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# Characterization of anthrax toxin

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The work of Cromartie et al. (4, 5, 18, 19) stimulated renewed interest in the role of the extracellular products of *Bacillus anthracis* in the pathogenesis of the disease caused by this organism. In 1954 Smith and Keppie (10) reported the presence of a specific toxin in the plasma of guinea pigs dying from anthrax. Intradermal injection of plasma from these animals into other guinea pigs or rabbits caused edematous lesions in the skin; intravenous or intraperitoneal injection into mice and guinea pigs resulted in death. Subsequently, it was demonstrated that material with similar biological activity is produced in vitro by both virulent and avirulent strains of *B. anthracis*. For detailed information on the development of current knowledge of anthrax toxin and the characterization of its three recognized components, the reader is referred to the work of Smith and co-workers (10–14), Thorne and associates (15, 17) and Beall et al. (1).

Some confusion has arisen from the use of different nomenclature by British and American authors for what appear to be identical materials. In this discussion we shall follow the recent suggestion of Dr. Harry Smith and use a combination of the two. The component which is essential for demonstration of the edema producing activity of toxin, called edema factor (EF) by American workers and Factor I by the British will be designated EF I. The second, apparently identical with the immunizing protective antigen, called either PA or Factor II, respectively, will become PA II. Similarly, the third component, necessary for the lethal effect of the toxin, will be referred to as LF III.

For definitive studies on the mode of action of the toxin large quantities of concentrated and highly purified material are required. Ideally, whole culture filtrates without prior fractionation should be used as starting material. Little is known of the physical and chemical properties of the biologically active material as originally produced by the organism or of the relative proportions of the recognized components existing in unaltered toxic filtrates. It is conceivable that the toxin may be a single, loosely bound entity, which is readily fragmented by the glass filtration usually employed in isolation procedures. In addition, the kinetics and mechanisms of toxigenesis remain to be elucidated. Therefore, the methods used for concentration, isolation and purification should avoid procedures known to alter characteristics of labile proteins and give high, quantitative yields of all active components. Both of these qualifications are essential for quantitative evaluation of the efficiency of a procedure in terms of the starting material. Such methods would also make possible basic studies on the kinetics and mechanisms of toxigenesis. A major objective of work on anthrax toxin in this laboratory has been to develop a procedure meeting these requirements.

Of several possible methods available for concentration of large volumes of culture filtrate, a simple ultrafiltration method was chosen. Small diameter dialysis tubing was used as the filtering membrane in a continuous flow system, under negative pressure. Cultures of the relatively avirulent Sterne (Weybridge) strain were grown in the casamino acids medium of Thorne and Belton (16). After a 24-hr incubation period, bacteria were removed by millipore filtration. In the ultrafiltration system used 40–60 liters of filtrate could be concentrated 500- to 1,000-fold in 4–5 days with minimum attention and personnel effort. All detectable toxic and antigenic activity remained in the sac. The ultrafiltrate residue, hereafter designated UFR, was harvested by "backwashing" with tris buffer. Yields of lethal activity, calculated on a volume dilution basis, and mean time to death in the Fischer no. 344 strain of rats, averaged 50–75%. For reasons as yet unknown, recoveries of edema producing and mouse lethal activity have been consistently lower. There is some loss in activity of the crude filtrates held at 4 C for the time period required for the ultrafiltration processing. However, the differential loss in edema producing activity and mouse lethality could not be accounted for on this basis.

Electrophoresis of the UFR on paper and cellulose acetate indicated that greatest resolution was achieved with the higher ionic strength buffers and higher currents used for peptides rather than conditions optimal for larger proteins. When UFR was incubated at 35 C, the number of protein staining bands increased initially, then decreased, after 4–6 hr, with a concomitant increase in ninhydrin positive bands. On disc electrophoresis at least 16 protein staining bands were observed in fresh UFR preparations. Upon incubation at 35 C an initial increase in number, followed by a loss of protein bands, was also seen on the disc gels. Biological activity rapidly decreased in preparations held at this temperature for more than 6 hr and was entirely lost after 24 hr. All of these changes occurred upon storage at 4 C but much more slowly.

