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Purification and Properties of In Vitro-produced Anthrax Toxin Components

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The three components of the toxin of *Bacillus anthracis*, edema factor (EF), protective antigen (PA), and lethal factor (LF), were purified 197-, 156-, and 1,025-fold, with 38, 78, and 11% recovery, respectively. Each purified component was serologically active, distinct, and free from the other components. The purified EF produced edema when mixed with PA, and the purified PA was an active immunogen. The components did not appear to be simple proteins by spectrophotometric analysis. As they were purified, the *p*H range in which they were most stable narrowed, centering between *p*H 7.4 and 7.8. Heat readily destroyed the biological activity of the components but not their serological activity. The rat lethality test showed that, with a constant amount of LF and an increasing amount of PA, the time to death reached a minimum and then was extended. When an increasing amount of LF was added to a constant amount of PA, the time to death became shorter as more LF was added. The biological, immunological, and serological properties of the components were shown to vary independently with storage and extent of purification so that serological activity was not always directly correlated with biological activity. Evidence is presented that the components can exist in different molecular configurations or as aggregates, and that this property is influenced by the state of component purity and by the environment.

As early as 1904, Bail (3, 4) demonstrated a "protective antigen" and an agressin from *Bacillus anthracis*, and in 1947 Cromartie et al. (8) extended this work. However, the presence of a diffusable cell-free toxin was not conclusively demonstrated until 1954 by Smith and Keppie (22). Once this in vivo-produced toxin was shown to be responsible for death of the host (23) and the same toxin was shown to be produced in vitro (14), the purification and characterization of the toxin of *B. anthracis* became extremely important to a full understanding of the organism and the disease.

The English workers demonstrated that in vivo-produced toxin was composed of two components (25, 26), and they later identified a third component from in vitro-produced toxin (24, 27). They called these components of the toxin factors I, II, and III (28). Other workers have called the same components (factors) edema factor (EF), protective antigen (PA), and lethal factor (LF), respectively (5).

Little is known about the biological and biophysical characteristics of anthrax toxin or its components, aside from the studies of Stanley, Sargeant, and Smith (24) on the stability of crude preparations of in vivo-produced factors I and II, and the work of Thorne, Molnar, and Strange (33) on *p*H and temperature sensitivity of "filter factor" (combination of EF and LF). The serological relationships between the various components were demonstrated by Sargeant, Stanley, and Smith (18). The best preparations of these three components reported in the literature to date have been purified 3.5- to 50-fold with 20 to 24% recovery (10), and all are still contaminated by one or more of the other components (18).

Purification of the components was undertaken as a first step for future studies on the physical and biological chemistry of the toxin per se, the variation of *B. anthracis* strains, the pathophysiology of the host, treatment of the disease, and immunization. Effective procedures for purification of the three components of the toxin are presented in this paper.

The three components, EF, PA, and LF, each free from the other components, were purified 197-, 156-, and 1,025-fold, with 38, 78, and 11% recovery, respectively.

The purified components were tested for *p*H, temperature, and chemical stability. The sensi-

tivity and interactions observed in the various assay systems are discussed, as well as our interpretation of data on the molecular weight of PA and molecular heterogeneity or aggregation of the whole toxin. A preliminary report of this work has been made (Fish, Bacteriol. Proc., p. 41, 1966).

MATERIALS AND METHODS

Production of whole toxin. Whole toxin was produced from the Sterne strain of *B. anthracis* by the procedure of Haines, Klein, and Lincoln (13).

Biological activity. Edema factor activity was titered in guinea pigs by injecting serial dilutions of EF in PA (33).

Rat lethality was quantitated by determining the time to death of Fischer 344 rats after intravenous injection (13). As both PA and LF are required for rat lethality, purification of LF was based entirely on Ouchterlony (serological) analysis.

