PRODUCTION OF TOXIN *IN VITRO* BY *BACILLUS ANTHRACIS* AND ITS SEPARATION INTO TWO COMPONENTS

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After a detailed study of anthrax in laboratory animals Cromartie *et al.* (1947*a*) concluded that the histopathological changes observed could be best explained by the production of a diffusible poison and they showed that crude extracts of anthrax lesions produced characteristic edematous reactions when injected intracutaneously into rabbits (Cromartie *et al.*, 1947*b*). The presence of a specific lethal toxin which was related immunologically to the protective antigen and which was presumably the same as the toxic material originally found in anthrax lesions was demonstrated in the plasma removed from guinea pigs immediately after death from anthrax (Smith *et al.*, 1955). The toxin consisted of two components which were separated by ultracentrifugation (Smith *et al.*, 1956). Initial attempts to produce toxin *in vitro* were unsuccessful but eventually toxin was produced in a medium containing a large amount of serum (Harris-Smith *et al.*, 1958).

In our laboratory we found that culture filtrates were toxic when *Bacillus anthracis* was grown under conditions for protective antigen production in the Casamino acids medium of Belton and Strange (1954) as modified by Thorne and Belton (1957) and supplemented with either 10 per cent (v/v) of normal horse serum or 1 per cent (w/v) of gelatin. Interest in the relationship between protective antigen and toxin was aroused when we found that the toxicity of culture filtrates, as evidenced by skin reactions in guinea pigs, showed some correlation with their protective antigen titers as determined by the agar diffusion assay method (Thorne and Belton, 1957). However, culture filtrates obtained after growth of *B. anthracis* in medium without added protein were completely nontoxic although the protective antigen titers were equivalent to those of toxic culture filtrates from medium containing serum or gelatin. It seemed probable that if the toxin consisted of two components and one of them was the protective antigen, then in the absence of serum or gelatin the other component was either (a) not produced, (b) destroyed as soon as it was formed, or (c) removed or inactivated during filtration of the growth medium. Possibilities (a) and (b) were eliminated by the finding that the addition of serum to culture fluid after growth and just before filtration resulted in a toxic filtrate. This report describes the isolation of a component of toxin from fritted glass filters used to sterilize supernatant fluid from cultures grown in the Casamino acids medium without added protein. The component was completely nontoxic by itself but it formed a toxic mixture with protective antigen.

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MATERIALS AND METHODS

Organisms. In the majority of experiments the avirulent strain of *B. anthracis* isolated by Sterne (1937) was used but several virulent strains including M-36, 994, and V1B were also tested for toxin production.

Medium and cultural conditions. The Casamino acids medium, which has been used previously for the production of anthrax protective antigen, was prepared as described by Thorne and Belton (1957). Phosphate was included and the concentration of glucose was increased to 0.2 per cent. In early experiments 0.6 per cent $NaHCO_3$ was used but increasing the concentration to 0.8 per cent resulted in higher yields of toxin. Glucose and $NaHCO_3$ were sterilized by filtration and added aseptically. When used, serum was also added aseptically to the sterilized medium. When gelatin was used it was sterilized in the medium by autoclaving at 121 C (15 lb) for 20 min.

Usually Fernbach flasks (3-L) containing 500 ml of medium were inoculated with 10^6 spores and incubated statically for 23 to 27 hr at 37 C. In some experiments 250-ml Erlenmeyer flasks

containing 50 ml of medium were inoculated with 10^7 spores and incubated statically 16 to 18 hr. After incubation the cultures were centrifuged at 0 C and the supernatant liquid was decanted and sterilized by filtration through fritted glass filters.

Assay of toxin. The ability to produce a skin reaction in guinea pigs (Smith *et al.*, 1955) was used routinely to determine the toxicity of samples. In the procedure followed, 0.2 ml of undiluted and serial 2-fold dilutions of the sample were injected into the shaven side of a guinea pig (4 injections per side) and the animals were observed after 16 to 20 hr. The highest dilution of sample which produced an edematous lesion was taken as the end point and toxin titers are expressed as dilution units, i. e., a sample which produced a lesion at a dilution of 1:8 but not at a dilution of 1:16 contained 8 units of toxin per ml. The lethality of toxin preparations was determined by injecting 0.5 ml of an appropriately diluted sample into the tail vein of a mouse weighing 14 to 18 g. The mice were observed for 7 days after injection. Dilutions for assay by either method were routinely made in 0.02 M phosphate buffer, pH 8.0, containing 0.5 per cent (w/v) of gelatin.

Preparation of antisera. Hyperimmune antiserum, referred to as spore antiserum, was prepared in a horse by injecting spores of the Sterne strain. The antiserum against purified protective antigen, referred to as protective antigen antiserum, was that prepared in a rabbit by Strange and Thorne (1958).