Early in this work, a proteinase, measurable by the Azocoll assay (8), was shown to be concentrated in the UFR and in partially purified protective antigen prepared by the ammonium sulfate fractionation method of Strange and Thorne (15). This enzyme is probably responsible for the rapid degradation of preparations held at 4 C and above.

Immunodiffusion of longitudinal sections of the acrylamide disc columns against antiserum prepared by hyperimmunization of a burro with spore vaccine showed that many of the 16 bands shared common antigenic determinants. At least three components formed continuous lines of immune precipitate down the length of the disc pattern. The presence of three or four antigen components identifying throughout the disc pattern was confirmed by reacting transverse sections of the column against burro antiserum in identity plates. These findings suggested that at least three components in UFR were in various states of polymerization, were complexing with other substances, or were extensively fragmented.

Initial chromatography studies of UFR employed the DEAE-cellulose-phosphate buffer programs described by Stanley and Smith (14). Recoveries of antigenic and biological activities of UFR chromatographed with this procedure were very low (25–40%) and all fractions contained significant cross-contamination. Under the conditions of these experiments, loss by enzymatic degradation was negligible. It was believed, and later demonstrated, that the $PO_4$ ion is in some manner detrimental to the UFR proteins. The heterogeneity of UFR proteins observed in column fractions also suggested enzymatic degradation, or protein-protein interaction, or both.

For further work the development of a chromatography system specifically designed for this mixture of proteins appeared essential. It was found that in ammonium acetate the UFR proteins retained their antigenic and biological activities for at least a week at 4 C. Both activities were significantly lost in $PO_4$ buffers. Since electrophoresis had shown that the mixture contained both strongly basic and strongly acidic proteins, a wide-range elution sequence was required. It was also found that Cellex celluloses gave more uniform results than the Eastman products.

At a ratio of 16 mg protein/g dry weight of cellulose, DEAE-cellulose was equilibrated with 0.02 M ammonium acetate at pH 7.0 and packed in a column of appropriate size to yield a column bed of 1:6 diameter-to-height ratio. Flow rate was adjusted to 1–1.5 ml/min without pressure. The sample of UFR, diluted 1:9 with distilled water, was applied and the column was eluted stepwise with increasing molar concentrations of ammonium acetate through 0.5 M. Volumes of buffer applied and eluate collected varied with the size of sample and column. As a final step, 0.1–0.5 N NaOH was applied to the column to remove a chromagen which will be discussed in detail later. All procedures were performed at 4 C. Protein was measured by the method of

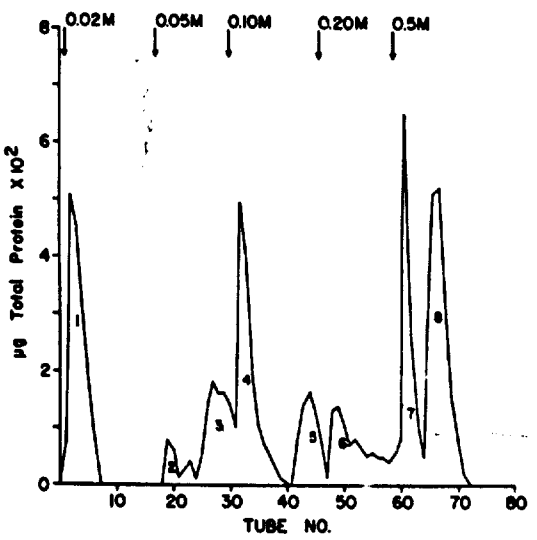


FIG. 1. Chromatography of 1 ml UFR (719 ×) on DEAE-cellulose eluted with ammonium acetate buffer gradient, pH 7.0.

