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TECHNICAL MANUSCRIPT 348

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CHARACTERIZATION OF ANTHRAX TOXIN

Donald C. Fish
Ralph E. Lincoln

MARCH 1967

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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CHARACTERIZATION OF ANTHRAX TOXIN

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Process Development Division
AGENT DEVELOPMENT AND ENGINEERING LABORATORY

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March 1967

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

BIOCHEMICAL AND BIOPHYSICAL CHARACTERIZATION OF ANTHRAX TOXIN

ABSTRACT

The literature on isolation and purification of anthrax toxin is discussed. The three major components of the toxin, edema factor, protective antigen, and lethal factor, were purified 197-, 156-, and 1025-fold, respectively. The final preparations were all serologically active. Edema factor remained biologically active and produced an edema when mixed with protective antigen; however, lethal factor was no longer lethal when injected in combination with protective antigen. Protective antigen remained immunogenic. Evidence is presented, with toxin produced both in vivo and in vitro, that the individual components and the whole toxin may exist in different aggregate or polymeric forms. Protective antigen and lethal factor were sensitive to hydrogen bond - disrupting reagents, but edema factor was resistant to these reagents. The molecular weight of protective antigen is 60,000 to 100,000. The influence of these data upon the course of future experimentation is discussed.

The toxin of Bacillus anthracis causes both immunizing responses against disease establishment and pathophysiological reactions leading to death. Unfortunately, the biochemical and biophysical characterization of the toxin has not kept pace with the other areas of anthrax research. We feel that this area is extremely important for (i) the study of the biological action of the toxin at the molecular level; (ii) the selection of superior immunizing antigens to be used either singly or in combination; (iii) to help provide some basis for selection of strains used for live vaccines or for controlled and variable virulence; (iv) a detailed study of the dynamics of toxin production both in vivo and in vitro; and (v) the enhancement of knowledge in the general fields of bacterial toxins, metabolic products, and pharmacodynamics.

This report covers the studies on purification of the toxin and its molecular configuration as it is known. We hope it will serve as a basis for discussion on the areas of investigation that will be most profitable.

The fact that sterile filtrates of edematous fluid or of macerated tissue containing the anthrax lesion caused an edematous response or some immunizing effect had been shown as early as 1904 by Bail¹ and was studied more extensively by Cromartie et al. in 1947.² In 1954 Smith and Keppie³ first demonstrated a toxin that was not cell-associated. This toxin, although present in all tissues, was most concentrated in edematous fluid and plasma. It was inactivated by antiserum prepared against avirulent spores, and essentially the identical toxin could be produced in vitro. We^{4,5} have since shown that toxin occurs in the blood of most animals dying of anthrax, although it is not demonstrable until very late in the course of the disease. Others⁶⁻⁸ have shown that essentially all strains of B. anthracis produce toxin, regardless of their relative virulence. However, a definitive quantitative and qualitative assay of the toxin from these different strains has not yet been undertaken.

The English workers first demonstrated that the coxin produced in vivo was composed of two components; later, from in vitro material, they identified a third component (Fig. 1). They called these components factors I, II, and III; the American workers have called the same factors edema factor (EF), protective antigen (PA), and lethal factor (LF), respectively. There is general agreement that (i) all three components are serologically distinct, (ii) EF and PA synergistically produce edema in guinea pigs and rabbits following subcutaneous injection, and (iii) LF and PA synergistically produce the lethal response.

American nomenclature

English nomenclature

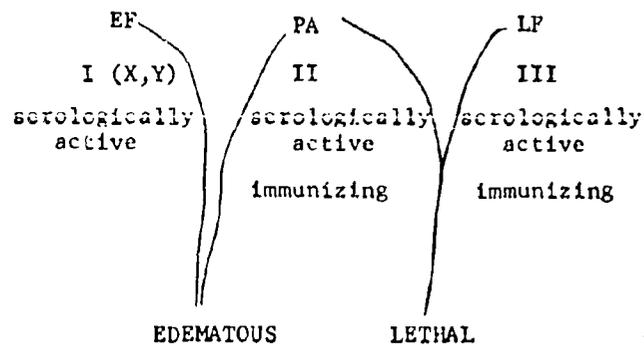


Figure 1. Activities Associated with Toxin Components.

