Isolation and Characterization of Staphylococcus Alpha-Hemolysin *

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Introduction

OF THE SEVERAL diffusible products of the staphylococcus aureus, the alpha-hemolysin is the most toxic in the experimental animal.[1, 14] In the rabbit this substance produces extensive dermal necrosis when given intradermally, renal cortical necrosis when given intravenously in sublethal doses and rapidly fatal circulatory collapse when given in large dose intravenously.[12] The local and general vascular changes appear to be mediated both by direct action of the toxin on the smooth muscle cell and by the liberation of serotonin and histamine from blood and tissue stores.[13] The common finding of antibody to the alpha-hemolysin in humans suggests that this toxin is commonly liberated in the human host. Some have ascribed an important role to this agent in human staphylococcus infection while others have regarded its role as minor. Experimental work in this area has been complicated by the use of crude culture filtrates.

It is the aim of the present paper to describe a method for the purification of alpha-hemolysin and to describe some of the chemical and physical properties of the purified substance.

Materials and Methods

Culture Filtrates

A four-hour brain-heart infusion broth of the Wood 46 strain of staphylococcus aureus was added to 2.5 to 7.5 L. quantities of media prepared by the method of Leonard and Holm.[5] The inoculated media were incubated at 37° C. with shaking and continuous aeration of a mixture of 50 per cent CO_2 and 50 per cent O_2 for 72–96 hours. Following incubation cultures were centrifuged at 15,000 rpm for 30 minutes at 5° C. and sterilized by pressure filtration through a Seitz pad of 0.1 micron pore size. Aqueous thimerosal was added to a final concentration of 1/10,000.

Bio-Assay

Hemolytic potency was determined by the spectrophotometric method of Jackson.[3] The toxin was titrated by adding one ml. of 1 per cent cell suspension to one ml. of toxin dilution in buffer (pH 6.9) and incubating for 15 minutes at 37° C. The hemolytic unit (minimal hemolytic dose, MHD) was defined as the reciprocal of the final dilution producing 50 per cent hemolysis.

Dermonecrotic activity was determined by injecting 0.1 ml. of various dilutions of the toxin intradermally into the shaved backs of adult albino rabbits. The dermonecrotic unit (minimal dermonecrotic dose, MDD) was defined as the reciprocal of the final dilution causing an area of necrosis 10 mm. in diameter after four days.

Lethal activity was measured by the intraperitoneal injection of 0.5 ml. of various dilutions of the toxin into groups of mice weighing from five to 25 grams. Sixteen hours later the number of deaths and survivals was recorded. The LD_{50} was calculated according to Reed and Muench.[10]

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Leucocidic Activity. Leucocytes were collected from albino rabbits following intraperitoneal injections of sterile saline. Eighteen hours after an initial injection of 300 ml., a second injection, also 300 ml., was administered by the same route. Three hours after the second injection, the remaining 100–200 ml. of fluid was aspirated from the peritoneal cavity. The suspension was filtered through cheesecloth and centrifuged. The sedimented leucocytes were resuspended and used to assay leucocidic activity as described by Wood.[16] The incubation period following addition of dye and KCN was 10 minutes. The leucocidic unit (minimal leucocidic dose, MLeD) was defined as the reciprocal of the final dilution in which the blue color was retained.

Analytical Methods

Estimates of protein concentration were made on the basis of ultraviolet absorption at 280 and 260 mu in a Beckman DU spectrophotometer.

Nitrogen content was determined by the micro-Kjeldahl method according to Ma and Zuazaga.[6]

Ultracentrifugal analyses of purified hemolysin were done in the Spinco Model E analytical ultracentrifuge. The synthetic boundary cell was used.

Moving boundary electrophoresis was carried out in the Spinco Model H electrophoresis-diffusion instrument. Acetate buffer, pH 5.0, and phosphate buffers, pH 6.8 and 8.0 respectively, were used. All were made up to 0.1 ionic strength.

Ultraviolet absorption spectra of purified hemolysin were determined in a Beckman DB spectrophotometer with a Sargent Model SRL recorder attachment. The scanning rate was 40 mμ/min.

