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TECHNICAL MANUSCRIPT 385

CHARACTERISTICS OF ANTHRAX TOXIN COMPONENTS PRODUCED IN VITRO

Donald C. Fish
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MARCH 1967

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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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TECHNICAL MANUSCRIPT 385

CHARACTERISTICS OF ANTHRAX TOXIN COMPONENTS PRODUCED IN VITRO

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AGENT DEVELOPMENT AND ENGINEERING LABORATORY

Project 1C522301A059 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.

ABSTRACT

Many of the biological and biophysical characteristics of the partially purified anthrax toxin components were measured. The components do not appear to be simple proteins. As the components are purified the pH range in which they are most stable narrows, centering between pH 7.4 and 7.8. Heat readily destroys the biological activity of the components but not their serological activity. All three components are serologically distinct. The rat lethality test showed that with a constant amount of lethal factor (LF) and an increasing amount of protective antigen (PA) the time to death reached a minimum and then was increased. When an increasing amount of LF was added to a constant amount of PA, the more LF that was added, the shorter the time to death. The biological, immunological, and serological properties of the components varied with storage and extent of purification so that serological activity was not always directly correlated with biological activity. Evidence is presented that the components could exist in different molecular configurations or as aggregates and that this property is influenced by the state of component purity and the environment.

I. INTRODUCTION

Little is known about the biological and biophysical characteristics of anthrax toxin or its components edema factor (EF), protective antigen (PA), and lethal factor (LF) aside from the studies of Stanley, Sargeant, and Smith[1] on the stability of crude preparation of in vivo - produced factors I and II and the work of Thorne, Molnar, and Strange[2] on pH and temperature sensitivity of "filter factor." The serological relationships among the various components were demonstrated by Sargeant, Stanley, and Smith.[3] This situation is understandable in that the major area of emphasis in studies of anthrax toxin has been on its use as an immunogen and on its mechanism of action in the host.

This paper presents (i) the results of our studies on pH, temperature, and chemical stability of the toxin; (ii) a discussion of the sensitivity and interactions observed in the various assay systems; and (iii) our interpretation of data on the molecular weight and molecular heterogeneity or aggregation of the molecule. We hope that these observations will stimulate further work on the characterization of this interesting and complex toxin.

II. MATERIALS AND METHODS

A. ANTHRAX TOXIN AND ITS COMPONENTS

The whole toxin and its components used for the experiments described in this paper were produced and purified by the procedure described by Fish et al.[4] Each time a particular component is mentioned, its state of purification as reported by Fish et al.[4] will be noted.

B. ASSAY CONDITIONS

The whole toxin and its components were quantitated by Ouchterlony assay,[5] rat lethal units,[6] or the guinea pig skin edema test.[2]

C. IMMUNOGENICITY OF THE COMPONENTS

Some of the samples of PA used for this experiment had been purified by methods additional to those reported by Fish et al.[4] The PA that had been isolated following ammonium sulfate precipitation and column

chromatography on polyacrylamide (P-2) gel was further treated with alumina C-gamma (Cγ) gel.* To 15 ml of the pooled fractions from the P-2 column in a 40-ml centrifuge tube were added 2.3 ml of alumina Cγ gel. The tube was maintained at 4 C with occasional stirring for 30 minutes and the gel was sedimented by centrifugation at 20,000 x g for 5 minutes. The supernatant fluid contained the PA activity, which was purified 128-fold. The recovery was 39% compared with original culture supernatant. A second treatment with alumina Cγ gel resulted in a product purified 8-fold and a recovery of 25%. The first and second supernatants were tested for their immunizing ability.

The immunity index (I) method of DeArmon et al.[7] was used to determine the degree of immunization. The guinea pigs (Hartley strain, 400 to 500 g) were immunized by five subcutaneous injections of the antigen on alternate days. They were then held for 7 days and challenged by intraperitoneal injection of 1.0 ml of 10^8 spores per ml of the virulent V1b strain of Bacillus anthracis.