The literature on anthrax toxin and antigen production is limited and confusing. The problem is that there has been no uniformity of strain used to produce toxin, and the methods of producing, processing, or assaying the products have been changed without quantitating the effect of these changes or relating them to a standard. Further confusion results in that there is evidence or suggestion that the molecule occurs in a polymeric or aggregate state that affects activity and any other parameter measured.

Table 1 summarizes the problem of critical evaluation of the experimental data. All four groups have only agar gel diffusion and antigenicity (immunogenicity) in common.

TABLE 1. METHODS OF EVALUATION

Criteria of Evaluation	British (Porton)	Research Groups		
		American (Ft. Detrick)		
		Wright	Thorne	Fish
Bacterial strain				
Sterne	+	-	+	+
Others	-	+	+	-
Purity				
Ultracentrifuge	+	+	+	-
Paper electrophoresis	+	-	+	-
Agar gel diffusion	+	+	+	+
Biological properties				
Guinea pig edema	+	-	+	+
Mouse lethality	+	-	+	-
Rat lethality	-	-	-	+
Antigenic activity	+	+	+	+

The majority of work on the effect of anthrax toxin on the host has been done with whole toxin. Whole toxin is the culture medium after removal of the bacteria by centrifugation and sterilization by filtration through a sintered glass filter. Table 2 characterizes six separate lots of whole toxin. Note the high ratio of solids to protein. This whole toxin is lethal, produces edema, and is serologically active.

TABLE 2. CHARACTERISTICS OF WHOLE TOXIN

Lot	Protein, mg/ml	Dry Weight ^{a/} mg/ml	Rat Lethality, units/ml	Guinea Pig Edema Titer	Ouchterlony Titer	
					LF	PA
1	1.24	15.8	32	1:50	1:8	1:32
2	1.46	17.4	36	1:8	1:8	1:64
3	1.07	13.6	37	1:16	1:8	1:16
4	1.36	12.9	21	1:32	1:8	1:32
5	1.41	14.0	28	1:4	1:16	1:64
6	1.33	13.0	22	-	1:8	1:32

a. The culture medium contained 7.5 mg/ml dry weight initially which included 2.2 mg/ml of glucose, 3.5 mg/ml of casamino acids, and 1.6 mg/ml of potassium phosphate.

Three of the four research groups used sintered glass filters to separate the three components. These results are shown in Table 3. Although there is some variability among the individual values, the following conclusions appear warranted: (i) EF in combination with PA is the component responsible for guinea pig edema; (ii) LF in combination with PA is the component responsible for rat lethality; (iii) none of the components is biologically active individually; and (iv) all three components are antigenically distinct.

From this point on, all four groups of investigators used different methods and assays for further purification. Purification is defined as the increase in specific activity, determined by dividing the Ouchterlony titer by the protein concentration.

Table 4 summarizes the results of studies on purification of edema factor. When guinea pig plasma was ultracentrifuged, the washed deposit was identified as EF. Stanley et al.⁹ purified this component about 50-fold with a 20% recovery by ammonium sulfate precipitation, DEAE-cellulose chromatography, and ultracentrifugation. This EF produced in vivo was still contaminated with 16% normal guinea pig plasma components. Stanley and Smith¹⁰ purified EF produced in vitro with two DEAE-cellulose columns and obtained 25% recovery of a material that was 25-fold purified, was still lethal for mice, and was contaminated by at least two other antigens. In these two instances, the assay method used was rabbit skin edema rather than Ouchterlony titer. On the basis of observations of Beall, Taylor, and Thorne¹¹ that EF is selectively adsorbed to sintered glass filters, we¹² have purified this component 197-fold with 38% recovery by DEAE-cellulose chromatography followed by dialysis.