Antigenic Analysis. The double diffusion methods of Oakley [8] and of Ouchterlony [9] were used. Micro-immunoelectrophoresis was done by the method of Schiedeger.[11]

Fractionation Procedures

Batches of culture filtrate (4.7 to 13.7 L, protein concentration 8–10 mg./ml., ionic strength 0.11–0.12, pH 6.5–6.8) were fractionated with methanol by the method of Wittler and Pillemer.[15] Precipitates were recovered by centrifugation at 15,000 rpm for 30 minutes at −5° C. A Spinco Model L preparative ultracentrifuge with a batch rotor was used for this purpose. Precipitates were extracted at 1–3° C. with 0.05 M sodium phosphate buffer, pH 6.8 and ionic strength 0.1. The extracts were then freeze-dried and stored at 4° C.

DEAE-Sephadex Chromatography. DEAE-Sephadex A-50,* coarse, (1 Gm. resin/200 mg. protein) was washed according to supplier's instructions and equilibrated with 0.05 M sodium phosphate buffer, pH 6.8, ionic strength 0.1. The freeze-dried extracts from methanol fractionation were pooled to make two samples. These were dissolved in water, dialyzed against buffer, and subjected to batchwise chromatography on DEAE-Sephadex. This was accomplished by adding the dissolved extracts to slurries of Sephadex and stirring for one hour. Recovery of unadsorbed material was effected by suction filtration through Whatman No. 1 filter paper. The filtrates were then freeze-dried.

CM-Sephadex Chromatography. CM-Sephadex C-50, medium, was washed according to supplier's instructions, packed in columns and equilibrated at 5° C. with 0.02 M phosphate buffer pH 6.1, ionic strength 0.026. The freeze-dried filtrates from DEAE-Sephadex chromatography were dissolved in water, dialyzed against buffer and adsorbed on the columns. Elution was by stepwise increases in buffer concentration at constant pH. Effluent was collected by the drop count method and assayed for ultraviolet absorption and hemolytic activity. Fractions demonstrating

* Pharmacia, Upsala, Sweden.

TABLE 1. *Properties of Several Batches of Culture Filtrate*

No.	Volume ml.	Prot. Con. mg./ml.	$\frac{OD_{280}}{OD_{260}}$	Hemolytic Activity MHD/ ml.	Hemolytic Activity MHD/ mg. prot.	Incubation Time hr.	pH
1	2,090	9.76	0.86	422.5	43.3	96	6.7
2	2,365	8.18	0.78	15.3	1.9	72	6.5
3	2,365	8.39	0.81	95.1	11.3	72	6.5
4	2,459	8.03	0.78	275.6	34.3	96	6.7
5	2,497	8.70	0.83	719.2	82.7	96	6.7
6	2,255	8.96	0.80	352.6	39.4	96	6.7
7	3,850	9.20	0.79	667.2	72.5	72	6.5
8	4,180	9.67	0.81	525.3	54.3	96	6.6
9	6,930	8.09	0.79	143.6	17.8	96	6.7
10	5,192	8.19	0.77	94.7	11.6	96	6.7
11	11,220	9.34	0.81	160.8	17.2	96	6.8
12	13,810	10.36	0.84	600.0	57.9	96	6.8

activity were pooled, concentrated, dialyzed against starting buffer, and readsorbed on columns of CM-Sephadex freshly prepared as described above. A linear ionic strength gradient from 0.026 to 0.126 (0.1 N NaCl in buffer) was then applied to the column. Effluent was collected as described above and assayed for ultraviolet absorption, conductivity, and hemolytic potency. Fractions demonstrating activity were pooled, freeze-dried and stored at 5° C.

Results

Toxin Production

Some properties of culture filtrates are listed in Table 1. The hemolytic potency of these filtrates did not compare favorably with that of filtrates produced by the small scale method described by Leonard and Holm.[5]

Fractionation

An outline of fractionation procedures is seen in Table 2.

When temperature conditions were rigidly controlled 80 to 90 per cent recovery of total activity and 30- to 38-fold increases in specific activity could be achieved from methanol fractionation.