Lowry (6) and antigen activity was determined by Ouchterlony's method (9) with the standard burro antiserum or the antisera of rabbits immunized with UFR with complete Freund's adjuvant.

Although rat lethality of LF-III is usually tested by recombination with an optimal number of units of partially purified PA-II (determined by titration), this method does not necessarily reflect combining proportions present in UFR or crude filtrates. Therefore, biological activity was measured by reconstitution, instead of recombination.

Figure 1 is a typical chromatogram of 1 ml of a 719 × concentrated UFR. The total volume of column eluate diluted the sample to 4.65 × concentration. Aliquots of 0.1 ml from each tube were combined and 2 ml of the mixture injected into each of three rats. Average time to death was compared with that of animals challenged with the original UFR diluted to 4.65 × concentration and the total recovery from the column was calculated in terms of the starting material. From these columns protein recoveries averaged 100% and rat lethal activity recovery averaged 80–85%.

If it is assumed that each peak is a discrete component, the volume of each peak could be diluted to 4.65 × concentration for testing alone, or any combination of peaks could be tested at the same dilution. Thus, specific rat lethal activity could be located and related quantitatively to the starting material. Frequently, combination of active fractions has had slightly higher activity than the whole column, suggesting an inhibitor which has not been further explored.

In the chromatogram shown in Fig. 1, peak 1 is a basic polypeptide which was weakly antigenic with antisera of rabbits immunized with UFR but did not react with the burro spore antiserum. It contained weak EF-1 activity when combined with the PA-II peak. Peaks 2, 3, 5, 6, and 8 contained no biological activity related to toxicity and were too weakly antigenic to be identified

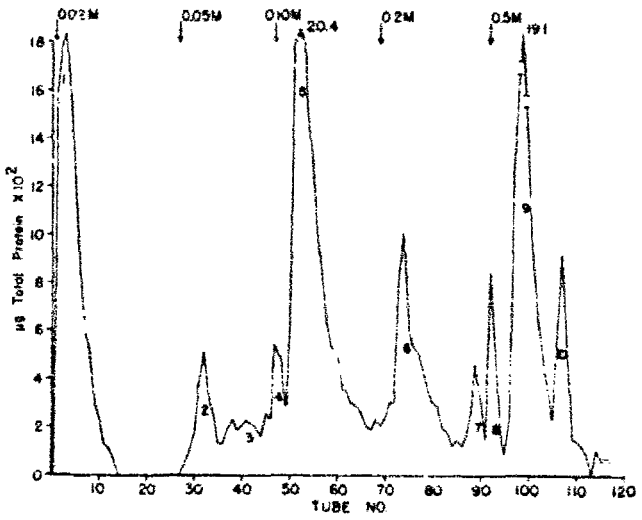


FIG. 2. Chromatography of 2 liters crude culture filtrate on DEAE-cellulose eluted with ammonium acetate buffer gradient, pH 7.0.

Peak 4 contained 90% of the PA-II component and was biologically inactive alone. In reactions with burro antiserum it presented one strong and one weak antigen band. On disc electrophoresis, this fraction showed a strong split band, reminiscent of C-1 and C-2 fractions of Strange and Thorne (15). Anodic to the main band was a weaker band and a chromagen band. Chromatography of peak 4 through ECTEOLA-cellulose with acetate buffers removed the weak band and some chromagen. The PA-II product gave a single antigen band with all antisera and only the strong split band on disc electrophoresis. Further purity studies are not complete. The fraction may still contain other materials not seen in this diluted state.

Peak 7 contained all of the LF-III activity and some PA-II by antigen analysis. It was lethal to rats only when combined with peak 4. Disc electrophoresis showed one major band with three or four minor ones. Bound PA-II could be removed by dilution and passage through the same column system. When this peak was chromatographed on an ECTEOLA-cellulose column, the LF-III product eluted by 0.5 M buffer gave a single antigen band with burro or rabbit antisera and disc electrophoresis of the fraction showed one strong band and a weak chromagen contaminant. Further analysis for purity has not been completed.