D. MOLECULAR WEIGHT DETERMINATION BY COLUMN CHROMATOGRAPHY

Sephadex G-75** was equilibrated and washed with 0.15 M KCl to remove the fines. The material was then equilibrated at 4 C and a 3 by 82 cm column was prepared. The following reference samples were used: cytochrome C (A grade), gamma globulin (bovine, fraction II),*** pepsin (3 x cryst.),**** and blue dextran 2,000.** For the molecular weight determination, 1 ml of a 2 mg per ml sample of the standard was carefully layer top of the column and allowed to absorb into the gel. The col was then eluted with 0.15 M KCl, using a flow rate of 1.5 ml per ho Fractions of 3 ml were collected and the protein concentration was det ined by the 215/225 mμ method of Waddell.[8] When the molecular weight of PA was determined, the PA component was assayed by Ouchterlony analysis as the major peak of protein was eluted from the column after about 120 ml of fluid had passed through the column.

E. ANTISERA

Three lots of antisera were used for these experiments: Detrick horse (lot DH-1-6C), and Sclavo lots 76 and 76 AQ.***** All three were prepared by repeated injections of B. anthracis strain Sterne spores into horses.

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*** California Biochemicals Corp., Los Angeles, Calif.
**** Nutritional Biochemicals Corp., Cleveland, Ohio.
***** Instituto Seraterapico Tuscano, Siena, Italy.

III. RESULTS

A. ULTRAVIOLET ABSORPTION

The ultraviolet (UV) absorption spectra were determined for whole toxin and for EF, PA, and LF in various stages of purification. The UV absorption was measured on a Beckman Model DU spectrophotometer, using the appropriate diluent for a blank. All spectra showed a sharp decline in absorbance above 215 mμ. The samples reached a minimum at 245 mμ and a maximum at 270 mμ before starting to decline again. The ratio of absorbance at 280/260 mμ ranged from 0.87 to 1.03, which corresponds to a 3 to 5% contamination by nucleic acid according to the method of Christian and Warburg.[9]

B. STABILITY

The stability of the individual components was tested with respect to pH, temperature, and various chemicals. The time interval and degree of purification of the fractions were deliberately varied in an attempt to visualize trends in the data rather than absolute values for one set of conditions. Unless specifically mentioned, only serological assays were used.

1. pH Stability

Crude PA, following passage through the final ultrafine filter, was stable at 4 C in the presence of 0.025 M potassium phosphate buffer during the 30 days for which tests were run over a pH range of 6.5 to 8.3. PA, purified 156-fold, showed an extremely narrow pH tolerance in the presence of 0.4 M potassium phosphate buffer. It was stable as long as 30 days between pH 7.1 to 7.5, but stability decreased rapidly at pH below 6.8 or above 7.9.

LF, purified 35-fold, was stable as long as 111 days over a pH range of 6.5 to 9.5 in either 0.025 M or 0.4 M phosphate buffer.

EF, purified 35-fold, had a narrow range of pH stability. It was stable for 15 days between pH 7.3 and 7.8 but was destroyed in 3 days at pH outside this range.

2. Temperature Stability

Samples of whole toxin, 320 and 160 rat lethal units per ml, respectively, were maintained in a shaking water bath at 30 C. After 48 hours, PA activity as measured by the Ouchterlony titer had dropped from 1:512 to 1:128 and 1:64 in the two experiments, respectively. The LF titer did not decrease significantly under these conditions (1:64 to

1:32 and 1:32 to 1:16, respectively). Assays in rats showed that, although these preparations were still active serologically (Ouchterlony), they were no longer biologically active (lethal for rats).

When these experiments were repeated with preparations of 40 and 80 rat lethal units of toxin per ml, the serological activity did not decrease even after maintenance at 30 C for 120 hours. However, these preparations had lost their rat lethality.

Serological and biological activity were both destroyed by placing the sample in a boiling water bath for 10 minutes.

The toxin and its components usually were stable following freeze-drying and storage at -20 C provided that the vials were not allowed to thaw during freeze-drying or to remain on the dryer for extended periods after they were dry. Biological and serological activity was unimpaired by storage of aqueous samples at -20 C.

3. Chemical Stability

The results of studies on stability of PA in the presence of various chemical agents are shown in Table 1. The initial pH of the sample was 7.8 and the pH range of the various mixtures was 6.8 to 8.1. PA appeared to be relatively stable in the presence of all reagents tested except for the hydrogen bond - disrupting reagents urea and guanidine acetate and the surface-active agent sodium lauryl sulfate. Crude PA, following the final filtration step, was relatively stable both in oxidizing reagents (0.15% potassium ferricyanide and 0.05% potassium iodate) and in reducing reagents (0.1% cysteine).