DEAE-Sephadex chromatography. Preliminary column chromatography experiments showed that the hemolysin was not adsorbed by DEAE-Sephadex A-50, medium, at low ionic strength in the pH range from 6.8 to 7.6. In the light of these results, batch processing with the coarse grade resin was carried out. Effluent from 0.05 M phosphate buffer pH 6.8 contained 99.8 per cent of the hemolysin added to the resins, purified 1.2–1.5 fold.

CM-Sephadex chromatography. Table 3 shows the results of stepwise elution of a DEAE-Sephadex product from a column of CM-Sephadex. A typical pattern is shown in Figure 1. Eluate from 0.03 M buffer contained 92.0 per cent of the hemolysin added to the column and yielded a 5.7 fold increase in specific activity. As seen in Figure 2 re-adsorption and gradient elution of the active fraction resulted in a single protein peak.

Bio-Assay of Purified Products

The results of assays for hemolytic, dermonecrotic, lethal and leukocidic activities are summarized in Table 5. The various activities were inseparable, but all did not exhibit parallel increases in specific activity. Increases in hemolytic activity, however, somewhat closely paralleled increases of leukocidic activity. Similarly, values for purification factors of dermonecrotic activity approximated those for lethal activity.

TABLE 2. *Fractionation Procedures*

Culture filtrate

Methanol fractionation
Glacial acetic acid to pH 4.0 at 0° C.
Methanol to 15 per cent at −5° C.
——— Supernate

Ppt.
Extracted with 0.05 M phosphate buffer, pH 6.8
Dialyzed vs. buffer

DEAE-Sephadex chromatography (batchwise)
——— Adsorbed material

Unadsorbed material
At 0.05 M phosphate buffer, pH 6.8
Concentrated
Dialyzed vs. 0.02 M phosphate buffer, pH 6.1

CM-Sephadex chromatography
(stepwise elution at pH 6.1)
——— Eluate from 0.02 M buffer
Adsorbed material

——— Adsorbed material

Eluate from 0.03 M buffer
Concentrated
Dialyzed vs. 0.02 M Phosphate buffer, pH 6.1

Rechromatography on CM-Sephadex
Gradient elution at pH 6.1
(0.02 M phosphate buffer and 0.1 M NaCl in buffer)

Purified alpha hemolysin

As shown in Figure 3, solutions of purified hemolysin (about 0.1%) were unstable at 5° C. in phosphate buffer pH 6.1. About 56 per cent of original hemolytic activity was lost after 24 hours and 80 per cent lost after 72 hours. Freeze-drying, dissolution and dialysis against phosphate buffers of pH 6.8 or 8.0 and acetate buffer pH 5.0 resulted in precipitation of much of the protein with irreversible activity losses. Instability of purified samples may thus somewhat account for the nonparallel specific activity increases since the four assay procedures were carried out in succession rather than simultaneously.

Homogeneity Studies

Antigenic analysis by diffusion yielded a single precipitin line when purified hemolysin (0.1 to 0.01%) was tested against commercial antitoxin * in a concentration of 10 to 30 units/ml. Figures 4 and 5 show results of diffusion analyses of culture filtrate and purified hemolysin by the methods of Ouchterlony and of Oakley respectively. Figure 6 shows results of immunoelectrophoresis of culture filtrate and purified hemolysin. The buffer used was Borate Phosphate pH 8.2, ionic strength 0.05. All three immunologic methods reveal the presence of a single antigenic component in purified hemolysin as opposed to the multiple components seen in crude culture filtrate.

Figure 7 consists of sedimentation patterns from ultracentrifugal analysis of a

* Lederle Laboratories, Pearl River, New York.

