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Production *in vitro* of the Toxin of *Bacillus anthracis* Previously Recognized *in vivo*

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SUMMARY: A lethal oedema-producing toxin has been made *in vitro* in serum cultures of *Bacillus anthracis* which is identical with the toxin originally recognized *in vivo*. Toxin is produced by the attenuated immunogenic Sterne strain as well as by the virulent N.P. strain. Previous non-recognition of the toxin in cultures was due to its early appearance and rapid disappearance under ordinary growth conditions. A toxin-destroying mechanism was demonstrable in organisms from cultures grown for more than a few hours. Attempts to maintain the toxin concentration or to increase it by addition of various nutrilites to batch culture failed; only by continuous culture have the organisms been kept in their toxigenic growth phase. Traces of the toxin appear in cultures in tryptic meat broth; these are increased by the addition of large molecular constituents of serum which appear to play an important role in toxin production.

Until recently the product of *Bacillus anthracis* which is responsible for death in anthrax was unknown. No lethal endo- or exotoxin had been found in cultures of the organism *in vitro* (Eurich & Hewlett, 1930; Sobernheim, 1931; King & Stein, 1950). As a result of working with organisms grown *in vivo* rather than with those from artificial culture, Smith, Keppie & Stanley (1955) showed that *B. anthracis* does form a specific lethal oedema-producing exotoxin. It was found in the plasma of guinea-pigs dying of anthrax. Although the characteristics of the toxin were clearly established it was not demonstrated in cultures *in vitro* until some time had elapsed. The failures to detect the toxin under these conditions was due to its exceptionally early appearance followed by its rapid disappearance, which meant that toxin was only detectable during a very short period, at an unusually early stage of the culture. The present paper describes the production of the toxin *in vitro*, also its properties and attempts to improve its yield and to prolong its activity under various culture conditions.

METHODS

Organisms used. The virulent N.P. strain of *Bacillus anthracis* and the attenuated Sterne and H.M. strains were used.

Guinea pig blood and serum. Blood was removed from guinea pigs by cardiac puncture and collected into heparin (10 i.u./ml.) or immediately defibrinated, under as aseptic conditions as possible. After removing the blood cells, the plasma contained a small amount of haemoglobin and was used for toxin production within 2 days of its collection.

Anthrax toxin produced in guinea pigs. This was obtained as described by Smith *et al.* (1955).

Albumin I (Armour). This was Fraction V of bovine plasma prepared by the Cohn & Edsall separation. It was not especially purified and formed a brown solution.

Albumin II. This was a special sample of bovine albumin which showed no evidence of heterogeneity when examined electrophoretically or in the ultracentrifuge.

γ -Globulin. This was Fraction II of bovine plasma prepared by the Cohn & Edsall separation, and supplied by L. Light and Co.

Guinea-pig haemoglobin. Well washed guinea-pig red blood corpuscles were haemolysed with distilled water; after centrifugation the solution was freeze dried.

Bovine and sheep haemoglobin. Prepared as described for guinea-pig haemoglobin.

Estimation of bacterial growth. The number of bacterial chains (2–8 bacilli)/ml. of culture was obtained from the average number of chains/microscope field of a standard film (Keppie, Smith & Harris-Smith, 1955); 1 chain/field was equivalent to 1.5×10^7 chains/ml. ($\pm 50\%$). In media consisting of mainly tryptic meat digest, *Bacillus anthracis* grew in longer chains than in plasma. For this reason an estimate of bacterial growth was obtained from the extinction of light by the turbid solutions, measured in an EEL spectrophotometer. Appropriate blanks were used in these estimations, and in Table 6 the readings of the EEL spectrophotometer are quoted as measures of bacterial growth.

Assay of toxin in culture filtrates. The skin test for oedema production described by Smith *et al.* (1955) was used for routine assay of samples of culture which had been filtered sterile through Millipore membranes. The average result of duplicate assays are recorded here in the manner described by these authors, i.e. 8:40 indicates a lesion of skin-fold thickness 8 mm. and diameter 40 mm. Tests for lethality in mice and guinea pigs were carried out as described by Smith *et al.* (1955).

RESULTS

Production of toxin from the virulent N.P. strain of Bacillus anthracis

The initial experiments were related to the fact that toxin appeared in the blood of guinea pigs dying of anthrax when the bacterial count was *c.* 1×10^7 chains/ml. and the animal was *c.* 7 hr. from death (Smith *et al.* 1955). In preliminary experiments, heparinized or defibrinated guinea-pig blood (9 ml.) was inoculated with a suspension (1 ml., 3×10^8 spores) of *Bacillus anthracis* strain N.P. spores in tryptic meat digest, which had been incubated at 37° for 30 min. to germinate the spores. The mixtures were shaken at 37° in a Warburg bath at 120 strokes/min. with air containing 20% (v/v) CO₂ continuously passing over them. Samples tested by the skin-assay showed that the toxin concentration was maximal at 4 hr. (size of skin lesion 8:35–10:40) and had disappeared by 7–8 hr. after the start of inoculation.