The results of studies on the stability of LF in the presence of various chemical reagents are shown in Table 2. The initial pH of the sample was 9.7 and the various mixtures had a pH range of 8.7 to 9.5 LF was not particularly sensitive to either oxidizing or reducing reagents. It was more stable than PA in the presence of hydrogen bond - disrupting reagents. In contrast to PA, it was relatively stable in the presence of sodium lauryl sulfate.

Crude preparations of EF, purified 35-fold, were stable in the presence of 0.1% cysteine and 0.05% potassium iodate but unstable in the presence of 0.15% potassium ferricyanide. These experiments were all performed in the presence of 0.05 M potassium phosphate buffer, pH 7.5.

TABLE 1. STABILITY OF PA, PURIFIED 156-FOLD, TREATED WITH SELECTED CHEMICAL REAGENTS

	Highest Positive Dilution on Ouchterlony Analysis after Indicated Days of Storage			
	0	12	27	48
Control (H_2O)	32	16	8	8
Oxidizing Agents				
0.05% K Iodate[a]	16	16	8	4
0.05% K Ferricyanide	16	16	8	2
Reducing Agents				
0.05% Cysteine	16	16	16	0
0.05% Na Glutathione	32	8	8	2
Disrupting Reagents				
6 M Urea	0	0	0	0
0.25 M Guanidine Acetate	16	8	0	0
0.05% Na Lauryl Sulfate	32	8	0	0
Miscellaneous				
0.05% EDTA	32	8	8	8
0.05 M Tris HCl	32	16	8	8

a. Final concentration of reagent.

TABLE 2. STABILITY OF LF, PURIFIED 35-FOLD, TREATED WITH SELECTED CHEMICAL REAGENTS

	Highest Positive Dilution on Ouchterlony Analysis after Indicated Days of Storage			
	0	12	37	111
Control (H_2O)	32	16	8	8
Oxidizing Reagents				
0.05% K Iodate[a]	32	8	8	8
0.05% K Ferricyanide	32	8	4	4
Reducing Reagents				
0.05% Cysteine	32	8	8	8
0.05% Na Glutathione	32	8	8	8
Disrupting Reagents				
6 M Urea	8	0	0	0
0.25 M Guanidine Acetate	32	4	2	0
0.05% Na Lauryl Sulfate	32	8	8	8
Miscellaneous Reagents				
0.05% EDTA	32	16	8	4
0.05 M Tris HCl	32	8	8	8

a. Final concentration of reagent.

IV. CONDITIONS FOR ASSAY

A. OUCHTERLONY ASSAY

Using the Ouchterlony technique and staining with azocarmine, it was possible to detect 2 to 4 μg protein of the purified PA and LF and 170 μg protein of EF per well. We were unable to obtain the sensitivity reported by Strange and Thorne[10] for PA. Neither the position of the lines nor their formation was hindered in the presence of up to 0.3 M potassium phosphate buffer, pH 7.4.

A line of precipitation specific for EF could not be distinguished when whole toxin was diluted and subjected to Ouchterlony analysis. The EF apparently formed a line that was masked by the PA present. However, its presence was established because EF could be isolated from the whole toxin and the whole toxin produced an edema when tested in the guinea pig. When samples of whole toxin were serially diluted, or when PA was titrated in the presence of LF or vice versa, it became increasingly difficult to recognize two distinct lines of precipitation; rather, the two distinct lines tended to merge into one line. Consequently, Ouchterlony titration of the components required that a complete series of dilutions be done at each step and that known components be included as controls.

Three different equine antisera prepared against repeated injection of Sterne spores were compared for their efficacy in the Ouchterlony technique. All three antisera had the same activity with antigen preparations of EF, PA, LF, and whole toxin. The lines of precipitation were sharper and of equal titer, and the need for washing the plates with saline prior to staining was avoided by using a 1:8 dilution of the antiserum in 0.2% gelatin in saline.