TABLE 3. *Chromatography of Alpha-Hemolysin on CM-Sephadex*

	Volume ml.	Prot. Conc. mg./ml.	Tot. Prot. mg.	Yield Prot. %	Hem. Titre. MHD/ml.	Tot. MHD	Yield MHD %	MHD / mg. Prot.	Purif. Factor	$\frac{OD_{280}}{OD_{260}}$
Before chromatography	355	7.67	2,722.8	—	9,000	3,195,000	—	1,173.4	—	1.40
Eluate from 0.02 M phosphate buffer	735	2.17	1,594.9	58.6	132.6	97,461	3.1	61.1	—	1.39
Eluate from 0.03 M phosphate buffer	780	0.56	436.8	16.0	3,767.4	2,938,572	92.0	6,727.5	5.7	1.77
Eluate from 0.04 M phosphate buffer	—	—	—	—	—	—	—	—	—	—
Eluate from 0.05 M phosphate buffer	—	—	—	—	—	—		—	—	—
Eluate from 0.5 M NaCl in 0.05 M phosphate buffer	480	0.84	412.8	15.2	47.9	22,992	0.7	55.7	—	1.30

TABLE 4. *Properties of Alpha-Hemolysin at Various Stages of Purification*

	Prot. Conc. mg./ml.	Tot. Prot. mg.	$\frac{OD_{280}}{OD_{260}}$	Yield Prot. %	Hem. Titre. MHD/ml.	Tot. MHD	Yield MHD %	MHD / mg. Prot.	Purif. Factor
Sample No. 1									
Culture filtrate	8.49	148,575	0.78		300	5,250,000		35.3	
After methanol fractionation	7.66	3,255	0.83	2.3	3,384.5	1,438,412	27.4	441.8	12.5
After DEAE-Sephadex chromatography	2.25	2,790	1.00	85.7	1,157.9	1,435,796	99.8	514.6	14.6
After CM-Sephadex chromatography	0.24	100.4	1.61	9.0	1,365.3	573,426	57.3	5,712.5	161.8
After rechromatography on CM-Sephadex by continuous gradient elution	0.64	76.8	1.78	77.8	4,627.4	555,288	99.8	7,230.3	204.8
Sample No. 2									
Culture filtrate	10.11	312,399	0.83		237.7	7,344,930		23.5	
After methanol fractionation	12.27	7,828	0.95	2.5	5,428.5	3,463,383	47.2	442.4	18.8
After DEAE-Sephadex chromatography	2.46	4,231	1.13	54.0	2,005.9	3,450,148	99.6	815.4	34.7
After CM-Sephadex chromatography	0.56	436.8	1.77	16.0	3,767.4	2,938,572	92.0	6,727.5	286.3
After rechromatography on CM-Sephadex by continuous gradient elution	1.38	179.4	1.86	44.5	11,250	1,452,500	57.6	8,153.6	346.9

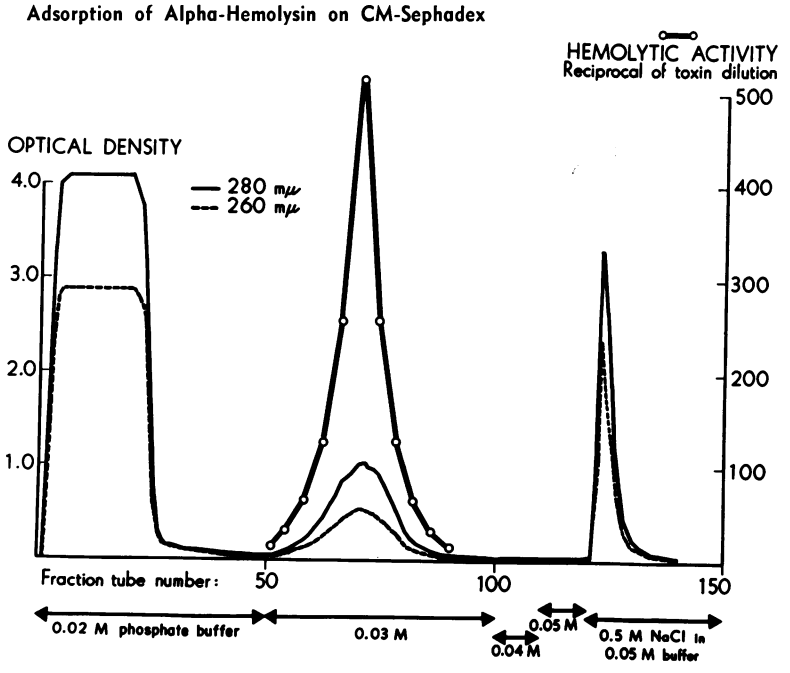

FIG. 1. Separation of alpha hemolysin from nontoxic components by stepwise elution from a column of CM-Sephadex C-50 medium at pH 6.1 Sample: 2.722 Gm, fractionated by methanol and DEAE-Sephadex. Column size: 2 × 7.2 cm.