Serological activity. The double diffusion in agar-gel (Ouchterlony) method (32) was used to quantitate the serological activity during purification. Equine antiserum (Detrick horse Lot DH-1-6C and Scalvo Lots 76 and 76 AQ, Institute Seraterapico Tuscano, Siena, Italy) was prepared by repeated injections of spores of the Sterne strain of *B. anthracis.* For all studies on purification, Detrick horse antiserum diluted 1:8 in 0.2% gelatin in saline was used. Antiserum was added to the center wells, and plates were incubated for 6 hr at 30 C; antigen then was added to the outer wells; the plates were reincubated at 30 C for 48 hr, and then were flooded with 0.1% azocarmine in 0.5 M acetate buffer (*p*H 4.0) for 3 to 4 hr. The stain was decanted, and the plates were destained by addition of 2% acetic acid for 1 hr. The titer, expressed as units per milliliter was the highest dilution that still yielded a visible line of precipitation (33). The extent of purification of each component was determined by following the increase in specific activity, which was determined by dividing the Ouchterlony titer by the protein concentration (milligrams per milliliter). When samples were concentrated and reassayed, the Ouchterlony titer was found to yield a linear response.

Immunogenicity of the components. Some of the samples of PA used for this experiment were purified by methods additional to those reported below. The PA (Table 4, procedure B) that had been isolated after ammonium sulfate precipitation and column chromatography on polyacrylamide (P-2) gel (Bio-Rad Laboratories, Richmond, Calif.) was further treated with alumina C-gamma ($C\gamma$) gel (Bio-Rad Laboratories). 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 $C\gamma$ gel. The tube was incubated at 4 C with occasional stirring for 30 min, and the gel was sedimented by centrifugation at 20,000 × *g* for 5 min. The supernatant fluid contained the PA activity, which was purified 128-fold. The recovery was 39% compared with the original culture supernatant fluid. A second treatment with alumina $C\gamma$ gel resulted in a product purified eightfold and a recovery of 25%. The first and second supernatant fluids were tested for their immunizing ability.

The immunity index (I) method of DeArmon et al. (9) 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 intraperitoneally with 10^3 spores of the virulent V1b strain of *B. anthracis* in a 1-ml volume of liquid.

Molecular weight determination by column chromatography. Sephadex G-75 (Pharmacia, Uppsala, Sweden) was equilibrated and washed with 0.15 M KCl to remove the fines. The material was then equilibrated at 4 C, and a column (3 × 82 cm) was prepared. The following reference samples were used: cytochrome *c* (A grade) and γ-globulin (bovine, fraction II) from Calbiochem, Los Angeles, Calif., pepsin (three times crystallized) from Nutritional Biochemicals Corp., Cleveland, Ohio, and blue dextran 2,000 from Pharmacia. For the molecular weight determination, 1 ml of a 2 mg/ml sample of the standard was carefully layered on top of the column and allowed to absorb into the gel. The column was then eluted with 0.15 M KCl, by use of a flow rate of 1.5 ml/hr. Fractions of 3 ml were collected, and the protein concentration was determined by the ratio of absorption at 215 mμ to that at 225 mμ (34). When the molecular weight of PA was determined, the PA component was assayed by Ouchterlony analysis, as the major protein peak was eluted after about 120 ml of fluid had passed through the column.

Analytical methods and preparation of column materials. Protein was determined spectrophotometrically by the 215/225 mμ ratio method of Waddel (34), with egg albumin and the 260/280 mμ ratio method of Christian and Warburg (7) as standards. Diethylaminoethyl (DEAE) cellulose (Schleicher and Schuell, Keene, N.H.) was washed with 5% (v/v) HCl in 10% NaCl, followed by 1% NaOH in 10% NaCl and then 10% NaCl to remove any impurities. NaCl was removed by extensive washing with water, and the DEAE cellulose then was stored under water at 4 C until used. Calcium phosphate gel was prepared according to the method of Main, Wilkins, and Cole (16).

RESULTS

Whole toxin and its separation into components. Analyses of six lots of whole toxin are presented in Table 1. The high ratio of dry weight to protein reflects the presence of salts, other culture medium components, and metabolic and soluble bacterial components in the whole toxin preparations. This whole toxin produced edema, was lethal, and was serologically active.

Beall et al. (5) reported that the three major components of anthrax toxin could be separated by selective adsorption and elution from sintered-glass filters. During the first phase of separation, we followed their procedures exactly, and the analysis of these individual components is shown

in Table 2. Guinea pig edema requires the presence of EF and PA, and rat lethality requires both LF and PA. The isolated components were then further purified by the procedures described below.