B. ANTISERUM NEUTRALIZATION

The ability of antiserum to neutralize toxin in vitro was determined. An identical amount of antigen was added to twofold serial dilutions of the antiserum, the mixture was maintained for 15 minutes at room temperature, and then samples were placed in the outer row of wells. The inner wells contained a 1:8 dilution of the antiserum. The highest dilution of antiserum that prevented the formation of a line of precipitation indicated its neutralizing ability. Table 3 shows that the neutralization observed is concentration-dependent and that 1 ml of concentrated antiserum neutralizes 128 Ouchterlony units of toxin. The antiserum appears to be as active against LF as it is against PA.

TABLE 3. ANTISERUM NEUTRALIZATION OF TOXIN IN VITRO

Fraction	Ouchterlony Titer	Highest Dilution of Antiserum that Prevented Formation of a Line of Precipitation[a]
PA, after final filtration	1:16	1:8
LF, purified 35-fold	1:8	1:16
Whole Toxin		
300 rat units	1:128	1:1
40 rat units	1:16	1:8

a. Antiserum used for this experiment was Sclavo 76 AQ.

C. GUINEA PIG SKIN EDEMA ASSAY

Using the procedure of Thorne et al.,[2] 18 μg protein of EF, purified 35-fold, could be detected. The minimal amount of PA needed to produce edema with EF was not determined.

D. RAT LETHALITY

Haines et al.[6] standardized a procedure for quantitating whole toxin by measuring the time to death following intravenous injection in the penial vein of Fischer 344 rats (200 to 300 g). These workers were concerned with quantitating only the whole toxin and not the components. Therefore, when studying the individual, separated components, the influence of each component had to be evaluated. The phosphate buffer concentration in the medium was 0.01 M. In our studies, injection of 2.0 ml of 0.1 M (20-fold) potassium phosphate buffer, pH 7.4, killed the rats; injection of 1.5 ml (15-fold) caused the rat to undergo tetani, muscle spasm, and coma, but the rat recovered from this in 5 to 10 minutes with no visible aftereffects. The greatest concentration used by Haines et al.[6] was 16-fold and part of the response curve used in deriving the equation was below 8-fold; therefore the salt effect reported above could not have affected their results. In the present paper, the concentration of the components was adjusted so that none of the rats would die as a result of the buffer and salts present.

The results from experiments in which the amount of LF was kept constant and the amount of PA varied are presented in Table 4. They show that the amount of LF and PA needed for lethality was a function of both components. The results also show that the time to death reached a minimum and then, with the addition of more PA, there was actually some extension in the time to death. This observation parallels that of Molnar and Altenbern.[11] The minimal lethal amounts of PA and LF were 32:4 or 16:8. When the amount of PA was kept constant and the amount of LF varied, the more LF that was present, the shorter the time to death (Table 4).

TABLE 4. EFFECT ON RAT LETHALITY OF VARYING ONE COMPONENT IN THE PRESENCE OF A CONSTANT AMOUNT OF THE OTHER COMPONENT

LF Constant, PA Varied			PA Constant, LF Varied		
Units[a]		Time to Death, min[b]	Units[a]		Time to Death, min[b]
LF	PA		PA	LF	
0	64	S[c]	0	32	S[c]
2	128	S	2	4	S
2	64	S			
			4	4	S
4	128	255	4	8	S
4	64	177			
4	32	336	8	4	S
4	16	S	8	8	S
4	8	S	8	16	S
4	4	S			
4	2	S	16	4	S
			16	8	331
8	128	124	16	16	227
8	64	112			
8	32	141	32	4	336
8	16	331	32	8	141
8	8	S	32	16	118
8	4	S			
			64	0	S
16	128	93	64	2	S
16	64	91	64	4	177
16	32	118	64	8	112
16	16	227	64	16	91
16	8	S			
			128	2	S
32	0	S	128	4	255
			128	8	124
			128	16	93

a. Units, total amount injected, based on Ouchterlony titer: LF, purified 35-fold and PA, after final concentration.
b. The time to death reported is the average of two animals.
c. Survived.

E. IMMUNOGENICITY

The capability of our preparation of PA, in various stages of purification, to protect guinea pigs against challenge with a lethal dose of virulent spores was tested. The results (Table 5) show that PA in all stages of purification is a good immunogen. The more purified material appears to be an even better immunogen than the cruder material, as evidenced by an increased immunity index (3.5 to 5.0) with a decreased amount of protein (1.47 to 0.01 mg) or Ouchterlony units (20 to 8) injected.

