of identifying the chemical compounds and processes responsible for the virulence of this and other pathogenic organisms (Smith, Keppie and Stanley, 1953*b*). An immediate approach to the identification of the chemical basis of the invasiveness of the organism was made. Agressins were easily demonstrated in extracts of the organisms (Smith *et al.*, 1953*b*) and extracellularly in the body fluids of the infected guinea-pigs (Keppie, Smith and Harris-Smith, 1953). These two crude products are being chemically fractionated for agressins (Smith and Zwartouw, 1954; Smith, Zwartouw and Gallop, 1954).

Unfortunately, in so far as the cause of death in anthrax is concerned, the literature (see Smith *et al.*, 1953*b*) had little to offer other than speculation. Neither the general nature of the lethal effect on the host nor even the products or processes of the organism mainly responsible for the characteristic symptoms of the disease were known. No study of the chemical basis of this aspect of pathogenicity was therefore possible until the nature of the fatal syndrome was established and the substance causing it recognised. Work leading to the solution of this problem, already briefly reported (Smith and Keppie, 1954; Smith, Keppie, Ross and Stanley, 1954), is described in more detail here and in two subsequent papers.

Up to the present, no lethal endo- or exotoxin has been found in *in vitro* cultures of *B. anthracis* (Eurich and Hewlett, 1930; Sobernheim, 1931; King and Stein, 1950), and our first attempt to demonstrate a lethal factor in the products of the organism growing *in vivo* was unsuccessful. Large quantities of a mixture of five parts thoracic exudate and one part plasma from guinea-pigs dying of anthrax were not lethal when injected intraperitoneally into guinea-pigs. The result was the same when bacterial extracts were injected equivalent to an amount of bacteria twice that estimated to be present in a guinea-pig when it dies of anthrax (Keppie *et al.*, 1953). These preparations, although non-lethal, did produce the mild oedematous skin reaction already associated with such material (Sobernheim 1931; Cromartie, Watson, Bloom and Heckly, 1947; Watson, Cromartie, Bloom, Heckly, McGhee and Weissman, 1947). It was pointed out that this lack of gross toxicity and the fact that very large numbers of *B. anthracis* grew in the blood of guinea-pigs before they died meant that death was due to harmful effects which were weak relative to the number of organisms producing them.

Such effects already suggested in the literature are that the massive bacteraemia might block the capillaries of the host, produce a fatal anoxaemia or a deficiency of essential nutrients. To assess the importance of such hypotheses it was essential to know whether the bacteraemia had to reach its possible maximum before death from anthrax followed. It is generally agreed that a pronounced bacteraemia is a usual feature in death from anthrax in many species, and we have found it invariably so in our work with guinea-pigs. However, a few reports state that partly immune guinea-pigs (Sterne 1953, private communication) and some animals of other species (Bloom, McGhee, Cromartie and Watson, 1947; Stockman, 1911) die of anthrax with only a slight bacteraemia. Such reports were the exception to the general rule and were in the main incidental to other studies. A thorough investigation of the significance of the final bacteraemia in the death of guinea-pigs seemed necessary.

This paper is mainly concerned with the bacteraemia, but observations have been made on the significance of obvious changes in the blood of guinea-pigs dying of anthrax (Keppie *et al.*, 1953) and on the validity of two previous hypotheses (Bloom *et al.*, 1947; de Moulin, 1936) on the cause of death. Opportunity has also been taken to analyse the distribution of organisms in the tissues of the host at different stages in the final bacteraemia.

EXPERIMENTAL.

The Relationship between the Degree of Bacteraemia and Death from Anthrax.

The plan was to use therapeutic measures calculated to terminate the final bacteraemia abruptly at known progressive stages in its development. The subsequent fate of the guinea-pigs would indicate the earliest stage at which irreparable damage to the host had occurred.

The infection.—The strain of *B. anthracis* (N.P.) and the breed of guinea-pigs (700 g. $\pm$ 50) used were the same as in previous work (Smith *et al.*, 1953*a*), but here an intradermal injection (2×10^3 spores) was used. The animals died between the 3rd and 5th days after injection.