Further experiments soon established that serum could be used as effectively as whole blood; also that the maximum concentration of toxin and its subsequent rapid disappearance were not greatly affected by varying the following

conditions of cultivation: the presence or absence of heparin; rate of shaking or aeration; temperatures of 28°, 34° or 37°; different batches of guinea-pig blood or serum. In all cases, as soon as a certain amount of growth had occurred (count *c.* $5-10 \times 10^7$ fully developed chains/ml.) the toxin concentration was maximal and then rapidly disappeared (at counts greater than $1-2 \times 10^8$ chains/ml.). The size of the initial inoculum was somewhat critical if a high maximal concentration of toxin was to be obtained. The results in the upper part of Table 1 show that an inoculum size lower than the optimal resulted in a slightly less rapid appearance and disappearance of the toxin, but the maximal toxin concentration was not as high as that obtained with the optimal size of inoculum. A large inoculum resulted in a very rapid appearance and disappearance of the toxin. The optimal inoculum size and the conditions of culture described in Table 1 were used for the production of toxin for all subsequent work.

*Production of toxin by the virulent non-capsulated immunogenic
Sterne strain of Bacillus anthracis*

The data in the lower part of Table 1 show that toxin was produced by the Sterne strain of *Bacillus anthracis*. The pattern of early appearance and rapid disappearance of toxin and the existence of an optimal inoculum size for maximal toxin production was the same as for the virulent strain. The capsulated attenuated H.M. strain of *B. anthracis* did not produce demonstrable toxin when tested under the conditions in Table 1.

Toxin concentrations similar to those quoted in Table 1 were obtained when the N.P. or the Sterne strains were grown for short periods in human, calf or sheep serum under the conditions described in Table 1.

*A comparison of the properties of the anthrax toxin prepared in vitro
and in vivo*

The toxins formed *in vitro* by the N.P. and Sterne strains of *Bacillus anthracis* were compared with that produced by the N.P. strain *in vivo*. The results shown in Table 1 demonstrate the difficulty of obtaining cultures at the peak of toxin production. For the following tests, cultures were taken at 15-30 min. intervals between 3.5 and 4.5 hr. after the start of incubation. Only in this way could toxin be obtained which approached in potency that invariably present in the plasma of guinea pigs dying of anthrax. When, in addition to the optimal conditions outlined in Table 1, cultures were grown in an atmosphere of 20% (v/v) CO₂ in air, the filtrates contained on the average slightly more toxin than those grown in air without added CO₂. The use of CO₂ appeared to some extent to broaden duration of the toxin peak, thus making it easier to obtain samples of maximum toxicity. Carbon dioxide + air was used whenever filtrates of maximum toxicity were required. On average, the Sterne strain of *B. anthracis* produced slightly higher concentrations of toxin than did the N.P. strain.

Oedema production. In the standard skin test for oedema production lesions of 7-10:35-40 were obtained after injecting toxic filtrates from N.P. and

Table 1. *The rapid appearance and disappearance of the anthrax toxin in cultures of virulent (N.P.) and avirulent (Sterne) strains of Bacillus anthracis: the effect of size of inoculum*

Culture conditions: fresh slightly haemolysed, defibrinated guinea pig serum (15 ml.) to which the inoculum is added as a suspension in tryptic meat digest (3.8 ml.) which had been incubated at 37° for ½ hr. to germinate the spores. Cultures were shaken at 37° in 250 ml. flasks with cotton wool plugs at 100 strokes/min.

Inoculum (no. germinated spores/ml.) N.P. strain (virulent) 3 × 10 ⁸	Age of culture (hr.)										
	0	2	2.5	3	3.5	4	4.5	5	6	8	10
Toxin* Count†	Nil (30)	5:27	7:36	6:34	6:30	5:19 (23)	Nil	Nil	Nil (80)	—	—
Toxin Count	Nil (4.5)	6:20 (4.5)	—	6:24	8:38	8:37 (9)	6:37	Trace	Nil (20)	Nil	Nil
Toxin Count	Nil (3)	Trace	—	—	6:27	6:28 (4.5)	6:35	7:38 (9)	7:34 (12)	Trace (24)	Nil
Toxin Count	Nil (1)	Trace	—	—	—	5:17 (1.5)	—	6:20	6:20 (8)	6:20	Nil (11)
Sterne strain (avirulent) 1.5 × 10 ⁸	Nil (15)	6:35	8:40	8:40	7:39	6:36 (15)	4:25	Nil	Nil (15)	Nil	Nil
Toxin Count	Nil (3)	6:28 (3)	—	7:32	8:38	9:42 (7)	6:27	Nil (12)	Nil (30)	Nil	Nil
Toxin Count	Nil (0.75)	5:16	—	5:18	—	7:30 (1.5)	—	8:35 (3)	5:22 (6)	Nil (15)	Nil

* Size of skin lesion (Smith *et al.* 1955): 5:27 = lesion of skin-fold thickness 5 mm. and lesion diameter 27 mm.