TABLE 3. CHARACTERISTICS OF TOXIN COMPONENTS

Component	Protein, mg/ml	Dry Weight, mg/ml	Ouchterlony Titer	Guinea Pig Edema Titer	Rat Lethality, units/ml
EF	0.393	16.7 ^a / ₁	1:2	1:40	0
	0.039	12.1	1:1	1:10	0
	0.273	-	1:64	1:256	25 ^b / ₁
	0.276	-	1:16	1:4096	0
	0.094	-	1:16	1:20	114
	0.026	-	1:8	-	0
PA	0.750	10.8 ^c / ₁	1:128	0	0
	0.985	12.4	1:32	0	0
	1.220	13.3	1:32	0	0
	1.263	13.8	1:16	0	0
	1.225	-	1:16	0	0
	0.558	-	1:32	0	0
	1.155	-	1:128	0	0
	1.640	-	1:32	0	0
LF	0.157	15.2	1:32	0	70
	1.090	28.7	1:8	0	7
	0.620	29.9	1:16	0	94
	0.422	29.5	1:16	0	142
	0.134	-	1:8	0	41
	0.445	-	1:32	0	60
	-	-	1:64	0	52
	-	-	1:256	0	138
-	-	1:16	0	156	

- a. Dry weight of the carbonate eluting buffer was 14.3 mg/ml in some experiments and 28.5 mg/ml in others.
- b. Shown to be contaminated with LF by Ouchterlony analysis.
- c. Dry weight of the initial medium was 7.5 mg/ml.

TABLE 4. PURIFICATION OF EDEMA FACTOR

Investigator	Method	Recovery, %	Purification, -fold
In vivo			
Stanley et al.	ammonium sulfate, DEAE-cellulose ultracentrifugation	20	50
In vitro			
Stanley and Smith	DEAE-cellulose	25	25
Fish	sintered glass filtration, DEAE-cellulose dialysis	38	197

The PA component (Table 5), because of American and Russian interest in it as an immunizing antigen, has been studied more than the other components. PA was the component Smith et al.¹³ found in the supernatant of terminal guinea pig serum after ultracentrifugation. Stanley⁹ obtained up to a 20-fold purification by passage through a DEAE-cellulose column, followed by dialysis and drying. Other procedures used have been (i) alum precipitation,¹⁴ (ii) trichloroacetic acid - citric acid precipitation,¹⁵ (iii) ammonium sulfate precipitation,¹⁶ (iv) sintered glass filtration,⁸ and (v) ethanol precipitation.¹⁷ All of the above preparations have been shown to be heterogeneous by Ouchterlony assay and/or contaminated with the other components.

Strange and Thorne¹⁸ purified PA by ammonium sulfate precipitation, followed by precipitation at pH 5.0 and chromatography on alumina C gamma gel. They recovered 25% of the activity but not enough information was given to calculate the purification. We¹² have reported purification of 156-fold with 78% recovery by ammonium sulfate precipitation followed by column chromatography on polyacrylamide gel. This material is not contaminated with extraneous antigens and has retained its serological and immunogenic properties. In some preliminary experiments we used a Diaflo pressure cell* and obtained results similar to those obtained with ammonium sulfate. The method is much easier, faster, and more economical for large quantities of material (i.e., vaccine production) than any other method studied so far.

* Amicon Corp., Cambridge, Mass.