TABLE 5. EFFICACY OF PA AS AN IMMUNOGEN FOR THE GUINEA PIG DURING DIFFERENT STAGES OF PURIFICATION

Fraction	Fold Purified[a]	Total Amount Injected			
		Volume, ml	Ouchterlony Units	Protein, mg	Immunity Index[b]
Filtrate	1	1.25	20	1.47	3.5
Filtrate	1	0.5	4	0.34	3.9
Filtrate	1	1.0	16	0.95	4.7
$(NH_4)_2SO_4$ ppt	67	2.5	320	0.37	5.9
Column Fractions	156	1.25	40	0.08	5.7
First CY Gel	128	0.5	16	0.02	5.4
Second CY Gel	8	1.0	8	0.01	5.0

a. Fold purification details given by Fish et al.[4]
b. Control value (nonimmunized guinea pig) is 0.0 based on MTD of 32 hours.

F. MOLECULAR WEIGHT AND MOLECULAR HETEROGENEITY

Allison and Humphrey[12] proposed a method for approximating the molecular weight of antigens based on their ability to migrate through various concentrations of agar. Figure 1 shows that both PA (following final filtration) and LF (purified 35-fold) migrated through 9% agar, indicating that their molecular weight was less than about 400,000.

Protective Antigen

Lethal Factor

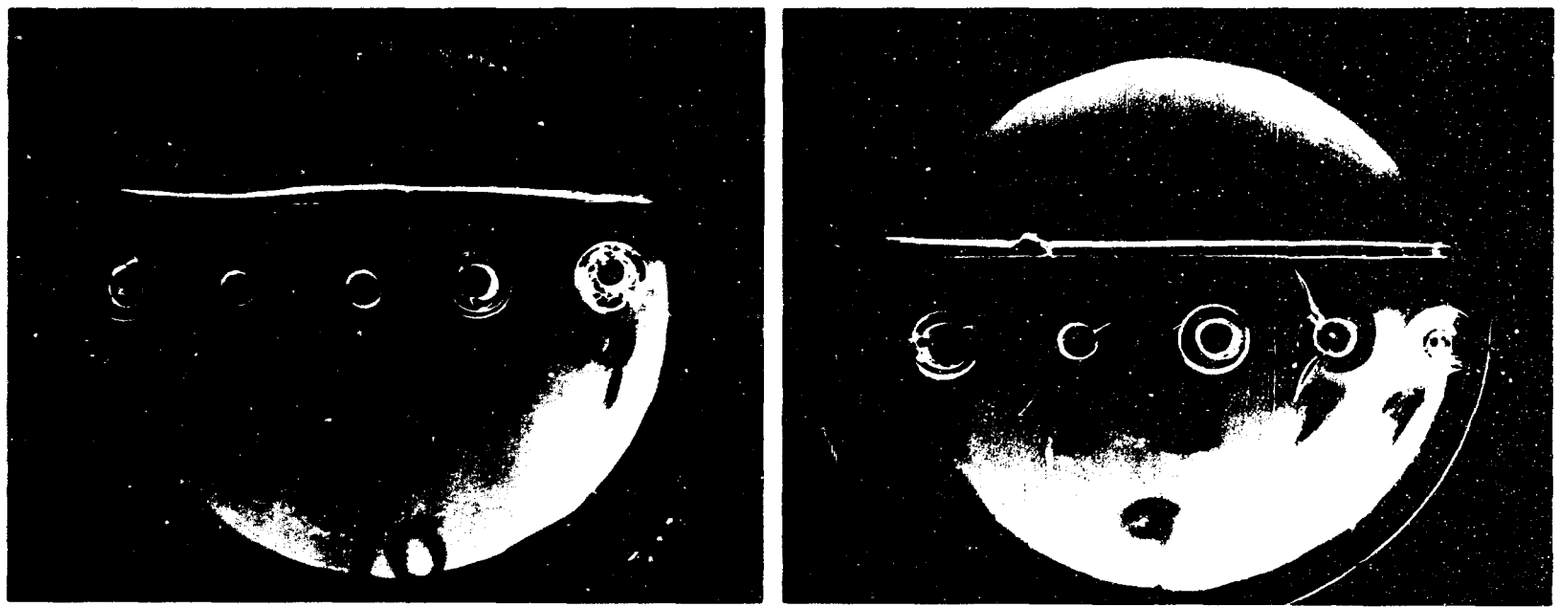

Figure 1. Migration of PA and LF Through 1 to 9% Agar.