The final bacteraemia.—In the 12 hr. preceding death, the number of organisms in the blood rose from approximately 3×10^5 chains per ml. to 1×10^9 chains per ml. The progress of this final bacteraemia could be followed conveniently by microscopic examination of blood films from the ear. One flat loopful (area enclosed by loop 2 sq. mm.) of blood was spread over 1 sq. cm. and stained with methylene blue. Examination of 50–100 fields (magnification $\times$ 600) gave the average number of bacterial chains per field. In 15 comparisons of this method of counting with haemocytometer counts of the organisms, the relationship with the absolute count was obtained (1 chain per field = $1{\cdot}5 \times 10^7$ per ml. of blood) and the error of maximum scatter was within $\pm$ 50 per cent. After the degree of bacteraemia had been determined in this manner the survival time of the guinea-pig subsequent to the making of the blood film was noted. In Table I, the results given in the 2 left-hand columns show that the degree of bacteraemia is closely related to the period of survival; each survival time given is the average of observations on 10–15 animals in 6 experiments.

Termination of the bacteraemia: subsequent fate of the guinea-pigs.—A rapid method for terminating the bacteraemia was essential, and a number of therapeutic agents were tested for this purpose. Hyper-immune anthrax antiserum prepared in the horse (30 ml.), chlortetracycline (25 mg.), oxytetracycline (20 mg.) and chloramphenicol (100 mg.), failed to stop the rapidly developing bacteraemia. Penicillin was toxic for guinea-pigs at a dosage which was adequately bacteriostatic. Streptomycin, however, proved very effective and was non-toxic for guinea-pigs. The dosage used was an initial injection of 80 mg. (half given subcutaneously and half intraperitoneally), followed 6 hr. later by a further 40 mg. subcutaneously. Thereafter daily doses of 25 mg. dihydrostreptomycin cerate were given

TABLE I.—*The Relationship between the Degrees of Anthrax Bacteraemia in Guinea-pigs and the Time to Death. The Results of Streptomycin Treatment.*

Infected guinea-pigs.				Streptomycin-treated guinea-pigs.†		
Organisms in blood.		Hours until death.			Hours until death.	
No. per field*	No. per ml. $\times 10^{-6}$ (± 50%).	Average.‡	Standard deviation.	Number dead. / Number treated.	Average.	Standard deviation.
1/80	0·2	12·7	0·6	0/2	—	—
1/40	0·4	11·5	0·7	0/6	—	—
1/20	0·8	9·5	1·2	2/12	—	—
1/10	1·5	8·5	1·7	1/12	—	—
1/5	3·0	8·1	1·3	11/20	—	—
1/2	7·5	6·9	1·0	36/37	43	15
1	15	6·1	0·8	19/19	39	12
2	30	4·9	1·1	16/16	36	12
4	60	4·4	0·7	9/9	24	8
8	120	3·6	1·0	14/14	17	6
16	240	2·4	0·5	12/12	10	4
32	480	1·5	0·5	7/7	6	2
64	960	Death	—	—	—	—

* Films prepared as described in the text and stained with methylene blue, magnification × 600. Numbers refer to chains.

† See text for details of streptomycin treatment.

‡ Average of 10–15 animals in 6 experiments.

intramuscularly. The antibiotic rapidly terminated the infection. Multiplication of the organisms had ceased within 1–1½ hr., by which time they were disintegrating; after 5–7 hr. no bacilli remained in the blood. This applied even in animals treated approximately 4 hr. before they would otherwise have died. At times later than this, only 50–75 per cent of the animals could be freed from infection. The fate of animals treated with antibiotic at selected stages of the developing bacteraemia was noted, together with the time of death, if this occurred. Throughout the experiments, detailed observations were made only on animals freed from active infection.