Edema factor. The purification of in vitro-produced EF is shown in Table 3. The eluates from the fine, ultrafine, and two further fine filters were pooled. These pooled filtrates (200 ml) were placed on a column (2.0 × 9.5 cm) of DEAE cellulose, and the eluate was discarded. The EF was eluted with 200 ml of 0.2 M phosphate buffer (*p*H 7.4) and dialyzed in an ultrafilter (LKB Produkter, Stockholm, Sweden) by removing 73 ml of the solution in four (16 to 23 ml) increments, replacing the volume each time with deionized water. The final preparation was 197-fold purified with respect to the original culture supernatant fluid, and the recovery was 38%. The original culture supernatant fluid had a guinea pig skin titer of 1:32, and the final purified material had a titer of 1:256. This final material was therefore still active biologically and serologically.

Protective antigen. After the initial separation, PA was purified by three methods which differed mainly in the procedure used for concentrating the culture filtrate.

In the first method (Table 4, A), 200-ml samples were placed in dialysis tubing and concentrated by pervaporation at 4 C for 7 to 10 days until the volume was reduced to 25 to 35 ml. This material was then chromatographed on a column (2 × 52 cm) of Sephadex G-25 equilibrated and eluted with 0.01 M NaCl. The serological activity appeared in a small protein peak (tubes 30 to 36) that preceded the major protein peak (Fig. 1). This procedure yielded PA in 90% recovery and 136-fold purification with respect to the original supernatant fluid.

TABLE 1. *Characteristics of whole toxin*

Lot	Protein	Dry wt[a]	Rat lethality	Guinea pig edema (titer)	Ouchterlony (titer)	
					LF	PA
	mg/ml	*mg/ml*	*units/ml*			
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 (dry weight) per ml initially; this included 2.2 mg of glucose, 3.5 mg of Casamino Acids, and 1.6 mg of potassium phosphate per ml.

TABLE 2. *Characteristics of toxin components*

Component	Protein	Dry wt	Ouchterlony (titer)	Guinea pig edema (titer)	Rat lethality
	mg/ml	*mg/ml*			*units/ml*
EF	0.393	16.7[a]	1:2	1:40	0
	0.039	12.1	1:1	1:10	0
	0.273	—	1:64	1:256	25[b]
	0.276	—	1:16	1:4096	0
	0.094	—	1:16	1:20	114[b]
	0.026	—	1:8	—	0
PA	0.750	10.8[c]	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[a]	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.

The second method (Table 4, B) for concentrating PA was by precipitation with ammonium sulfate (30). PA was precipitated by the addition or solid $(NH_4)_2SO_4$ until the solution was saturated. Periodic additions of NH_4OH were necessary to maintain the *p*H at 7.4 to 7.8. The saturated solution was stirred constantly and held at 4 C for 72 hr to insure complete precipitation. The precipitate was collected by centrifugation at 20,000 × *g* for 30 min and redissolved in cold water. The PA, in 25-ml amounts, was further purified by chromatography on a column (2 × 52 cm) of polyacrylamide (P-2) gel (Calbiochem) equilibrated and eluted with 0.01 M potassium phosphate buffer (*p*H 7.4). This method yielded PA in 78% recovery with 156-fold purification.

The last method tested (Table 4, C) used a Diaflo (Amicon Corp., Cambridge, Mass.) ultrafiltration cell. The cell had a capacity of 65 ml and was operated at 45 psi of N_2 with the use

TABLE 3. *Purification of edema factor*

Fractionation procedure	Vol	Ouchterlony (titer)[a]	Recovery	Protein	Specific activity[b]	Purification
	ml		%	*mg/ml*		
Culture supernatant fluid..	1,000	1:8	100	1.190	6.7	1.0-fold
Fine, ultrafine, fine, and fine filter eluate........	200	1:64	160	0.273	235	35-fold
Phosphate (0.2 M) eluate of DEAE cellulose........	200	1:32	80	0.045	710	106-fold
LKB dialysis.............	190	1:16	38	0.0121	1320	197-fold

[a] Highest positive dilution.
[b] Titer divided by protein (mg/ml).