TABLE 5. PURIFICATION OF PROTECTIVE ANTIGEN

Investigator	Method	Recovery, %	Purification, -fold
In vivo Stanley et al.	DEAE-cellulose dialysis, lyophilization	20	20
In vitro Strange and Thorne	ammonium sulfate precipi- tation, pH 5.0 precipitation, alumina C gamma gel column	25	-
Fish	sintered glass filtration, ammonium sulfate precipi- tation, polyacrylamide gel column	78	156

LF (Table 6) was identified by Smith and Stanley in 1962¹⁰ and purified 3.5-fold with 24% recovery by DEAE and hydroxyapatite chromatography. Again, following the observations by Beall et al.¹¹ of selective adsorption on sintered glass, we were able to purify this component 1,025-fold with 11% recovery by chromatography on Sephadex G-25 followed by adsorption and elution from calcium phosphate gel. The resulting product showed only one line when tested by double diffusion in agar.

TABLE 6. PURIFICATION OF LETHAL FACTOR

Investigator	Purification Process	Recovery, %	Purification, -fold
Smith and Stanley	sintered glass filtration, DEAE-cellulose hydroxyapatite column, ammonium sulfate precipi- tation	24	3.5
Fish	sintered glass filtration, Sephadex G-25 column calcium phosphate gel adsorption	11	1025

During the course of our studies on purification it appeared that the toxin molecule could exist in several different forms. What was not clear, though, was whether this represented aggregation or polymerization.

Smith and Gallop⁹ found evidence for compounds X and Y in EF precipitated with ethanol and NaCl. Y was postulated to be a converted form of EF, losing its tissue-damaging and virulence-enhancing activity. Later, Stanley, Sargeant, and Smith⁹ reported that EF had a tendency to aggregate and lose activity; they noted that on ultracentrifugation the peaks depended upon the freshness of the original material and could be altered by very mild treatment. The high-molecular-weight material could be increased at the expense of the main peak. In 1961, Stanley and Smith¹⁰ called attention to the remarkable chelating action of EF on most metals and speculated on the possibility that the three components might at one time have been joined in a loose complex. We¹² showed (Table 7) that after a 4-day storage at -20 C, the number of lines (Ouchterlony) increased without measurable decrease in final titer, indicating dissociation rather than destruction. The biological activity, death of rats, and edema in guinea pig skin were markedly less after freezing and storage.

The observation of additional lines of precipitation from PA (Ouchterlony) by Strange and Thorne¹⁸ was interpreted to indicate degradation of this component by a proteolytic enzyme present in the medium. The possibility that this heterogeneity is dissociation of a complex, rather than destruction of a single antigen, cannot be discounted. Upon immunoelectrophoresis of their "highly purified protective antigen" Wright and Luksas²⁰ found three immunologically related components differing in electrophoretic mobility. They postulated degradation of the basic antigen. The amounts of each compound varied with the age of the culture. Smith and Stanley¹⁸ reported that their final preparation of LF, serologically distinct from EF and PA, contained two closely associated antigens that had different rates of migration upon agar gel diffusion.

We have found, during the course of purification of the toxin components, that often the lines formed by LF and by EF show partial identity while always being distinct from PA. The PA preparations sometimes yield two or three lines that form closer to the antigen well, indicating a greater molecular weight upon storage in concentrated aqueous solutions or in solutions containing a high concentration of ammonium sulfate. Upon chromatography of either PA or LF on Sephadex G-25 we have occasionally observed the presence of multiple peaks that were still antigenically distinct from the other two major components of toxin. These may represent polymers of different molecular weight.

TABLE 7. EFFECT OF FREEZING AND THAWING ON ANTHRAX TOXIN PRODUCED IN VIVO

Ouchterlony's 2 Lines 1 Line	Fresh Serum		Frozen and Thawed Serum					Rat, $\frac{c}{f}$ min.
	Guinea Pig ^b	Rat, $\frac{c}{f}$ min.	4 Lines	3 Lines	2 Lines	1 Line	Guinea Pig	
Lost	1:80	75	- ^d	1:2	1:16	1:16	NE ^e	1,110
-	>1:32	61	-	-	1:16	1:16	NE	75
-	1:16	50	-	-	-	1:32	NE	73
1:4	1:16	50	1:4	1:8	>1:32	-	NE	113
-	1:8	101	-	1:16	-	-	NE	Survived
-	>1:32	63	-	>1:32	-	-	NE	Not Done

- a. Highest titer at which indicated number of lines are visible.
- b. Highest titer that still gives visible edema.
- c. Average time to death of two rats following IV injection.
- d. No lines formed.
- e. No edema.