† No. of chains × 10⁷/ml. of culture.

The almost equal counts/ml. of bacterial chains at 0 and 2 hr. are somewhat misleading; there was an increase of bacterial protoplasm during this period from the small germinated spore to the mature chain of *B. anthracis* containing 2-8 organisms.

Sterne strains of *Bacillus anthracis*. The lesions formed by toxin produced *in vivo* were 8–10:40–45. The null point (Smith *et al.* 1956) of the toxin prepared *in vivo* was at a dilution of 1/256; the corresponding dilutions for toxic filtrates from the Sterne and N.P. strains were 1/256 and 1/128, respectively.

Lethality to mice and guinea pigs. Results of lethality tests on toxic filtrates from subcultures of N.P. and Sterne strains of *Bacillus anthracis* are shown in Table 2.

Table 2. *The lethality of Bacillus anthracis toxic filtrates for mice and guinea pigs and its specific neutralization*

Antiserum (i) was prepared by hyperimmunizing horses with living avirulent *B. anthracis* spores; 1/5 of volume of toxic filtrate was added.

Antiserum (ii) was prepared by hyperimmunizing horses with the immunizing antigen of Belton & Strange (1954); 1/5 of volume of toxic filtrate was added.

Toxic filtrates came from cultures in air containing 20 % (v/v) CO₂.

All injections made intravenously, and for guinea pigs, freeze-dried material was used at double the original strength.

Sample	Skin lesion 0.2 ml.	Mice* (deaths/no. injected)			Guinea pigs* (deaths/ no. injected)	
		1.5 ml.	1 ml.	0.5 ml.	20 ml.	10 ml.
Toxin from N.P. strain						
Alone†	7:38	37/46	24/41	0/10	7/9	1/3
With antiserum (i)	Nil	0/5	—	—	0/2	—
With antiserum (ii)	Nil	0/5	—	—	—	—
Toxin from Sterne strain						
Alone†	8:40	24/29	57/73	11/15	9/9	9/15
With antiserum (i)	Nil	0/14	0/24	—	0/5	—
With antiserum (ii)	Nil	0/10	0/10	—	—	—

* For comparison, the approx. L.D.₅₀ of the anthrax toxin produced *in vivo* for the mouse and the guinea pig was 0.4 ml. and 6 ml. respectively (Smith *et al.* 1955).

† When normal horse serum (1/5 volume of toxic filtrate) was mixed with the toxic filtrates it did not affect either lethality or oedema production.

Specific neutralization by antiserum. The anthrax toxin produced *in vivo* was specifically neutralized by the serum of horses hyperimmunized against anthrax by using live avirulent *Bacillus anthracis* spores (Sterne strain) or a non-toxic immunizing antigen produced *in vitro* by our colleagues Messrs Belton & Strange (1954). Similarly, the oedema production and lethality of toxic filtrates from cultures of N.P. and Sterne strains of *B. anthracis* were also neutralized by these two types of antiserum (see Table 2.)

Specific precipitation in Ouchterlony plates. Toxin produced *in vivo* formed a maximum of three precipitation lines when examined in a standard Ouchterlony diffusion system against either of the two sera described above (Stanley & Smith, personal communication). When compared on the same Ouchterlony plate with the toxin formed *in vivo*, toxin filtrates from cultures of N.P. and Sterne strains of *Bacillus anthracis* when concentrated fivefold, formed identical lines against both types of antiserum.

Presence of at least two components forming a synergic mixture. The anthrax toxin produced *in vivo* could be separated by ultracentrifugation into two

components—Factor I (deposit) and Factor II (supernatant fluid) (Smith *et al.*, 1956). When injected alone, Factor I had a much lower activity than the original toxin and Factor II (when free from Factor I) was devoid of activity; mixed together, they formed a fully toxic mixture. Our colleague Mr J. L. Stanley ultracentrifuged the toxic culture filtrates from both strains of *Bacillus anthracis* as described by Smith *et al.* 1956; after one ultracentrifugation for 16 hr. the unwashed deposit (Factor I) was separated from the supernatant fluid (Factor II approx. 8 ml.) and dissolved in saline (3 ml.). The results of skin tests for synergic action between these two factors and with the respective components of the toxin prepared *in vivo* are shown in Table 3. It is clear that Factors I and II are present in the toxin prepared *in vitro*.