TABLE 4. *Purification of protective antigen*

Fractionation procedure	Vol	Ouchterlony (titer)[a]	Recovery	Protein	Specific activity[b]	Purification
	ml		%	*mg/ml*		
Initial separation						
Culture supernatant fluid...	1,000	1:16	100	1.190	13.4	1-fold
F, UF filtrate[c]..............	950	1:16	95	1.175	13.6	1-fold
F, UF, F filtrate............	916	1:16	92	1.215	13.2	1-fold
F, UF, F, UF filtrate.......	916	1:16	92	1.225	13.1	1-fold
Final separation A						
Concentrate by pervaporation......................	214	1:64	86	5.40	11.2	1-fold
G-25 pooled fractions.......	449	1:32	90	0.017	1850	136-fold
Final separation B						
Ammonium sulfate precipitate......................	117	1:128	94	0.147	873	67-fold
P-2 pooled fractions........	97	1:128	78	0.062	2080	156-fold
Final separation C						
Diaflo ultrafiltration cell....	87	1:256	138	3.14	82	6-fold
P-2 pooled fractions........	133	1:64	53	0.23	278	21-fold
Concentrate by Diaflo cell..	29	1:256	47	0.30	854	64-fold

[a] Highest positive dilution by Ouchterlony double diffusion.
[b] Titer divided by protein (mg/ml).
[c] F is fine, UF is ultrafine pore filter.

of a UM 1 membrane, which retained components having a molecular weight of 10,000 or greater. The flow rate was approximately 0.5 ml/min, which allowed the processing of large volumes of material in a relatively short time. The concentrated material then was purified by chromatography on polyacrylamide gel (as in method 4 B), and the peak tubes were reconcentrated with the Diaflo cell. PA was obtained with 47% recovery and 64-fold purification. Of the three methods tested, this last method offers the best possibility for future work in purification of large volumes of material (i.e., vaccine production), as it is rapid, economical, and simple. The final preparations of PA were active serologically and biologically, both when combined with EF or LF and as immunogens (10; Fish et al., J. Infect. Diseases, *in press*).

Lethal factor. The results from the purification of LF are given in Table 5. The carbonate-eluate from the final ultrafine filter was mixed with 31 ml of calcium phosphate gel and allowed to stand for 30 min at 4 C. The gel was removed by centrifugation at 20,000 × *g* for 5 min, and the supernatant fluid was chromatographed on a column (2 × 52 cm) of Sephadex G-25 equilibrated and eluted with 0.01 M pyridine. The pooled fractions then were mixed with 16 ml of calcium phosphate gel and allowed to stand for 30 min at 4 C. The gel was washed once with 0.05 M phos-

phate buffer (pH 7.4), and the LF was eluted with 0.2 M phosphate buffer (pH 7.4). LF was obtained in 11% yield with a purification of 1,025-fold over the initial culture supernatant fluid. The low recovery was due chiefly to adsorption and loss of LF onto the filters used to remove EF. The biological assay for LF was not attempted because of the low concentration of LF and the interfering high concentration of phosphate buffer (see *Rat lethality* below; Fish et al., *in press*).

The carbonate eluate from the ultrafine filters has been concentrated three- to fivefold with 51% recovery by use of the Diaflo ultrafiltration cell. However, column chromatography of this material has not yet been attempted.

Serological relationships and purity of the components. Purified toxin components placed in adjacent wells on an Ouchterlony plate resulted in each purified component giving rise to one line of precipitation that intersected the line formed by the other two components (Fig. 2). The initial culture filtrate (WT) contained two lines: the one that formed farther from the antigen well was identical with PA and the one that formed closer to the antigen well was identical with LF. Although known to be present (guinea pig skin edema), EF could not be detected in the whole toxin by Ouchterlony analysis, as its line of precipitation formed at the same location as that of PA. The EF used in Fig. 2 was a crude preparation that still contained approximately 10% LF. This LF contaminant formed a line of precipitation nearer to the antigen well and exhibited partial idenity with the line of precipitation formed by the purified LF. However, the major line, which corresponded to EF, was distinct from PA and LF. Molnar and Altenbern (17) reported similar observations with one of their preparations which contained both components.