When the three purified components were incubated with urea and guanidine acetate (Table 8), PA was destroyed readily by both agents, LF was more slowly destroyed, and EF was stable. These observations indicate that the antigenic activity of PA and of LF is sensitive to hydrogen bond - disrupting reagents.

TABLE 8. EFFECT OF UREA AND GUANIDINE ACETATE ON ANTHRAX TOXIN COMPONENTS

Days at 4 C	Components ^{a/}	Titer by Ouchterlony Assay		
		H ₂ O	Guanidine 0.25 M	Urea 6 M
Day 0	EF	1:16	1:16	1:16
	PA	1:16	1:16	1:1
	LF	1:4	1:4	1:4
Day 17	EF	1:16	1:16	1:8
	PA	1:8	- ^{b/}	-
	LF	1:4	1:2	-

a. Component diluted 1:1 with reagent to indicated concentration.

b. No line of precipitation formed.

Cromartie et al.² associated FA activity with a component that migrated between the beta and gamma globulins on paper electrophoresis. The antigen of Bjor and Tresselt¹ was characterized as a gamma globulin - like protein by measurement of precipitation and electrophoretic limits. The possibility of serum components was not excluded. We found that all components of toxin are retained by a Diaflo membrane that retains molecules of molecular weight 10,000 or greater. PA has been shown to have a molecular weight in the order of 100,000 by chromatography on Sephadex G-75 (Fig. 2). The antigenic activity is eluted shortly after the void volume (elution of blue dextran). Because PA migrates through 9% agar, the molecular weight is probably no greater than 100,000. The PA produced in vitro by Strange and Belton¹⁶ had a sedimentation constant of 3, which indicates a molecular weight of 40,000 to 60,000. Preliminary work by Gruber and Wright,²² using the ultracentrifuge, indicated a molecular weight of 80,000 for PA. The molecular weight of LF appears to be of the same order as that of PA. The molecular weight determinations will no doubt have to be reevaluated after we more fully understand the molecular configuration of the toxin.