Table 3. *Synergic action of Factors I and II from the toxic filtrates of Bacillus anthracis produced in vitro with each other and with Factors I and II from toxin produced in vivo.*

Strain of <i>Bacillus anthracis</i> from which toxic filtrate prepared	Skin tests for toxin				
	Ultracentrifugal fractions injected alone				
	Unwashed supernatant deposit		Synergic mixtures of Factor I (0.1 ml.) + Factor II (0.1 ml.)		
	Factor I* (0.1 ml.)	Factor II (0.1 ml.)	<i>in vitro</i> I <i>in vitro</i> II	<i>in vitro</i> I <i>in vivo</i> II	<i>in vivo</i> I <i>in vitro</i> II
<i>In vitro</i>					
Strain N.P.	8:24	Nil	11:30	9:40	10:40
Strain Sterne	9:35	Nil	11:40	11:38	10:35
<i>In vivo</i> †					
Strain N.P.	9:32	Nil	—	—	—

* These crude preparations of Factor I inevitably contain some Factor II which accounts for the relatively high inherent activity. Factor II can be freed from I by ultracentrifugation.

† For method of preparation see Smith *et al.* 1956.

Production of active immunity. Anthrax toxin prepared *in vivo* will actively immunize guinea pigs against subsequent infection with *Bacillus anthracis*. A 1/8 or 1/16 dilution of it will protect *c.* 50% of the animals in the test for immunizing activity described by Smith & Gallop (1956). A similar degree of protection in this test was obtained from 1/4 and 1/16 dilutions of toxic filtrates from 4 to 4½ hr. cultures of the N.P. and Sterne strains, respectively. After 7 hr. growth, when the culture filtrates were no longer toxic, they could still immunize although less effectively. Thus, an undiluted filtrate from a late culture of N.P. strain and a 1/2 dilution of a similar filtrate from Sterne strain protected 50% of the animals. This partial deterioration parallels the behaviour of the *in vivo* toxin when treated with various chemical reagents; it rapidly loses its toxicity but retains some ability to immunize.

Selective inhibition of toxin synthesis by 2-thiouracil. Tempest & Smith (1957) showed that *in vivo* 2-thiouracil interfered with toxin production by *Bacillus*

anthracis without significantly affecting its rate of growth. This antimetabolite had a similar action on toxin production by both N.P. and Sterne strains *in vitro* (Table 4).

Table 4. *The effect of 2 thiouracil on toxin synthesis in vitro by Bacillus anthracis*

Medium and conditions (optimal inoculum) of incubation as described in Table 1. The number of bacterial chains/ml. in the growing culture at the different times was not significantly different in control and experimental samples.

Medium	Organism	Duration of incubation		
		3.5 hr	4.5 hr.	6 hr.
		Skin reaction		
Control	Sterne	6:37	7:42	Nil
+ thiouracil (0.1 %)	Sterne	4:21	4:20	tr
Control	N.P.	6:32	6:35	Nil
+ thiouracil (0.1 %)	N.P.	5:30	5:27	Nil

The toxin-destroying action of Bacillus anthracis

The effect of bacterial population on toxin production. The results in Table 1 show that toxin production and destruction were related to the number of bacteria in the culture. As soon as a critical amount of growth had occurred (c. $5-10 \times 10^7$ fully developed chains/ml.), the amount of toxin was maximal and at greater amounts of growth it rapidly disappeared. This was confirmed by the following experiment. When non-toxic, 6-7 hr. cultures of the N.P. or Sterne strains of *Bacillus anthracis* were centrifuged and the deposited organisms resuspended in the same volume of medium (1 vol. tryptic meat broth + 9 vol. plasma), no toxin appeared on incubating this relatively high concentration of organisms. On the other hand, toxin was produced when the medium was inoculated with these organisms, when the inoculum was decreased to that normally used for toxin production. Thus, after 2-2.5 hr. of incubation, the toxin gave skin tests of 7-9:35-40. The toxin soon disappeared on further incubation, as the number of bacteria increased.

The toxin-destroying mechanism in organisms from cultures aged 7 hr. or more. It seemed likely that organisms found in the older cultures of *Bacillus anthracis* (N.P. and Sterne strains) could destroy their own toxin. Extracts of organisms from 2.5, 7 and 12 hr. cultures and the non-toxic filtrates from 7 and 12 hr. cultures were tested for their ability to destroy toxin when incubated with it (see Table 5). In contrast to toxigenic organisms from 2.5 hr. cultures, organisms from 7 and 12 hr. cultures contained a toxin-destroying mechanism which appeared to be essentially intracellular; at 7 hr. it was not present in the culture medium to any appreciable extent, but by 12 hr. it was clearly demonstrable, probably due to lysis of some organisms. This apparent change in the organisms between 2.5 and 7 hr. was supported by a difference in their infra-red spectra; these examinations were made by our colleague Dr K. Norris to whom we are indebted.