Ultraviolet absorption. The ultraviolet absorption spectra were determined for whole toxin and for EF, PA, and LF in various stages of purification. The ultraviolet absorption was measured on a Beckman model DU spectrophotometer, with the appropriate diluent used 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 was 0.87 to 1.03, which corresponds to a 3 to 5% contamination by nucleic acid determined by the method of Christian and Warburg (7).

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.

pH stability. Crude PA, after passage through the final ultrafine filter, was stable at 4 C in the

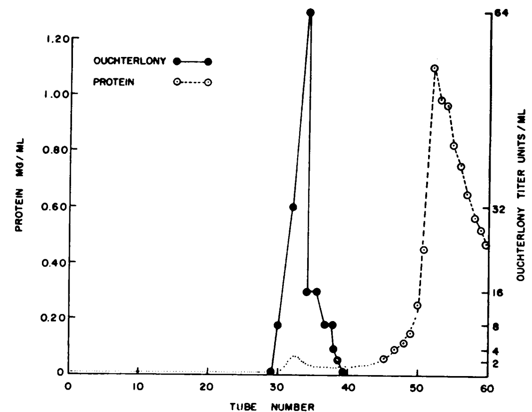

FIG. 1. *Comparison of Ouchterlony titer and protein distribution during Sephadex column chromatographic purification of PA.*

TABLE 5. *Purification of lethal factor*

Fractionation procedure	Vol	Ouchterlony (titer)[a]	Recovery	Protein	Specific activity[b]	Purification
	ml		%	*mg/ml*		
Culture supernatant fluid	1,000	1:2	100	1.190	1.7	1-fold
F, UF filtrate	950	1:2	95	1.175	1.7	1-fold
Eluate final UF filter	77	1:8	32	0.134	60	35-fold
$Ca_3(PO_4)_2$ supernatant fluid	108	1:8	45	0.085	95	56-fold
G-25 pooled fractions	77	1:4	16	—	—	—
$Ca_3(PO_4)_2$ eluate with 0.2 M phosphate	26	1:8	11	0.0046	1740	1,025-fold

[a] Highest positive dilution by Ouchterlony.
[b] Titer divided by protein (mg/ml).

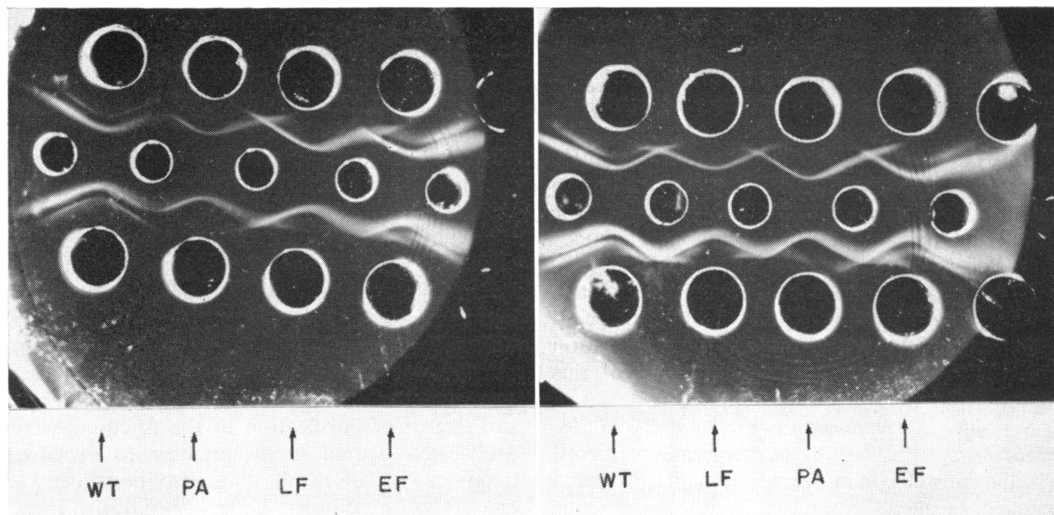

FIG. 2. *Ouchterlony analysis of the purified anthrax toxin components. WT = whole toxin.*

presence of 0.025 M potassium phosphate buffer during the 30 days for which tests were run over a *p*H range of 6.5 to 8.3. PA, purified 156-fold, showed an extremely narrow *p*H tolerance in the presence of 0.4 M potassium phosphate buffer. It was stable as long as 30 days between 7.1 and 7.5, but stability decreased rapidly at *p*H values below 6.8 or above 7.9.