The results of six experiments are summarised in the right-hand columns of Table I. Removal of the infection with streptomycin saved the guinea-pigs, provided the bacteraemia had not increased beyond *ca.* 3×10^6 chains/ml. of blood. All guinea-pigs with a bacteraemia greater than this critical value died although they were free from active infection. The time interval (6–72 hr.) between antibiotic treatment and death was much longer than that which would have elapsed had the bacteraemia not been stopped; it decreased regularly as the level of bacteraemia at which the animals were treated increased.

Observations on Two Recorded Hypotheses on the Cause of Death.

The study of the bacteraemia and the effective use of streptomycin to control it provided an opportunity to investigate previous hypotheses on the cause of death.

Interference with calcium metabolism.

Bloom *et al.* (1947) compared the symptoms of anthrax in rabbits (lassitude, drowsiness and excessive response to stimuli) with those of calcium deficiency, and noted the work of Weinstein (1938) who protected mice against anthrax by

injections of parathyroid extract. They showed that injections of calcium gluconate and laevulinate would to some extent protect rabbits against anthrax. The calcium salts and the parathyroid extract of Weinstein were given from the time of challenge onwards; their effect may have been on the initial invasion rather than on the killing power of the organism. Govaerts (1951) and Renaux (1952) noted the adverse effect on the virulence of *B. anthracis* when calcium salts were present in cultures *in vitro*.

In our work with guinea-pigs, no excessive response to stimuli was apparent. Administration of large quantities of calcium borogluconate (1 g. every 12 hr.) and parathyroid extract (0·5 mg.) separately or together, did not save life; furthermore it did not prolong the survival time of guinea-pigs (4 for each treatment) given streptomycin at a time immediately beyond the critical level of bacteraemia which predetermined the death of the animals 1–3 days later. The results of these experiments suggest that any action of the calcium or the parathyroid injections of Bloom *et al.* (1947) and Weinstein (1938) was not directed against the killing mechanism of the organism.

An attack on the central nervous system.

de Moulin (1936) reported that lesions occurred in the central nervous system of guinea-pigs infected with *B. anthracis*. He attributed the symptoms of anthrax and the death of the host to damage of this system. Our colleague Dr. Joan M. Ross has examined the histopathology of the tissues of guinea-pigs dying of untreated anthrax, and of those in which active infection had been stopped by streptomycin injection after the critical point in the bacteraemia. She could not detect the changes in the central nervous system described by de Moulin.

The Significance of the More Obvious Changes in the Blood of Guinea-pigs Dying of Anthrax.

Following earlier work (Sobernheim, 1931; Bloom *et al.*, 1947), Keppie *et al.* (1953) noted abnormalities in the blood of guinea-pigs dying from untreated anthrax. It was suggested that these abnormalities might have some bearing on the cause of death. Therefore a study was made of the R.B.C. agglutination, the slight haemolysis, the increased red-cell fragility, and the delayed clotting which occurs, with a view to deciding whether such abnormalities were present in the blood at the critical point of the bacteraemia or at the death of guinea-pigs freed from infection by streptomycin treatment 1–3 days previously. The general conclusion from this work was that such changes in the blood only occurred very late in the untreated disease and were not of primary importance. A summary of the results is given below. Observations were made on individual samples of heart blood from at least five animals.

Haemolysis.—The blood of guinea-pigs dead from untreated anthrax was 10–20 per cent haemolysed. This is a late phenomenon. Blood from animals approximately 6 and 1½ hr. before death from untreated anthrax was 1 per cent and 1–3 per cent haemolysed respectively. Animals dead after the streptomycin treatment described above had blood which was less than 1 per cent haemolysed.

Red cell fragility.—In the usual R.B.C. fragility test the concentration of NaCl solution in which haemolysis first occurs with cells from normal guinea-pigs is 0·40–0·45 per cent. The same concentration was necessary with the R.B.C.

from untreated animals approximately 6 hr. before death. This also applied to cells from animals treated with streptomycin at a time beyond which their lives could be saved. In fact, the only slight change in sensitivity was observed with cells from animals dead of untreated anthrax; here an NaCl concentration of 0·45–0·52 per cent gave first evidence of haemolysis.