The presence of multiple lines of precipitation in these preparations that showed only one line of precipitation under standard conditions of Ouchterlony analysis indicated the presence either of aggregates of various sizes of the individual components or of extraneous antigens that were not detectable under the previous assay conditions.

A comparison of the elution volume following column chromatography on Sephadex has yielded a reasonably reliable value for the molecular weight of many proteins.[13]

A composite elution diagram from a Sephadex G-75 column of several proteins whose molecular weight is known is shown in Figure 2. The PA preparation used for this determination was a sample that had been concentrated in the Diaflo ultrafiltration cell (purified 64-fold). The indicated molecular weight of PA was between 35,000 and 171,000 and appeared to be about 100,000.

During purification of PA and LF, when desalting on Sephadex G-25, both components were eluted near the void value of the column, indicating a molecular weight greater than 10,000.

During the purification of the toxin components, the lines of precipitation corresponding to LF and EF occasionally showed partial identity but always were distinct from that of PA. When PA preparations that had been concentrated by pervaporation or by ammonium sulfate precipitation and still contained a rather high concentration of ammonium sulfate were stored at 4 C for as long as 60 days, not only did the titers decrease but several additional lines appeared, forming closer to the antigen well. These lines were still serologically distinct from those of LF and EF and could represent PA aggregates of different molecular weight. Recently, Winstead and Wold[14] showed that rabbit muscle enolase dissociated reversibly in the presence of ammonium sulfate and that it underwent "a concentration-dependent association to higher molecular weight asymmetrical aggregates." Upon chromatography of either PA or LF on Sephadex G-25 we occasionally observed the presence of multiple peaks that were still antigenically distinct from the other two major components of toxin. These most likely represent aggregates of different molecular weight.