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

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

Temperature stability. Samples of whole toxin, 320 and 160 rat lethal units/ml, were maintained in a shaking water bath at 30 C. After 48 hr, PA activity, as measured by the Ouchterlony titer, had dropped from 1:512 to 1:128 and 1:64, respectively, in the two experiments with 320 and 160 units/ml. 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 hr. However, these preparations had lost their rat lethality. It is possible that these results were influenced by surface effects caused by shaking rather than to the effects of temperature.

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

The toxin and its components usually were stable after 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 of time after they were dry. Biological and serological activity were unimpaired by storage of aqueous samples at −20 C.

Chemical stability. The stability of 156-fold purified PA in the presence of various chemical agents is shown in Table 6. The initial *p*H of the sample was 7.8, and the *p*H 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, after the final filtration step, was relatively stable in both oxidizing reagents (0.15% potassium ferricyanide and 0.05% potassium iodate) and reducing reagents (0.1% cysteine).

LF (Table 7) 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. The initial *p*H of the sample was 9.7, and the various mixtures had a *p*H range of 8.7 to 9.5.

Crude preparations of EF, 35-fold purified, were stable in the presence of 0.1% cysteine and 0.05% potassium iodate but were unstable in the presence of 0.15% potassium ferricyanide. These

TABLE 6. *Stability of PA, purified 156-fold, treated with selected chemical reagents*

Reagent	Highest positive dilution on Ouchterlony analysis after indicated days of storage			
	0	12	27	48
Control (water)	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-chloride	32	16	8	8

[a] Final concentration of reagent.

TABLE 7. *Stability of LF, purified 35-fold, treated with selected chemical reagents*

Reagent	Highest positive dilution on Ouchterlony analysis after indicated days of storage			
	0	12	37	111
Control (water)	32	16	8	8
Oxidizing reagents				
0.05% 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-chloride	32	8	8	8

[a] Final concentration of reagent.

experiments were all performed in the presence of 0.05 M potassium phosphate buffer (*p*H 7.5).

Ouchterlony assay. The Ouchterlony technique detected 2 to 4 μg of protein of the purified PA and LF and 170 μg of protein of EF per well. We were unable to obtain the sensitivity reported by Strange and Thorne (30) 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 (*p*H 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.

Antiserum binding. The ability of antiserum to bind toxin in vitro was determined. An identical amount of antigen was added to twofold serial dilutions of the antiserum; the mixture was kept at room temperature for 15 min, 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 binding ability. Table 8 shows that the binding observed was concentration-dependent and that 1 ml of undiluted antiserum bound

TABLE 8. *Antiserum binding to 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 76AQ.

128 Ouchterlony units of toxin. The antiserum appeared to be as active against LF as against PA.

Guinea pig skin edema assay. By use of the procedure of Thorne et al. (32), 18 μg of protein of EF, 35-fold purified, could be detected. The minimal amount of PA needed to produce edema with EF was not determined.

Rat lethality. Haines et al. (13) standardized a procedure for quantitating whole toxin by measuring the time to death after intravenous injection of toxin in the penial vein of Fisher 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 (*p*H 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 min with no visible after effects. The greatest concentration used by Haines et al. (13) was 16-fold, and the part of the response curve used in deriving the equation was below 8-fold; therefore, the salt effect reported here 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 was varied are presented in Table 9. 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 some extension in the time to death. This observation parallels that of Molnar and Altenbern (17). The minimal lethal amounts of PA and LF were 32:4 or 16:8. When PA was kept constant and the amount of LF was varied, the time to death became shorter as the amount of LF present increased (Table 9).

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 10) show that PA, in all stages of purification, was a good immunogen. The more purified material appeared 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.

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

TABLE 9. *Effect on rat lethality of varying one component in the presence of a constant amount of the other component*

Units of PA injected	Units of LF injected					
	0	2	4	8	16	32
0						S
2			S[a]			
4			S	S		
8			S	S	S	
16			S	331	227	
32			336	141	118	
64	S	S	177	112	91	
128		S	255	124	93	

[a] S = sublethal.