The chromatography method described was also applicable to toxic whole crude culture filtrates. The filtrate was diluted 1:6 with distilled water and applied to the column. Fractions were eluted from the column by the same buffer programs and were evaluated by the reconstitution method described.

Figure 2 is a chromatogram of 1 liter of crude filtrate. There were striking similarities to the UFR graph. The basic polypeptide was seen in peak 1; the large peak 5 contained PA-II, and the large peak 9 contained LF-III. But, there were also significant differences. EF-I in good yield was found in the same fraction as PA-II in peak 5 and peak 9 contained only LF-III activity. The PA-II in peak 5 could be further resolved on ECTEOLA-cellulose columns. The nonantigenic, biologically inactive contaminants in peak 9, detected by disc electrophoresis, could also be removed by rechromatography. Protein recoveries from crude filtrates averaged 27 ± 0.6 mg liter, excluding the chromagen eluted by NaOH. Total biological activity recoveries, calculated as rat lethality, were approximately 85%.

In 5 μliter antigen wells in microimmunoelectrophoresis, at least 1.02 μg PA-II and 0.95 μg LF-III could be detected with the use of the standard burro antiserum. Two milliliters of crude filtrate, which killed rats in 73 min, contained 17.4 μg PA-II and 6.48 μg LF-III, a ratio of about 3 to 1.

The UFR preparative procedure recovered approximately 11 mg protein liter or 43.4% of the protein in the crude filtrate (excluding chromagen). Chromatography of the biologically inactive dialysis filtrate remaining after UFR harvest yielded 17.7 mg protein liter (excluding chromagen protein) or 55% which accounted for the protein apparently lost in the ultrafiltration procedure.

A dialysis filtrate chromatogram presented the same outline of peaks as that of the UFR or crude filtrate, except for the complete absence of protein in the PA-II peak area. None of the dialysis filtrate proteins had detectable antigenic or biological activity. This filtrate apparently contains a large proportion of the low molecular weight proteins or peptides synthesized. The relationship of these dialyzable components to those which are nondialyzable but have similar chromatographic characteristics has not yet been explored.

Not shown in Figs. 1 and 2 and extending beyond peaks eluted by 0.5 M acetate buffer, is the peak of the chromagen mentioned earlier. This material could not be eluted by increasing buffer concentrations to 1.0 M or lowering pH to 4 or both, but could be removed with 0.1–0.5 N NaOH. This protein peak had more than twice the area of the others and represented at least 40% of the protein of samples of UFR, crude filtrate, or dialysis filtrate. The substance existed both in a dialyzable form and in a sequence of aggregates up to visible precipitates in UFR or filtrates. The protein complex was nonantigenic and biologically inactive, showed a strong ultraviolet absorption at 280 mμ, had an isoelectric point at pH 3.8, and dissociated into a free chromophore group and free apoprotein below that pH.

This material seemed to be made up of protein-bound intermediates of melanin formed in culture filtrates, dialysis filtrates, and UFR on standing. Alkaline hydrolysis of a UFR yielded an ether-soluble compound with strong ultraviolet absorption at 280 mμ which turned pink on oxidation and was identical with the protocatechuic acid first reported by Chao et al. (3). This dihydroquinone is probably the precursor for the melanin intermediates.

The chromagen complex, which is a ubiquitous con-

taminant in column fractions, is in a highly kinetic oxidation-reduction state, is highly acidic, readily polymerizes, and combines strongly with amine groups and cellulose. Its relationship to or effects upon other proteins in the toxic filtrates are as yet unknown. Since the chromagen or its chromophore group was shown to be capable of $Ca^{++}$ chelation, this substance may be responsible for the ultraviolet absorbing, chelating activity in EF-I preparations described by Stanley and Smith (14).