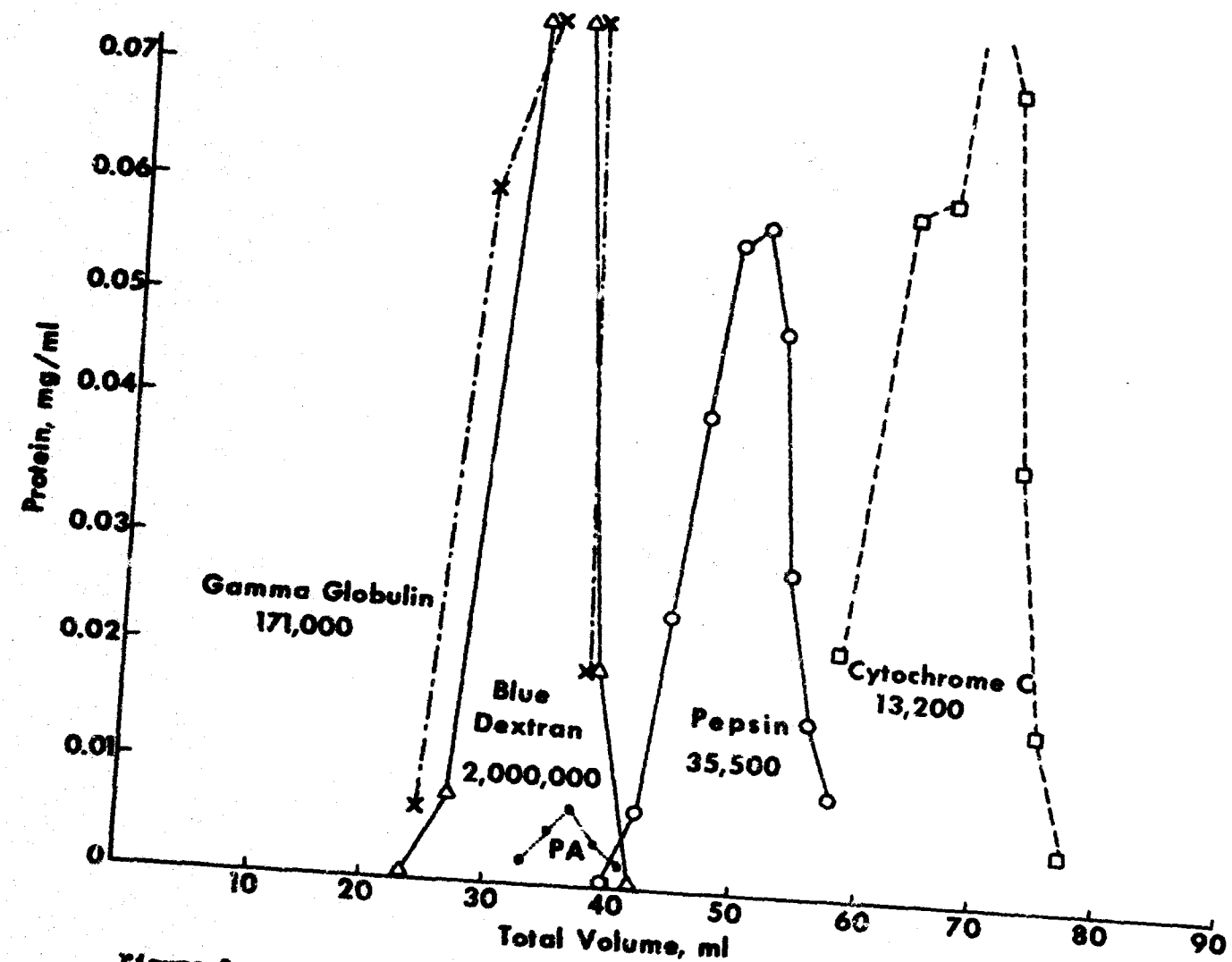

Figure 2. Composite Elution Diagram of PA and Various Known Proteins from Sephadex G-75.

V. DISCUSSION

In this discussion, literature references to toxin produced both in vivo and in vitro and its components are included. Sargeant et al.[8] found several serological differences between toxins produced in vivo and in vitro, and so the reader is reminded, again, that all our work was done with in vitro - produced toxin and that the two types of toxin may or may not be identical in their composition and/or any specific response.

The exact chemical composition of the toxin components is unknown. Smith,[15] working with in vivo - produced toxin that was later shown to be heavily contaminated with guinea pig serum components,[1] stated that factor I (EF) was a lipoprotein containing carbohydrate residues, and that factor II (PA) was a protein. Based on its chemical composition, factor III (LF) was also reported to be a protein.[16] Although EF appeared to be contaminated with 6 to 8% nucleic acid based on its absorption at 260 mμ, the presence of RNA or DNA could not be demonstrated.[17] Strange and Thorne[10] showed that purified PA had the UV absorption spectrum of a typical protein. Our results indicate that none of the purified components is pure protein; rather, all appear to contain from 3 to 5% nucleic acid or some other base that accounts for their absorption maximum at 270 mμ and the ratio of their absorbance at 280/260 mμ. The observation by Tempest and Smith[18] that pyrimidines and nicotinamide are necessary for in vivo toxin formation is of interest in this connection.

Thorne et al.[8] reported that their in vivo - produced "filter factor" (EF and LF) was stable over a rather wide pH range. Stanley and co-workers[1,17] reported a narrow range of pH stability, pH 7.4 to 7.7, for their in vivo - produced factor I (EF). We found EF to be stable between pH 7.3 and 7.8; LF was stable over a wide pH range. Our crude PA was stable over a wide pH range, 6.5 to 8.3, but the highly purified PA showed an extremely narrow range of pH stability, 7.1 to 7.5. This observation contrasts with the increased stability of PA at pH 8.7 observed by Wright, Hadberg, and Slein,[19] which may have been due to the presence of EF and/or LF that had not been removed by filtration.[20] Apparently there is a general trend of inverse relationship between stability and degree of purification. However, it is clear that the relationship is not simple.