purified preparation. The presence of only one sedimenting boundary throughout the run suggests homogeneity.

Moving boundary electrophoresis revealed the presence of a major and a minor peak, both of which migrated cathodally at pH 5.0. At pH 6.8 neither migrated. At pH 8.0 very slow anodal migration of both was observed.

These results suggest the presence of an impurity with electrochemical properties very similar to those of the hemolysin. Both substances appear to have isoelectric points in the vicinity of pH 6.8.

Characterization Studies

Purified hemolysin contained 15.2 to 15.8 per cent nitrogen and exhibited maximum ultraviolet absorption at 280 mu (Fig. 8).

The presence of carbohydrate in purified hemolysin was established by the Molish

TABLE 5. *The Properties of Purified Alpha-Hemolysin*

	Culture Filtrate	Purified	Purification Factor
Sample 1			
Hemolytic activity	96.2 MHD/mg. N	45,761.4 MHD/mg. N	475.7
Dermonecrotic activity	12.8 MDD/mg. N	1,582.3 MDD/mg. N	123.6
Lethal activity (LD_{50})	38.8 μg N	0.47 μg N	82.5
Leucocidic activity	40.9 MLeD/mg. N	12,658.2 MLeD/mg. N	309.5
Sample 2			
Hemolytic activity	75.4 MHD/mg. N	53,645.9 MHD/mg. N	711.5
Dermonecrotic activity	12.7 MDD/mg. N	2,459.3 MDD/mg. N	193.6
Lethal activity (LD_{50})	39.3 μg N	0.19 μg N	206.8
Leucocidic activity	20.2 MLeD/mg. N	12,147.5 MLeD/mg. N	601.4

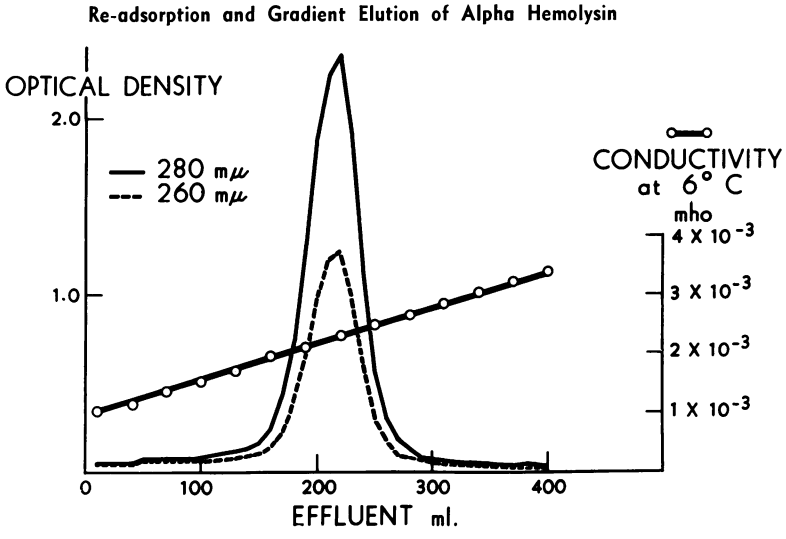

FIG. 2. Gradient elution of the active fraction shown in Figure 1 after it was readsorbed on a freshly prepared column of CM-Sephadex. Gradient device: two identical vessels in hydrostatic equilibrium containing respectively, 0.02 M sodium phosphate buffer and 0.1 N NaCl in buffer. Sample: 403 mg. Column size: 1 × 10.5 cm, fraction volume: 10 ml., flow rate 11 m/hr.

test and the Schiff test. A Sudan-black test for lipid was negative.