Red cell agglutination.—The R.B.C. of guinea-pigs which had died from untreated anthrax were distorted and agglutinated in large masses. Agglutination occurs very late, commencing about 3 hr. before death. No agglutination had occurred in guinea-pigs about 6 hr. before death from untreated anthrax, or in those animals dying 1–3 days after removal of the infection with antibiotic.

Increased clotting time.

The fact that the blood of animals infected with *B. anthracis* clots poorly is well known (Bloom *et al.*, 1947) and this was readily confirmed in guinea-pigs. Blood (2 ml.) obtained by cardiac puncture was expelled immediately into a small tube at 37° in a water bath. The time for clotting was taken from the moment the blood first entered the syringe. Sixteen samples of normal blood clotted in 4–8 min. Samples of blood from 10 guinea-pigs dying from untreated anthrax clotted in 60, 24, 18, 10, 23, 6, 37, 14, 7 and 10 min.; some of the clots were incomplete. Samples of blood from 5 guinea-pigs dying 1–3 days after removal of the infection with streptomycin clotted in 13, 14, 17, 15 and 14 min., which is slightly slower than for normal blood.

Watson *et al.* (1947) found their crude "inflammatory factor" from rabbit oedema fluid had an anti-coagulant activity. We have been unable to find any pronounced anti-coagulant activity in the crude intra- and extracellular products of *B. anthracis* growing *in vivo* (Smith *et al.*, 1953*a*, *b*) or in purified fractions from them (Smith and Zwartouw, 1954; Smith *et al.*, 1954). The recalcification test of Foster (1941) was used, but citrated guinea-pig plasma was substituted for the sheep plasma. In a test in which heparin (0·0006 per cent) was active the following materials were inactive: dialysed plasma exudate (4 per cent), as well as the purified polyglutamic acid (1 per cent) and the highly active aggressin fraction (1 per cent) from it; ballotini bacterial extract (4 per cent); ammonium carbonate bacterial extract (4 per cent) and the following fractions from it, diffusate (1 per cent), purified polyglutamic acid (1 per cent), protein fractions (1 per cent), and purified polysaccharide (1 per cent).

The Distribution of B. anthracis *in the Tissues of Guiuea-pigs during the Final Bacteraemia.*

In these experiments the object was to determine if any particular tissue or organ harboured organisms in such selectively high numbers as to suggest a site for toxic action.

The organisms in the blood and major organs of the body were counted in tissue samples from groups of five guinea-pigs infected as described above and representative of the following three stages of the bacteraemia: pre-critical point when the blood contained 0·8–3 $\times$ 10^6 chains per ml.; post-critical point when the blood contained 4–15 $\times$ 10^6 chains per ml.; and at death. Suspensions for haemocytometer counts were prepared from spleen, liver, kidney, lung, skin and muscle by grinding the tissue with sand and water. Table II shows the relative

distribution of anthrax bacilli in these tissues. The numbers found in the muscle, the skin lesion, and the oedema of the primary lesion which extended into the peritoneal cavity were too low to be of any significance and are omitted from Table II. The total number in the guinea-pigs at death agrees with that obtained previously (Keppie *et al.*, 1953) for guinea-pigs infected by a different route. The most striking fact about the distribution of organisms is that at the critical point in the bacteraemia, the bacilli are concentrated in the spleen which contains over half the total number of organisms in the host; at death most of the organisms are in the blood stream.

TABLE II.—*Distribution of* B. anthracis *in the Tissues of Guinea-pigs* (700 *g.*) *during the Final Bacteraemia.*

Stage of bacteraemia at which samples were taken.*		Blood (Est. 40 ml.).	Spleen.	Lung.	Liver.	Kidney.	Total.
Pre-critical point (approx. 9 hr. from death)	1	1·0	4·5	0·9	0·8	0·1	7·3
	2	0·4	3·6	0·6	0·3	0·1	5·0
	3	0·4	2·2	0·2	0·1	0·1	3·0
	4	1·1	4·3	1·0	1·4	0·2	8·0
	5	1·4	2·8	0·9	0·3	Pr. nil	5·4
Post-critical point (approx. 6 hr. from death)	1	3·1	9·7	5·5	0·2	0·4	18·9
	2	1·7	10	1·6	0·1	1·2	14·6
	3	3·3	14	2·5	2·3	0·2	22·3
	4	1·7	13	5·2	2·9	0·2	23·0
	5	4·6	15	0·1	1·0	0·1	20·8
At death	1	440	110	25	7	13	595
	2	200	70	20	45	3	338
	3	260	40	8	4	13	325
	4	360	64	6	37	2	469
	5	440	96	37	44	6	623