Assay of protective antigen. The agar diffusion method was used as described by Thorne and Belton (1957). Further evidence for the specificity of this method has recently been obtained (Strange and Thorne, 1958). Titers are expressed as arbitrary units; a solution of antigen which just gave a visible precipitation line with standard spore antiserum in an Ouchterlony plate contained 1 unit per ml.

RESULTS

Toxin production in serum or gelatin medium. Protective antigen, 8 to 32 units per ml, but no toxin was detected in filtrates of the Sterne strain grown in the Casamino acids medium. Filtrates from cultures grown with 10 per cent (v/v) of serum in the medium contained 32 to 128 units of toxin and 16 to 32 units of protective antigen per ml and were lethal when injected into mice (table 1). Similar results were obtained with several virulent strains including M-36, 994, and V1B. When 2 per cent (w/v) of gelatin was substituted for serum in the medium, culture filtrates contained 16 to 32 units of toxin and 8 to 16 units of protective antigen per ml. Lower concentrations of gelatin resulted in lower yields of toxin.

TABLE 1

*Effect of horse serum or gelatin in the medium on toxicity of filtrates**

Addition to Casamino Acids Medium	Protective Antigen	Toxin		
		Guinea pig skin test	Mouse lethality test†	
			Ml of filtrate injected:	
			0.5	0.25
	units/ml	*units/ml*		
None	8	0	0/5	
Horse serum, 10% (v/v)	16	32	5/5	3/5
Gelatin, 2% (w/v)	8	32		
Gelatin, 1% (w/v)	8	16	3/10	
Gelatin, 0.5% (w/v)	8	2		

* The avirulent Sterne strain was grown in 50 ml volumes of medium containing 0.6 per cent $NaHCO_3$ with incubation for 16 hr. Similar results were obtained with several virulent strains including M-36, 994, and V1B.

† The values indicate the number of dead mice over the number of mice in the test.

Neutralization of toxin with antisera. The data in table 2 show that toxicity as measured by the guinea pig skin test and mouse lethality test was neutralized with spore antiserum as well as with antiserum prepared in a rabbit against purified protective antigen. The failure of the latter antiserum to inhibit the skin reaction completely in this experiment is a reflection of its antibody titer. As determined by the agar diffusion method of measuring antibody (Thorne and Belton, 1957) this antiserum had a lower titer against protective antigen than did the spore antiserum.

Fractionation of filtrates from medium containing gelatin. By treatment of filtrates from the gelatin medium with $(NH_4)_2SO_4$ it was possible to separate the toxin into two fractions. Neither fraction was significantly toxic when tested alone but when the two were combined the resulting mixture was toxic. One of the fractions contained

TABLE 2

*Neutralization of toxin by antisera**

Test Solution	Toxin Activity	
	Guinea pig skin test†	Mouse lethality test‡
Toxin +		
Normal horse serum	4/4	5/5
Spore antiserum	0/4	0/5
Protective antigen antiserum	1/4	0/5

* Three volumes of a solution of crude toxin having a potency of 256 units per ml in the skin test were mixed with one volume of serum or antiserum. The antisera are described under Materials and Methods.

† Values represent number of positive reactions over the number of tests.

‡ Values represent number of dead mice over the number of mice in the test.

protective antigen and could be replaced with partially purified protective antigen in forming toxic mixtures with the second fraction. The procedure for this fractionation is not given here since the filtration process presented below for separating the two components was much more satisfactory.

Effect of adding serum to culture supernatant fluid before filtration. Results of experiments designed to determine whether serum or other protein was essential for toxin production or whether it was functioning as a protective agent during filtration and subsequent handling of the filtrate are given in table 3. When the culture supernatant fluid was filtered directly through fritted glass filters, the filtrate was nontoxic when tested with or without the addition of serum. However, when 10 per cent (v/v) of serum was added before filtration filtrates were toxic. Although not shown in the table, when the concentration of serum was reduced to 1 per cent (v/v) the filtrate was nontoxic.

Since the amount of protective antigen passing through the filters was not affected by the addition of serum, these results indicate that in the absence of serum the other component of the toxin was adsorbed on the glass filter or was inactivated by the process. These results also demonstrate that serum or other protein is not necessary for toxin production.