Discussion

A number of attempts have been made to purify staphylococcal hemolysin. Recently Kumar *et al.*[4] reported isolating the toxin by subjecting it to continuous flow paper curtain electrophoresis at pH 8.6 followed by another run at pH 5.6. Goshi [2] purified the toxin by successive steps of cold trichloroacetic acid and methanol fractionation followed by column chromatography on DEAE cellulose and calcium hydroxylapatite. Madoff and Weinstein [7] employed zinc acetate precipitation, gel filtration on Sephadex, curtain electrophoresis, and column chromatography on DEAE-cellulose.

During preliminary investigations in this laboratory it was observed that considerable activity losses occurred when culture filtrate was subjected to starch block elec-

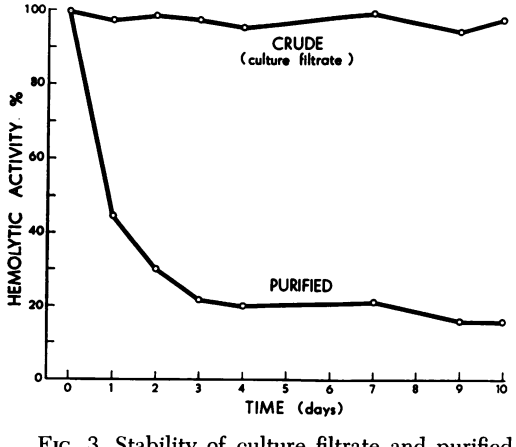

FIG. 3. Stability of culture filtrate and purified hemolysin at 5° C. Culture filtrate: 10.11 mg./ml. Purified: 1.38 mg./ml.

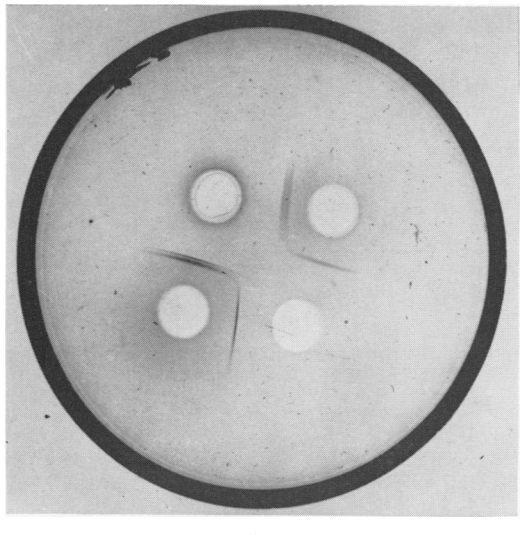

FIG. 4. Antigenic analysis of culture filtrate and purified hemolysin by the method of Ouchterlony. Upper left: culture filtrate (20 mg./ml.). Lower right: purified hemolysin (0.6 mg./ml.). Lower left: antitoxin (30 units/ml.). Upper right: antitoxin (20 units/ml.).

trophoresis. Combined active fractions yielded only 10 to 30 per cent of the original total hemolytic units and specific activity values were decreased by 75 to 86 per cent. It is not known whether these losses were due to the electrophoretic process, to some property of the starch, or to separation of the toxin from stabilizing agents. The use of electrophoresis as a preparative technic was subsequently abandoned in favor of ion exchange chromatography.

Ion exchange chromatography has been used successfully for purification of a variety of proteins. During preliminary investigations the authors had occasion to employ CM-cellulose and calcium hydroxylapatite. It was observed that alpha-hemolysin trailed considerably on both columns and was eluted from CM-cellulose over a fairly wide range of parameters. It was subsequently found that DEAE-Sephadex A-50 and CM-Sephadex C-50 resulted in low nonspecific adsorption and elution over narrow ranges with superior yield and reproducibility. In addition, specific activity values of samples processed on the Sephadex resins were 28 to 33 times greater than those of samples processed on cellulose and apatite. Hemolytic, dermonecrotic, leucocidic and lethal activity were inseparable which is in accord with the findings of Goshi,[2] of Kumar [4] and of Madoff.[7]