The application of the chromatographic procedure to studies of the kinetics of toxigenesis has been initiated only recently; the results are still very preliminary. Single chromatograms of 12-, 16-, and 48-hr culture filtrates have been compared in peak location, yield, and activity to the standard 24-hr filtrate chromatograms. In the chromatogram of the biologically inactive 12-hr culture filtrate, early synthesis of basic protein, PA-II, and chromagen were seen, but no LF-III was detected. The chromatogram of the fully active 16-hr filtrate presented a confusing, but highly suggestive and interesting picture. Basic protein, PA-II, and chromagen had increased in quantity and a large peak in the LF-III area was well developed. However, antigenic activity was found in other areas of the graph and the LF-III protein area was biologically inactive in all combinations with PA-II from the same preparation or others known to be fully active. The chromatogram of the nontoxic 48-hr filtrate showed extensive fragmentation of PA-II with antigenic activity in several peaks.

To continue meaningful studies of kinetics of production, more rapid preparation of larger quantities of the purified fractions is necessary. A preliminary survey of application of the chromatographic procedure to batch preparations has been made. The dry DEAE-cellulose was stirred in the diluted crude culture filtrate overnight. A column packed with this material was eluted with the stepwise sequence of buffers. It was found that the active fractions could be obtained from crude filtrates within 36 hr by this method in yields comparable to those obtained with the standard chromatography procedure. The full capacities of the system are still under investigation.

In summary, an efficient preparative column chromatography procedure was developed which was adaptable to CFR, crude filtrates, or batch collection. Yields of 80–85% of the initial biological activity were obtained in the fractions studied. The PA-II and LF-III fractions prepared by this method appeared to be pure by immunochemical analysis and disc electrophoresis, but they must be subjected to further analyses in order to meet the standard criteria of purity. The EF-I has been recorded only qualitatively in this presentation. A similar quantitative analysis for this substance by chromatography requires a more precise bioassay procedure.

Free chromophore groups, seen in the dialysis filtrate, may be synthesized in excess of the protein moiety of the chromogen component. Since the chromagen and its chromophore are actively aggregating substances, they may act as absorption centers for other proteins and this capability may account for chromagen contamination in chromatographic fractions. The influence of chromagen upon the physicochemical behavior of those proteins is not known.

The presence of considerable amounts of a basic polypeptide in these culture filtrates might contribute to aggregation with the more acidic chromagen or LF-III protein. While this aggregation might contribute to that observed in ultracentrifugal fractions (2), it apparently does not exert a significant effect upon column fractions from DEAE-cellulose. The basic protein is readily dissociated from acidic proteins by the ion-exchange procedure.

In none of the preparations studied has there been evidence to indicate that the components responsible for lethality in the rat exist as a single chemical entity. The preliminary studies on the kinetics of toxigenesis suggest that they are synthesized at different rates in vitro. The dissociation of LF-III activity from the protein eluted by 0.5 M acetate in chromatography of both the nontoxic dialysis filtrate and the fully toxic 16-hr culture filtrates suggests a kinetic extracellular assembly to yield the protein peak with full LF-III activity at 24 hr. This observation makes it tempting to speculate that the protein may serve as an inert carrier for a small, toxic hapten. The work of A. J. Gaspar (unpublished data) and Molnar and Altenbern (7) demonstrated that separate injections of PA-II and LF-III (within 1–4 hr depending upon which was given first) were fully lethal for the rat. There is the suggestion, then, that these two components act sequentially, rather than in combination, in this species. The sum total of all of the above observations provides basis for further speculation and the hypothesis that PA-II activates some mechanism (possibly enzymatic) in the rat which acts to release a toxic hapten from its protein carrier. The requirement for a contribution by the host of some factor essential for full toxic activity is compatible with the observed differences in response to toxin in different species of test animals.

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