TABLE 10. *Efficacy of PA as an immunogen for the guinea pig during different stages of purification*

Fraction	Purification[a]	Total amt injected			
		Vol	Ouchterlony units	Protein	Immunity index[b]
		ml		*mg*	
Filtrate	1-fold	1.25	20	1.47	3.5
Filtrate	1-fold	0.5	4	0.34	3.9
Filtrate	1-fold	1.0	16	0.95	4.7
$(NH_4)_2SO_4$ precipitate	67-fold	2.5	320	0.37	5.9
Column fractions	156-fold	1.25	40	0.08	5.7
First Cγ gel	128-fold	0.5	16	0.02	5.4
Second Cγ gel	8-fold	1.0	8	0.01	5.0

[a] Purification details given in Materials and Methods under *Immunogenicity.*

[b] Control value (nonimmunized guinea pig) is 0.0 based on a mean time to death of 32 hr.

The presence of multiple lines of precipitation in these high concentrations of agar from purified samples which 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 after column chromatography on Sephadex has yielded a reasonably reliable value for the molecular weight of many proteins (2). A composite elution diagram from a Sephadex G-75 column of several proteins whose molecular weight is known is shown in Fig. 3. The PA preparation used for this determination was a sample that had been concentrated in the Diaflo (Amicon Corp.) 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 volume 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 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 LF and EF, and could represent PA aggregates of different molecular weight. Recently, Winstead and Wold (35) 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 weights.

DISCUSSION

The three components of anthrax toxin have been purified from both in vivo (23, 25, 26) and in vitro (5, 14, 24, 27, 29, 33) sources. Although the assay methods have differed from one research group to another, certain generalizations have been made about the state of purity of the various components (10). The procedures described here yielded the three separated components in a more highly purified state than any method in the literature to date. The purified EF was free from

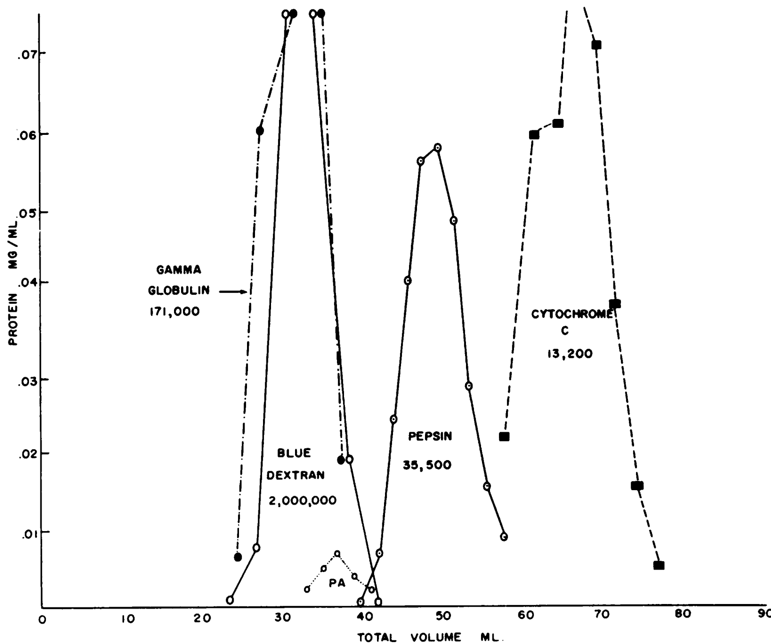

FIG. 3. *Composite elution diagram of PA and various known proteins from Sephadex G-75.*

both LF and PA, and when mixed with PA produced an edema in guinea pigs. The purified PA was serologically distinct from the other two components and was an active immunogen. However, the identification of PA solely on the basis of its ability to protect animals against spore challenge is dangerous. For example, the PA used by Gladstone (12), Wright, Hedberg, and Slein (36), and Boor and Tresselt (6) was almost certainly the complete toxin, since either serum, which prevents the retention of the components on the filter (5, 33), was added at some stage either of production or of processing, or the *p*H was raised to 8.7, which could prevent EF or LF from being adsorbed. In our procedure, based on the observations of Beall et al. (5), a *p*H of 9.3 eluted these components from the filter.