With the exception of two observations,[8,16] there is general agreement that all of the components appear to be extremely sensitive to heat.[1,8,16,17] The assay system is especially important because serological activity is not always a good criterion for biological or immunological activity.[8]

We have not been able to maintain consistently either the biological or the serological activity of EF during freeze-drying or storage at 4 or -20 C. The most stable preparation was an ammonium sulfate precipitate stored at 4 C. We have found that carefully controlled freeze-drying of

the whole toxin, PA, or LF yields products that retain their biological and serological activity after long periods of storage at -20 C. Biological and serological activity were unaffected by storage at -20 C for 2 years. Again, as in the case of pH stability, the effect of temperature depends on the concentration of the components.

Results from Ouchterlony analyses must be interpreted carefully. Although Thorne and Belton[5] obtained a good correlation between Ouchterlony titer and immunizing ability, Smith[21] and Beall et al.[20] pointed out that the toxin components readily lost toxicity but retained their serological activity. Molnar and Altenbern[11] found that two of four preparations of PA were unable to form a lethal mixture when combined with LF but were serologically indistinguishable from active PA. We often observed that biological activity was lost prior to loss of serological activity. The serological relationship among these components indicates that they could, at some time, have shared a common form or architecture, although EF and LF appear to be much more closely related to each other than to PA.

Sargeant et al.[3] concluded that there were at least three distinct serological components in toxins produced both in vivo and in vitro, although minor differences were found between the in vivo and in vitro toxins. They also demonstrated the presence of biologically inactive but serologically active factor II (PA) in some preparations of factor I (EF). Separation of what had appeared to be one line of precipitation into two lines occurred during heat inactivation studies. We found that, although we could clearly distinguish among the three purified components when they were placed in adjacent wells, it was often difficult to identify the three components when mixed. PA always seemed to be serologically distinct from both EF and LF, but, during various stages in their purification, EF and LF often showed partial identity or at least some sort of interaction. In addition, storage of PA resulted in the appearance of new lines of precipitation. These observations indicate the ease with which these components may aggregate or polymerize.

Molnar and Altenbern[11] pointed out that injection of PA into animals that had been injected with a lethal dose of toxin could considerably extend their time to death. They therefore stated that PA was the site of attachment of the toxin to the tissue. Our results, obtained by adding an increasing amount of PA to a constant amount of LF, verify their work, but the explanation is probably too simple, because increasing amounts of LF added to a constant amount of PA decreases the time to death and addition of more PA to a constant amount of whole toxin also decreases time to death. We again feel that not enough consideration has been given to the ability of these components to exist in different aggregate states and also in different degrees of activity (i.e., biological, serological, immunological). Molnar and Altenbern themselves speculate that PA undergoes some subtle physiochemical changes rather than extensive splitting of the molecule. The PA studied by Strange and Thorne[10] and Wright and

Luksas[22] was composed of more than one molecule. The English workers[1,23,24] have repeatedly reported that EF dissociates and reaggregates with the slightest treatment. Stanley and Smith[17] reported the chelating action of EF and speculated that the whole toxin might have existed as a loose complex.

These data from the literature and our results reported here and in other places[25,26] all indicate that the toxin components, and the whole toxin itself, exist as different molecular complexes whose biological, immunological, and serological activities are greatly affected by and/or related to their degree of purity and immediate environment. Further knowledge of these molecules and their configuration should enhance our understanding of anthrax toxin in particular and of other bacterial toxins in general.

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13. ABSTRACT

Many of the biological and biophysical characteristics of the partially purified anthrax toxin components were measured. The components do not appear to be simple proteins. As the components are purified the pH range in which they are most stable narrows, centering between pH 7.4 and 7.8. Heat readily destroys the biological activity of the components but not their serological activity. All three components are serologically distinct. The rat lethality test showed that with a constant amount of lethal factor (LF) and an increasing amount of protective antigen (PA) the time to death reached a minimum and then was increased. When an increasing amount of LF was added to a constant amount of PA, the more LF that was added, the shorter the time to death. The biological, immunological, and serological properties of the components varied with storage and extent of purification so that serological activity was not always directly correlated with biological activity. Evidence is presented that the components could exist in different molecular configurations or as aggregates and that this property is influenced by the state of component purity and the environment.

14. Key Words

in vitro	Edema	Temperature
Toxins	Lethal	Stability
Characteristics	Assay	
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