Table 5. *The toxin-destroying mechanism in organisms from late samples of cultures of Bacillus anthracis (strains N.P. and Sterne)*

Mixture	Skin lesions produced by the mixtures (0.4 ml.) after 2 hr. incubation at 37°	
	Strain N.P.	Sterne strains
Sterile toxin* (1 vol.) mixed with 1 vol of:		
Saline	7:33	7:32
Streptomycin solution (2000 u./ml.)	7:35	7:33
Extract† of organisms from 2.5 hr. culture‡	7:29	7:31
Non-toxic filtrate from 7 hr. culture	7:35	6:31
Extract of organisms from 7 hr. culture	Nil	Nil
Non toxic filtrate from 12 hr. culture	Nil	Nil
Extract of organisms from 12 hr. culture	Nil	Nil
(a) Soluble (filtered sterile)	Nil	Nil
(c) Insoluble	Nil	Nil

* Toxic filtrates from 3½–4 hr. cultures under conditions as in Table 1.

† Bacterial extracts were prepared by shaking in a Mickle shaker with ballotini beads for ½–¾ hr. No organisms could be cultured from the disintegrates but streptomycin (2000 units per ml.) was added as a precaution since the turbid whole extracts could not be filtered sterile. The concentration of organisms used was equal (on the basis of turbidity) to the concentration in a normal 7 hr. culture prepared under the conditions described in Table 1.

‡ Conditions of culture as described in Table 1.

Attempts to increase yields of toxin and prolong its life in cultures

Quasi-continuous culture. Cultures (24 ml.) of both the N.P. and Sterne strains of *Bacillus anthracis* were incubated for 4 hr. at 37° under the optimal conditions described in Table 1, i.e. until toxin production was near its peak. Half (12 ml.) of the culture was then removed for assay and the medium replenished over the next hour by the addition, at 10 min. intervals, of six 2 ml. portions of warm medium. After 5 hr. of incubation, half (12 ml.) the culture was again removed and the same mode of addition of fresh medium repeated. The whole process was continued until the 8th hr. of incubation. The bacterial count of all samples of both strains did not depart significantly from 10⁷ bacterial chains/ml. At 4, 5, 6, 7 and 8 hr. after the start of incubation the sizes of the skin lesions in the assay for toxin were 6:37, 6:36, 6:37, 6:44 and 6:42 for the N.P. culture and 7:42, 7:40, 7:41, 7:46 and 8:47 for the Sterne culture. A comparison of these results with those given in Table 1 shows that when fresh medium was continually added and the bacterial count retained approximately the same by occasional removal of culture, then the high content of toxin which normally disappeared quickly in these cultures was maintained.

Batch culture. Apart from the application of this quasi-continuous culture system, we were not able to maintain peak toxicity or significantly to increase toxin concentration in the standard type of culture where the bacterial population was continually increasing. Attempts in this direction are described below.

(1) *Added nutrients.* The following four mixtures of amino acids, purine/pyrimidines and vitamins produced no significant difference from the control culture when added either alone (at the concentrations given in brackets and at 1/10, 1/50 and 1/500 of these concentrations) or when mixed together.

A. Glycine (20 mM), DL-alanine (2 mM), DL-valine (0.4 mM), DL-leucine (1 mM), DL-isoleucine (1 mM), DL-proline (1 mM), hydroxyproline (1 mM) DL-serine (2 mM), DL-threonine (0.4 mM), DL-norleucine (1 mM).

B. L-cystine (0.4 mM), DL-methionine (0.4 mM), DL-aspartic acid (1 mM), DL-glutamic acid (2 mM), DL-lysine (1 mM), L-arginine (1 mM), L-histidine (0.4 mM), DL-phenylalanine (1 mM), DL-tyrosine (1 mM), DL-tryptophane (0.2 mM).

C. Thiamin (2 μ M), riboflavine (2 μ M), nicotinamide (10 μ M), Ca pantothenate (2 μ M), pyridoxine (4 μ M) choline chloride (40 μ M), inositol (200 μ M), biotin (2 μ M) pimelic acid (200 μ M), folic acid (1 μ M) *p*-aminobenzoic acid (10 μ M).

D. Adenine (100 μ M), guanine (100 μ M), xanthine (100 μ M), hypoxanthine (100 μ M), cytosine (100 μ M), thymine (100 μ M), uracil (100 μ M), glutamine (1000 μ M), haemin (80 μ M).