A second danger in characterizing PA solely by its ability to protect animals against spore challenge was shown by Mahlandt et al. (15), who observed (i) that immunization with LF protected rats against either toxin or spore challenge and guinea pigs against spore challenge, (ii) that PA protected both species against spore challenge but neither against toxin challenge, and (iii) that EF had an additive effect in certain combinations.

In this discussion, literature references to toxin produced both in vivo and in vitro, and its components, are included. Sargeant et al. (18) found several serological differences between toxin 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 similar in their composition or in any specific response.

The exact chemical composition of the toxin components is unknown. Smith (20), working with in vivo-produced toxin that was later shown to be heavily contaminated with guinea pig serum components (26), stated that factor I (EF) was a lipoprotein containing carbohydrate residues, and that factor II (PA) was a protein. On the basis of its chemical composition, factor III (LF) also was reported to be a protein (24). Although EF appeared to be contaminated with 6 to 8% nucleic acid, based on its absorption at 260 mμ, the presence of ribonucleic acid or deoxyribonucleic acid could not be demonstrated (27). Strange and Thorne (30) showed that purified PA had the ultraviolet absorption spectrum of a typical protein. Our spectrophotometric results indicated that none of the purified components was pure protein; rather, all appeared to contain from 3 to 5% nucleic acid or some other base, which accounted for their absorption maximum at 270 mμ and the ratio of their absorbance at 280/260 mμ. The observation by Tempest and Smith (31) that pyrimidines and nicotinamide are necessary for in vivo toxin formation is of interest in this connection.

Thorne et al. (33) reported that their in vivo-produced "filter factor" (mixture of EF and LF) was stable over a rather wide *p*H range. Stanley and co-workers (26, 27) reported a narrow range of *p*H stability (*p*H 7.4 to 7.7) for their in vivo-produced factor I (EF). We found EF to be stable between *p*H 7.3 and 7.8; LF was stable over a wide *p*H range. Our crude protective antigen was stable over a wide *p*H range (6.5 to 8.3), but the highly purified PA showed an extremely narrow range of *p*H stability (7.1 to 7.5). This observation contrasts with the increased stability of PA at *p*H 8.7 observed by Wright, Hedberg, and Slein (36), which may have been due to the presence in their PA of edema factor or lethal factor, or both, that had not been removed by filtration (5). 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 (18, 24), there is general agreement that all of the components are extremely sensitive to heat (20, 26, 27, 33). The assay system is especially important, because serological activity is not always a good criterion for determining biological or immunological activity (18).

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 2 years of storage at −20 C. Again, as in the case of *p*H stability, the effect of temperature depends on the concentration and purification of the components.

Results from Ouchterlony analyses must be interpreted carefully. Although Thorne and Belton (32) obtained a good correlation between Ouchterlony titer and immunizing ability, Smith (19) and Beall, Taylor, and Thorne (5) pointed out that the toxin components readily lost toxicity but retained their serological activity. Molnar and Altenbern (17) 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. (18) concluded that there were at least three distinct serological components in toxin produced both in vivo and in vitro, although minor differences were found between the in vivo and in vitro toxin. 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 protective antigen resulted in the appearance of new lines of precipitation without loss of biological activity. These observations indicate the ease with which these components may aggregate or polymerize or be degraded. Similar results have been obtained with in vivo-produced toxin (11).

Molnar and Altenbern (17) 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 protective antigen 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 their explanation is probably too simple because increasing amounts of LF added to a constant amount of PA decrease the time to death, and the 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 (30) and Wright and Luksas (Federation Proc. **23**:191, 1964) was composed of more than one molecule. The English workers (21, 25, 26) have repeatedly reported that EF disassociates and reaggregates with the slightest treatment. Stanley and Smith (27) 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 elsewhere (10, 11; Fish et al., *in press*) 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 or related to their degree of purity and immediate environment.

Use of these purified components and knowledge of their configuration will allow more critical studies to be undertaken to characterize the toxin and to determine the pathophysiological changes that it induces in the host. The use of defined materials will also result in more uniformity of results among workers.

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