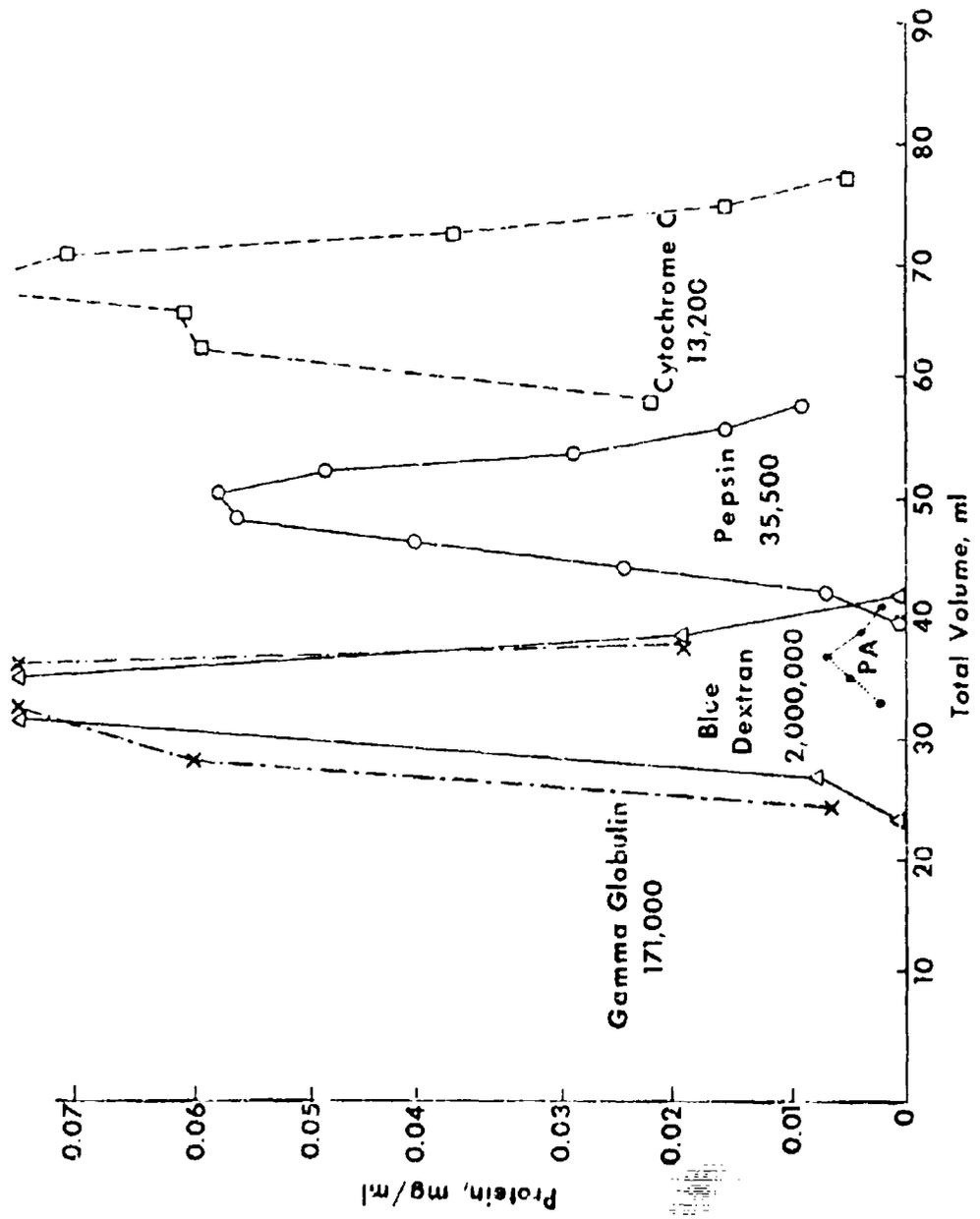


Figure 2. Composite Elution Diagram of PA and Proteins of Known Molecular Weight.

It is obvious that the work in this field has presented more questions than it has solved. With the exception of the work on the isolation and purification of the toxin components, all the results should be considered to be more of a preliminary nature than final and definitive statements.

Despite these drawbacks, we feel that certain advances have been made: (i) the three major components (EF, PA, and LF) have been partially purified and freed of all extraneous antigens for which tests were made; (ii) the molecular weights of PA and of LF have been approximated to be between 60,000 and 100,000; (iii) the work on chemical bonding of the separate components indicates that PA and LF depend upon hydrogen bonds for their molecular configuration, but EF is not dependent on hydrogen bonds; and (iv) the configuration necessary for biological activity of the components appears to be different from that needed for serological and antigenic activity.

The fact that dissociation occurs with toxin produced both in vivo and in vitro under favorable storage conditions makes it imperative that standard techniques for both field and laboratory work be established and closely followed.

In summary, then, we are now at the point where definite questions can be asked about the contribution of each toxin component in the over-all picture of the disease. We can prepare specific antibodies for each component that can be used in elucidating the action of each one, in treatment of the disease, and as a rational basis for strain selection and screening. We can also speculate as to what properties this toxin molecule should have that will enable it to penetrate the blood-brain barrier. If we can understand the molecular composition and spatial relationships of the toxin and its components, we will have come a long way toward understanding not only anthrax but many other diseases in which a toxin or some other extracellular product is involved in initiating the disease syndrome.

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*Anthrax	Molecular weight	Freezing
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