The work of Tempest & Smith (1957), on the metabolic background to the production of the anthrax toxin *in vivo*, suggested that addition of coenzyme I (0.0025 %), coenzyme II (0.0025 %), uracil (0.1 %), or nicotinamide (0.1 %) might increase toxin production. The addition of nicotinamide and uracil did slightly increase toxin production but this increase was hardly significant.

(2) *Constant pH value.* The anthrax toxin is sensitive to changes in pH value (Smith, *et al.* 1955). The pH value of cultures did not fall outside the range 7.4–7.8 during 8 hr., and no change in the toxin production and destruction pattern was obtained by adding sodium phosphate to maintain a constant pH value. The addition of sodium bicarbonate (0.5 and 0.1 %) retarded the growth rate somewhat so that toxin was maintained for a slightly longer period.

(3) *Oxidizing and reducing substances.* Mr J. L. Stanley and Dr H. Smith (personal communication) have found that Factor I of the anthrax toxin is sensitive to oxidation and reduction. Addition of cystine (0.1 and 0.01 %), cysteine (1, 0.1 and 0.01 %) and KIO₃ (0.1 %) with a view to counteracting any oxidizing or reducing action of the organism, produced no significant change from normal. Anaerobic culture resulted in some toxin production of the same pattern as that in normal culture, but possibly slower.

(4) *Miscellaneous.* Addition of egg-yolk broth (1/5 vol.), arachis oil containing cholesterol (1/5 vol.), soya-bean meal containing trypsin inhibitor (0.5 %), and charcoal (0.1 %) produced no significant change from the normal pattern.

The influence on toxin production of high molecular weight components of serum

The experiments described above gave no clue as to the function of serum and tryptic meat digest medium in producing the right nutritional conditions for toxin production. The effectiveness of the added mixtures of smaller

molecular nutrilites suggested that it was the larger molecular components of serum which were important in toxin production. The following experiments support this view and also indicate the part played by tryptic meat broth.

When serum alone was used in the standard procedure, toxin production and disappearance was delayed but the maximal content was not significantly lower than when the normal mixture of serum and 10 % tryptic meat digest was used; the function of tryptic meat digest seemed to be to accelerate growth (see top of Table 6). Tryptic meat digest in the standard medium can be replaced by its ultrafiltrate without affecting toxin production or growth (see Table 6), showing that it is the small molecular components which are important. On the other hand, toxin was not produced on a medium of tryptic meat digest or its ultrafiltrate + the ultrafiltrate of serum, although normal growth of the organism occurred. Hence the large molecular weight components of serum appeared to be most important in toxin production.

Production of small amounts of toxin in tryptic meat broth; the influence of serum and other material of high molecular weight

Only the Sterne strain of *Bacillus anthracis* regularly produced a small amount of toxin in tryptic meat digest (see Table 6). The N.P. strain produced a similar quantity only in certain batches of tryptic meat digest. A significant increase in toxin production was brought about by the addition of serum (20 %, v/v; see Table 6), but the amount of toxin was not equal to that in the normal mixture of serum and 10 % (v/v) tryptic meat digest even when the proportion of serum was increased to 50 %.

The nature of the high molecular weight components in serum which may be of importance is not clear. Addition of a sample of albumin which had not been specially purified, significantly increased toxin production but a pure sample of albumin and a sample of γ -globulin did not. The impure sample of albumin was brown and its absorption at 410 m μ indicated the presence of haemoglobin. The addition of guinea-pig haemoglobin (not specially purified) increased toxin production but no more than the impure sample of albumin or serum. A fivefold decrease of the haemoglobin concentration did not significantly change the effect. Horse and bovine haemoglobin acted similarly, but haemoglobin could not be replaced by haematin or iron salts.

At no time in this work with media composed mainly of tryptic meat digest, did the concentration of toxin reach that obtained during growth in the conventional mixture of serum with 10 % (v/v) of tryptic meat broth. The rather low concentrations of toxin obtained were however maintained in the presence of far higher bacterial populations than in the conventional mixture (see Table 6).