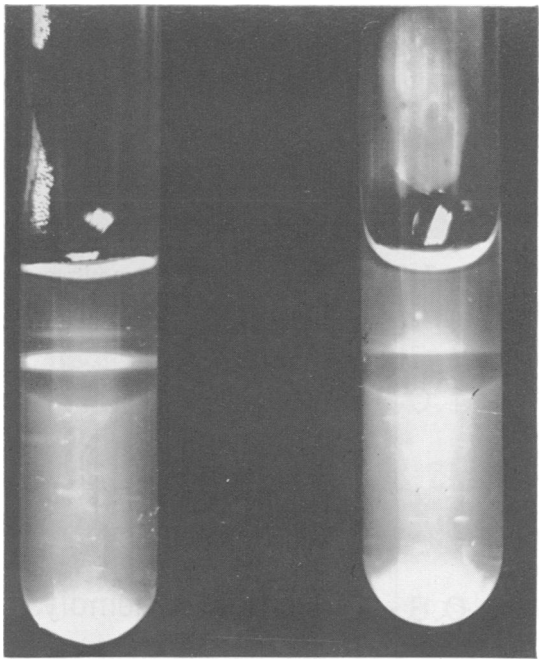

FIG. 5. Antigenic analysis by the method of Oakley. Left: culture filtrate (30 mg./ml.) vs. antitoxin (40 units/ml.). Right: purified hemolysin (0.6 mg./ml.) vs. antitoxin (30 units/ml.).

Culture filtrates containing low ratios of active-to-total protein and the marked instability of purified preparations constituted major obstacles throughout this investigation.

Certainly a substantial reservoir of a

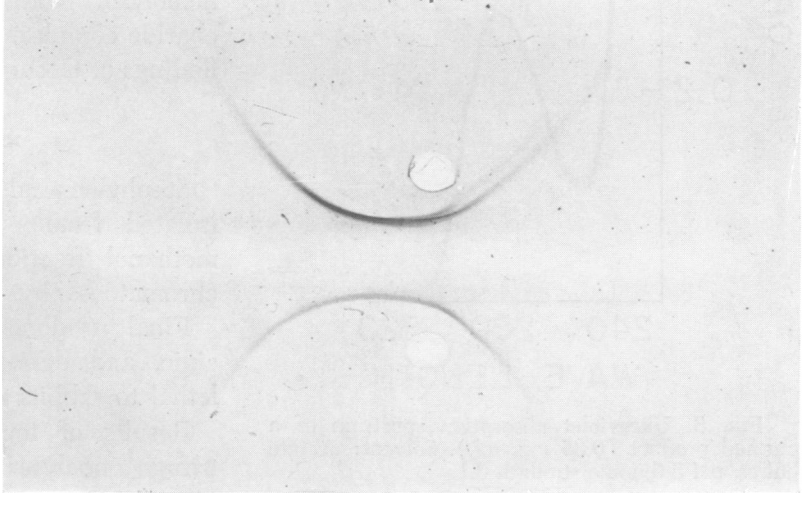

FIG. 6. Immuno-electrophoresis of culture filtrate and purified hemolysin. Upper well: culture filtrate, 40 mg./ml. Bottom well: purified, 0.6 mg./ml. Center elongated well: antitoxin, 30 units/ml.

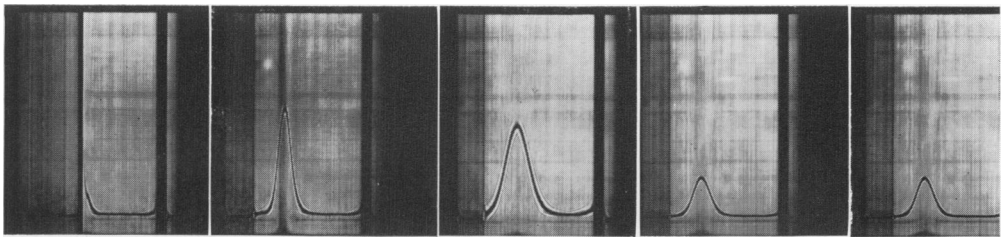

FIG. 7. Sedimentation patterns from ultracentrifugation of purified hemolysin in a synthetic boundary cell. Photographs from left to right were taken at 4", 20", 35", 65", and 67" following start of run. Solvent: 0.1 M phosphate buffer pH 6.8. Protein concentration: 0.78 mg./ml. Rotor speed: 15,220 RPM.