Elution of active component from fritted glass filters. Preliminary attempts to elute active material from glass filters by means of solutions containing 10 per cent (v/v) of serum, surface active agents, or organic solvents were unsuccessful. However, active eluates were obtained when glass filters were treated with 0.1 M $NaHCO_3$-Na_2CO_3 buffer, pH 9.7. In initial small scale experiments 100 to 300 ml of culture medium, freed from most of the cells by centrifugation, were passed through a fritted glass filter (40 mm diameter, ultrafine fritted disc; Corning no. 33990, Corning Glass Works, Corning, New York). The filter was washed with 10 ml of 0.05 M phosphate buffer, pH 7.0, and eluted with 10

TABLE 3

*Effect of adding serum to culture supernatant fluid before filtration**

Expt No.	Treatment of Culture Fluid	Protective Antigen	Toxicity of Filtrate			
			Guinea pig skin test	Mouse lethality test† Ml of filtrate injected:		
				0.5	0.25	0.125
		units/ml	*units/ml*			
1	*a.* Filtered	16	0			
	b. 10% (v/v) of horse serum added after filtering	16	0			
	c. 10% (v/v) of horse serum added before filtering	16	128			
	d. Filtered (10% (v/v) of horse serum was added at time of inoculation)	16	128			
2	*a.* 10% (v/v) of horse serum added after filtering	16	0	0/5		
	b. 10% (v/v) of horse serum added before filtering	16	64	4/5	5/5	1/5

* The Sterne strain was grown for 26 hr in 3-L Fernbach flasks with 500 ml of medium containing 0.8 per cent $NaHCO_3$.

† Values indicate the number of dead mice over the number of mice in the test.

ml of 0.1 M $NaHCO_3$-Na_2CO_3 buffer. In later experiments 0.3 M buffer at pH 9.7 was used. The eluate and the filtrate were each nontoxic. However, when the eluate was mixed with either the filtrate or partially purified protective antigen, toxic mixtures resulted. The active material in the eluate is subsequently referred to as filter factor.

TABLE 4

*Preparation of filter factor**

Preparation No.	Description	Volume	Toxicity† (Guinea Pig Skin Test)	
		ml	*units/ml*	*total*
1a	Culture supernatant fluid	300	128	38,400
1b	Culture filtrate from 1a	300	0	0
1c	Eluate from glass filter used to obtain 1b	10	2560	25,600
2a	Culture supernatant fluid	8500	16	136,000
2b	Culture filtrate from 2a	8500	0	0
2c	Eluate from glass filter used to obtain 2b	110	1280	140,800
3a	Culture supernatant fluid	8500	64	544,000
3b	Culture filtrate from 3a	8500	0	0
3c	Eluate from glass filter used to obtain 3b	110	4800	528,000

* The Sterne strain was grown in 3-L Fernbach flasks with 500 ml medium. Cultures for preparations 1 and 3 were grown for 26 hr in the Casamino acids medium with 0.8 per cent $NaHCO_3$ and the culture for preparation 2 was grown for 23 hr in the same medium with 0.6 per cent $NaHCO_3$.

† To determine toxin units in culture supernatant fluid, 10 per cent (v/v) of horse serum was added to a sample which was then sterilized by filtration through fritted glass and assayed. The eluates (filter factor) were completely nontoxic when tested alone but formed toxic mixtures with protective antigen. For assay purposes they were diluted in culture filtrate which was diluted 1:2 in gelatin-phosphate to contain 8 units of protective antigen per ml.

TABLE 5

*Toxicity of filter factor when tested with purified protective antigen and with culture filtrate containing protective antigen**

Final Dilution of Filter Factor	Skin Test in Guinea Pigs		
	Filter factor + culture filtrate	Filter factor + purified protective antigen	Filter factor alone
1:100	+++	+++	−−−
1:200	+++	+++	−−−
1:400	+++	+++	
1:800	+++	+++	
1:1600	+++	+++	
1:3200	+++	+++	
1:6400	+±±	±±−	
1:12,800	−−−	−−−	

* The culture filtrate was that from which the filter factor used in this experiment was isolated. The final concentration of protective antigen used with all dilutions of filter factor was 8 units per ml (agar diffusion assay). Culture filtrate, purified antigen, and filter factor were each nontoxic when tested alone. Each dilution was tested in three guinea pigs. "+" indicates the formation of an edematous lesion, "−" indicates no reaction, and "±" indicates a questionable reaction.

For large scale preparation of filter factor, batches of 20 Fernbach flasks were used with the nonprotein Casamino acids medium as described above. The supernatant fluid from the Sterne strain was filtered through fritted glass filters of fine porosity and 600-ml capacity (Corning no. 36060). Two filters were used for each large batch. Upon completion of filtration each filter was washed with about 100 ml of 0.05 M phosphate buffer, pH 7.0. The active material was then eluted with 0.3 M $NaHCO_3$-Na_2CO_3 buffer, pH 9.7. This was done by adding about 40 ml of buffer to each filter, applying suction until the solution began to pass through the filter, and then releasing the vacuum and allowing the buffer to remain on the filter for about 30 min. Filtration was then completed by applying suction and each filter was finally washed with an additional 10 ml of buffer. Since the fine porosity filters used for this process did not remove all organisms, the eluate was finally sterilized by filtering through an ultrafine fritted glass filter. At pH 9.7 the active material was not adsorbed by the glass.