* See text for details of these stages in the bacteraemia. Figures quoted are the total number ($\times 10^{-8}$) of bacterial chains (4–8 bacilli) in each tissue as determined by haemocytometer counts.

DISCUSSION.

In guinea-pigs a bacteraemia is intimately associated with death from anthrax, but in fact the death of the guinea-pig is determined when the bacterial invasion was still only about 1/300th of its possible maximum. This is experimental proof in a species which normally dies with a massive bacteraemia of a situation which was suspected on more limited observation in other species (Bloom *et al.*, 1947; Stockman, 1911). In small-scale experiments, we have been able to confirm the observation of Sterne (1953) that partially immune guinea-pigs may die after infection with *B. anthracis* with a mild degree of bacteraemia comparable to that existing at the critical point described above. There is a remarkably constant relationship between the increasing degree of bacteraemia and the decreasing period of survival in untreated guinea-pigs. This fact coupled with the quite large variation in the time to death after infection indicates that the animals are equally susceptible to the final killing stage of the disease but vary more in their ability to combat the initial infection. The relationship between the degree of bacteraemia and the extent of pathological damage is emphasised by the behaviour of animals treated with streptomycin. Any delay in chemo-

therapy subsequent to the critical point in the bacteraemia is reflected in a proportionally diminished survival time.

These experiments render untenable the hypotheses that death from anthrax is due to overwhelming numbers of organisms blocking the capillaries of the host or producing a deficiency in it of O_2 or essential nutrients (*e.g.*, glucose). Experiments reported here also shed doubt on the hypotheses of Bloom *et al.* (1947) and of de Moulin (1937) on the cause of death from anthrax. Furthermore, the more obvious changes in the blood of dying animals noted in previous work (Sobernheim, 1931 ; Bloom *et al.*, 1947 ; Keppie *et al.*, 1953) are terminal effects in untreated anthrax and largely disappear in those guinea-pigs dying of anthrax but free from infection as a result of streptomycin treatment. Hence a renewed search was made for the nature of the fatal syndrome, and in view of the relatively low level of bacteraemia which sealed the fate of a guinea-pig, for a toxic product of the organism which would produce this syndrome. Two subsequent papers describe the results of these investigations.

An analysis of the distribution of organisms in the host during the final bacteraemia had some bearing on the problem. It showed that at the critical point the spleen appeared to be fulfilling its function as part of the reticulo-endothelial system in removing large numbers of bacteria from the blood during the earlier period of active proliferation and spread. At this stage of the bacteraemia over half the total organisms were concentrated in the spleen. However, the figures for guinea-pigs at death showed that the spleen was unable to cope with the bacteraemia and soon the blood contained the majority of the organisms. The death of the host accompanies this final overflow. It is obvious, therefore, that any search for harmful effects or products of *B. anthracis in vivo* should pay particular attention to the blood and to the spleen.

SUMMARY.

A bacteraemia is intimately associated with death from anthrax in the guinea-pig. The fate of the animal is determined at a time when the bacterial proliferation in the blood is still only about 1/300th of the maximum obtaining at death. This finding renders untenable a number of hypotheses on the cause of death from anthrax which depend on the presence of a massive bacteraemia.

Evidence is presented against hypotheses of Bloom *et al.* (1947) and de Moulin (1936) on the cause of death from anthrax. Several abnormalities in the blood which were noted in previous work have been shown to be secondary side-effects.

An analysis of the distribution of organisms in guinea-pigs in the final phase of anthrax has focussed attention on the blood and spleen as possible sites for the production of harmful products.

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