DISCUSSION

The toxin responsible for death from anthrax has now been produced *in vitro* from a virulent strain and from an attenuated immunogenic strain of *Bacillus anthracis* growing in a mixture of serum + tryptic meat digest. The toxin appears identical with that found *in vivo*, and cultures of the Sterne strain have a

Table 6. *Toxin production in mixtures of tryptic meat broth with guinea pig serum and other materials of high molecular weight*

Medium		Skin tests for toxin and turbidity* measurements of bacterial growth at 37° by EEL Spectrophotometer (in brackets)											
Tryptic meat digest % v/v.	Other constituent of mixture	N.P. Strain						Sterne strain					
		Age of culture (hr.)						Age of culture (hr.)					
		3	4	5	6	7	8	3	4	5	6	7	8
10%	Serum	7:35 (3)	7:35 (5)	tr (7)	—	Nil (22)	—	6:35 (3)	7:40 (6)	6:30 (7)	tr (11)	Nil (19)	—
Nil	Serum	5:20	5:25 (1)	7:32 (2)	6:30 (2)	6:31 (3)	—	tr (4)	6:32 (1)	8:35 (1-5)	6:29 (2)	tr. (7)	—
10% ultrafiltrate	Serum	7:30 (4-5)	6:36 (5)	5:23 (7)	Nil (12)	—	—	6:37 (5)	7:44 (5)	7:35 (5)	tr. (9)	—	—
10% ultrafiltrate	Serum ultrafiltrate	Nil (3)	Nil (7)	Nil (13)	Nil (12)	—	—	—	Nil (7)	Nil (16)	Nil (14)	—	—
80%	Serum ultrafiltrate	Nil (2)	Nil (2)	Nil (5)	Nil (8)	—	—	—	Nil (6)	Nil (10)	Nil (11)	—	—
80%	Saline	Nil (13)	Nil (14)	tr. (19)*	tr. (20)	Nil (26)	Nil (30)	tr. (12)	4:18 (15)	4:20 (20)	3:14 (28)	3:13 (29)	tr. (33)
80%	Serum	5:18 (15)	5:23 (17)	5:26 (20)	6:28 (21)	6:25 (28)	5:24 (31)	tr.	5:17 (7)	5:26 (9)	5:29 (10)	5:25 (18)	5:23 (21)
80%	Albumin I (impure) 10% (w/v) in saline	tr. (15)	5:20 (16)	5:24 (18)	6:25 (21)	5:26 (26)	4:18 (31)	tr. (12)	5:22 (14)	5:26 (14)	5:28 (17)	5:26 (18)	tr. (18)
80%	Albumin II (pure) 10% (w/v) in saline	—	Nil (13)	Nil (20)	Nil (23)	Nil (29)	Nil (33)	—	4:15	4:18 (20)	tr.	Nil (20)	Nil (27)
80%	γ -Globulin 10% (w/v) in saline	—	Nil (12)	Nil (20)	Nil (18)	tr. (23)	tr. (29)	—	—	tr. (20)	tr.	4:22 (24)	tr. (28)
80%	Haemoglobin† 1.25% (w/v) in saline	—	tr. (10)	4:18 (24)	5:21 (28)	4:18 (35)	tr. (42)	4:23 (11)	5:22 (14)	5:24 (19)	3:16 (21)	3:21 (31)	tr. (36)

Results are the average of 3-7 experiments. A representative selection of these reactions were specifically neutralized by anthrax antiserum.

Conditions of culture see Table 1; inoculum of recently germinated spores: 6×10^7 N.P., 3×10^7 Sterne.

* Occasional batches of tryptic meat broth produced culture filtrates with a significant skin reaction.

† Guinea-pig haemoglobin. A reduction of 1/10 in its concentration did not significantly alter the pattern of toxin production.

maximum toxin content approximately equal to that produced *in vivo*. The toxicity of cultures of the virulent strain is $\frac{1}{2}$ – $\frac{1}{4}$ that of toxic plasma.

The early appearance and rapid disappearance of the toxin in artificial culture probably explains the failure of earlier workers to demonstrate it. In culture, *Bacillus anthracis* organisms change rapidly from toxin producers to toxin destroyers as the result of the production of an intracellular toxin-destroying enzyme system. This change occurs when the bacterial population increases beyond a critical concentration. The appearance of this enzyme was prevented by continuous culture in a mixture of serum + tryptic meat digest where the population and nutritional conditions remained more or less constant; under these conditions, the maximum toxin content of the culture was maintained. Hence, the concentration of toxin does appear to cause the change of organisms from toxin producers to destroyers; the change may be due to a nutritional deficiency. Continuous culture is not unlike the conditions which exist *in vivo*, where any depletion of plasma constituents would be replaced by compensatory mechanisms. One important difference however, is that the bacterial population *in vivo* increases to $c. 1 \times 10^9$ chains/ml. blood, at which concentration complete destruction of the toxin would have occurred *in vitro*. The compensatory mechanisms of the host appears to be able to supply the correct nutritional environment for a toxigenic bacterial population in excess of that supported by normal serum in artificial culture.

We have been unable to identify in detail the nutritional requirements for toxin production and maintenance. The addition of many nutritives and the use of various stabilizing procedures have not increased the maximal yield of toxin or prevented its destruction. The large molecular components of serum play an important part in toxin production, and it is interesting to speculate whether these components protect the toxin against the action of the toxin-destroying system by acting as competitive enzyme substrates, or whether they provide essential large molecular intermediates for toxin production.