TABLE 6

Toxicity of filter factor plus protective antigen to mice and neutralization of toxicity with antiserum

Material Injected		No. of Dead Mice/ No. of Mice Injected
Protective antigen	Filter factor	
units	*units*	
0	32	0/5
16	0	0/5
16	32	5/5
16	16	5/5
16	8	1/5
16	4	0/5
8	32	2/5
4	32	3/5
2	32	1/5
16	32 + AS*	0/5

* Twenty per cent (v/v) of spore antiserum was added to the mixture before injection. Addition of the same amount of normal horse serum to samples before injection had no effect.

Examples of yields of filter factor are given in table 4. Apparently all the factor was adsorbed on the filters during filtration of the culture supernatant fluid and recovery of material in the eluate was good. The eluted material was nontoxic when tested alone and for assay purposes dilutions of it were mixed with a filtrate containing protective antigen to give a final concentration of 8 units of antigen per ml. Mixtures of the two components were rendered nontoxic to both guinea pigs and mice when spore antiserum was added.

Replacement of crude filtrates with purified protective antigen. Since filter factor was toxic only when mixed with protective antigen, crude filtrates obtained by filtering culture supernatant fluid through fritted glass filters to remove filter factor were used routinely as a source of protective antigen. To confirm that the protective antigen was the active component in culture filtrates, an experiment was carried out in which crude filtrate and purified protective antigen (Strange and Thorne, 1958) were compared as to their effectiveness in producing toxic mixtures with filter factor. The results are shown in table 5. Toxic mixtures were formed when filter factor was added to either filtrate or purified antigen and the titers obtained in the guinea pig skin test were the same for both combinations. These results provide conclusive evidence that the active component in culture filtrates was the protective antigen.

Toxicity of filter factor plus protective antigen to mice. The toxicity for mice of mixtures of filter factor and protective antigen is shown in table 6. The protective antigen was a partially purified preparation. Neither of the components alone killed mice but mixtures were lethal. Further experiments on the effect of various proportions of the two components in mixtures have not been done. Table 6 also shows that the toxicity of the combined factors was neutralized with spore antiserum.

Properties of filter factor. Filter factor solutions contained protein and on agar diffusion plates various preparations produced from 1 to 3 lines of precipitation with spore antiserum. When filter factor and protective antigen were placed in adjacent reservoirs on agar diffusion plates, the line formed by protective antigen crossed the lines formed by filter factor preparations. This is evidence that the antigens in filter factor preparations were different from the protective antigen.

The filter factor was nondialyzable and was inactivated by freeze-drying. Potencies of preparations were reduced upon storage at 4 C or −20 C. Results of stability tests at 37 C and 4 C indicated that the factor was most stable over a range of pH from 7 to 9, inactivation occurring more rapidly at pH values lower and higher than these.

DISCUSSION

The results of the present study are in agreement with the findings of Smith *et al.* (1956) with respect to the presence of two components in the toxin of *B. anthracis*. Harris-Smith *et al.* (1958) describe conditions for the elaboration of toxin *in vitro* and they report that a trace of toxin was produced in tryptic meat broth medium but that higher and more reproducible yields were obtained when 90 per cent (v/v) of serum was included in the medium. They suggested that the large molecular components of serum play an important part in toxin production. The present studies show unequivocally that serum is not required for toxin production *in vitro*, and failure to recognize this earlier is due to the fact that filtration of growth medium containing toxin to free it from bacteria results in the removal of

one of the toxin components. When serum or other protein is present, presumably the factor is preferentially adsorbed by it and thus passes through the filter. Elution of the factor from fritted glass filters can be achieved using alkaline buffers and the eluted material is toxic only in the presence of protective antigen.

Purification studies of filter factor are now in progress and we plan to investigate the nature of the combination or reaction occurring between it and protective antigen. Studies are also under way in this laboratory on the mechanism of action of the toxin.

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SUMMARY

Bacillus anthracis produced toxin in a Casamino acids medium without added serum or other protein. The toxin was separated into two components by filtering through fritted glass filters. One component, which appears to be the protective antigen, was present in the filtrate. The other component, referred to as filter factor, was adsorbed on the glass filter and could be eluted with alkaline buffer. Each of the factors was nontoxic when tested alone but when added together they formed a toxic mixture as evidenced by skin reactions in guinea pigs and lethality tests in mice. The toxin was neutralized by *B. anthracis* antiserum.

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