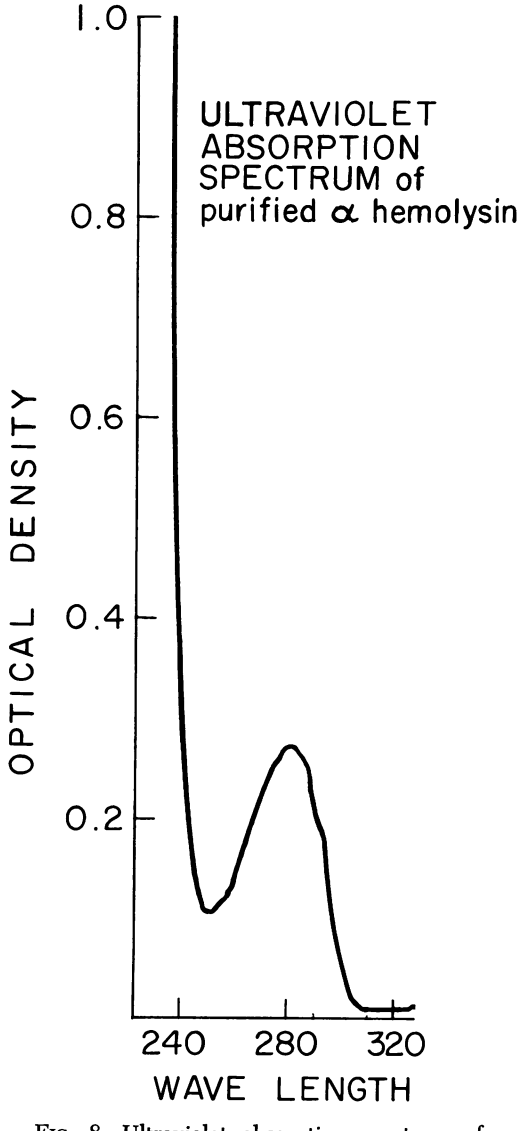

FIG. 8. Ultraviolet absorption spectrum of a purified product (0.33 mg./ml.). Solvent: acetate buffer, pH 5.0, ionic strength 0.1.

stable purified product is needed prior to undertaking extensive physico-chemical characterization studies.

While nonsynthetic media have in general been shown to yield the most potent filtrates, their use considerably complicates purification. Conversely, synthetic media, ideal for purification purposes, seem inadequate in terms of the content of active protein which can be recovered in pure form.

Rapid deterioration of purified samples considerably hampered characterization studies. Purified hemolysin retained antigenic properties following deterioration resulting in loss of biological activity. A mixture of toxin and toxoid may thus account for the presence of two distinct mobile components during boundary electrophoresis.

In the present study results of characterization studies indicate that the alpha-hemolysin consists of a protein-polysaccharide complex. This is in accord with the findings of Goshi [2] and of Kumar.[4]

Summary

Staphylococcal alpha-hemolysin has been isolated from culture filtrates following methanol fractionation and ion-exchange chromatography.

Final products were hemolytic, leucocidic, and dermonecrotic to rabbits and lethal to rabbits and mice.

Results of immunologic and ultracentrifuge analyses indicate homogeneity.

Moving boundary electrophoresis studies revealed a major and a minor component both with isoelectric point in the vicinity of pH 6.8.

These observations together with the results of qualitative carbohydrate tests and ultraviolet absorption analysis lead to the conclusion that alpha-hemolysin consists of a protein-polysaccharide complex with isoelectric point in the vicinity of pH 6.8.

Marked instability of pure hemolysin temporarily prevented more precise physico-chemical characterization.

Acknowledgments

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