The formation of toxin by the avirulent uncapsulated Sterne strain of *Bacillus anthracis* fits in with our views on the virulence of this organism and on immunity to anthrax. Virulence is due to at least two factors: the toxin and a capsule which contains polyglutamic acid (Smith, Zwartouw & Harris-Smith, 1956). Both factors must be present for full virulence; they both act as aggressins and later in the disease the accumulation of toxin proves fatal to the host. The toxin is the complete natural antigen for stimulating active immunity to anthrax. The Sterne organism lacks a capsule and is therefore almost avirulent, but it is a well-known immunogenic strain, either as a spore vaccine or in the form of a filtrate of cultures grown under special conditions (Belton & Strange, 1954). This strain should therefore produce the toxin in suitable cultures, and this has now been observed. On the other hand, the HM strain is heavily capsulated but since it lacks the capacity to form significant quantities of toxin it is an attenuated strain and non-immunogenic.

Now that the *in vitro* production of the anthrax toxin in serum has been achieved, it should be possible to succeed also with simpler media. The formation of a trace of toxin in tryptic meat digest, which can be increased by the

addition of impure serum albumin or haemoglobin is encouraging in this respect. Furthermore, a product closely connected with the anthrax toxin has already been prepared in a chemically defined medium (Wright, Hedberg & Slein, 1954; Belton & Strange, 1954). It is a non-toxic protective antigen, which on injection leads to the production of antitoxin capable of neutralizing the anthrax toxin from *in vivo* (Smith, *et al.* 1955) or *in vitro* (see above) sources; it also forms common precipitin lines with the toxin in serological diffusion plate analysis (Mr J. L. Stanley and Dr H. Smith, personal communication) and will, to some extent, replace factors I and II in the tests for oedema production and lethality (Smith *et al.* 1956).

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REFERENCES

- BELTON, F. C. & STRANGE, R. E. (1954). Studies on a protective antigen produced *in vitro* from *Bacillus anthracis*; medium and methods of production. *Brit. J. exp. Path.* **35**, 144.
- EURICH, F. W. & HEWLETT, R. T. (1930). In *A System of Bacteriology*, Vol. 5, chap. 10. (Medical Research Council.) London: H.M.S.O.
- KEPPIE, J., SMITH, H. & HARRIS-SMITH, P. W. (1955). The chemical basis of the virulence of *Bacillus anthracis*. III. The role of the terminal bacteraemia in death of guinea pigs from anthrax. *Brit. J. exp. Path.* **36**, 315.
- KING, H. K. & STEIN, J. H. (1950). The non-toxicity of *Bacillus anthracis* cell material. *J. gen. Microbiol.* **4**, 48.
- SMITH, H. & GALLOP, R. C. (1956). The chemical basis of the virulence of *Bacillus anthracis*. VI. An extracellular immunising aggressin isolated from exudates of infected guinea pigs. *Brit. J. exp. Path.* **37**, 144.
- SMITH, H., KEPPIE, J. & STANLEY, J. L. (1955). The chemical basis of the virulence of *Bacillus anthracis*. V. The specific toxin produced by *B. anthracis in vivo*. *Brit. J. exp. Path.* **36**, 460.
- SMITH, H., TEMPEST, D. W., STANLEY, J. L., HARRIS-SMITH, P. W. & GALLOP, R. C. (1956). The chemical basis of the virulence of *Bacillus anthracis*. VII. Two components of the anthrax toxin: their relationship to known immunising antigens. *Brit. J. exp. Path.* **37**, 268.
- SMITH, H., ZWARTOUW, H. T. & HARRIS-SMITH, P. W. (1956). The chemical basis of the virulence of *Bacillus anthracis*. VIII. Fractionation of the intracellular material of *B. anthracis*. *Brit. J. exp. Path.* **37**, 361.
- SOBERNHEIM, G. (1931). *Handbuch der Pathogenen Micro-organismen*, Vol. 3, pt 2, p. 1041. Jena: Gustav Fischer, Urban and Schwarzenburg.
- TEMPEST, D. W. & SMITH, H. (1957). Studies on the metabolism of *Bacillus anthracis* growing in the bloodstream of a guinea pig: The effect of metabolite analogues on growth and synthesis of virulence determining factors. *J. gen. Microbiol.* **17**, 739.
- WRIGHT, G. C., HEDBERG, M. A. & SLEIN, J. B. (1954). Studies on immunity in anthrax. III. Elaboration of protective antigen in a chemically defined, non protein medium. *J. Immunol.* **72**, 263.

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