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Purification and Properties of Staphylococcal Beta Hemolysin

II. Purification of Beta Hemolysin[1]

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Staphylococcal beta hemolysin from the 681 strain of *Staphylococcus aureus* grown in a Heart Infusion dialysate semisolid medium under 10% carbon dioxide was obtained in an immunoelectrophoretically pure form by a combination of procedures of precipitation with 2 volumes of acetone followed by chromatography on diethylaminoethyl cellulose at *p*H 6.0. The acetone precipitation procedure did not show any deleterious effect on the hemolytic activity of the beta hemolysin unless the precipitate was left in contact with the acetone for at least 4 hr. The crude preparations contained two types of beta hemolysin. One of these represented the major portion of the total activity of beta hemolysin and behaved as a cation. The other represented a minor (1/5,000) portion of the total beta hemolysin activity and behaved as an anion. These active principles were designated as cationic and anionic beta hemolysins, respectively. An unexpected increase in the total beta hemolysin activity of the crude preparations was noted when these were concentrated by dialysis against polyethylene glycol (20 M). This effect was probably due to polyethylene glycol. A further unexpected increase in the titer of the acetone-precipitated preparations occurred when these were lyophilized. The reason for this incremental increase is not known. It may be due to fragmentation of the beta hemolysin.

Certain strains of *Staphylococcus aureus* are capable of producing a hemolytic macromolecule with characteristic ability to cause "hot-cold" lysis of sheep erythrocytes. This hemolysin, designated *beta* hemolysin by Glenny and Stevens (5), is most readily identified by the characteristic zone of darkening or discoloration that it produces on sheep blood-agar plates.

Until recently, "hot-cold" lysis of sheep erythrocytes was the only activity attributed to beta hemolysin. Chesbro et al. (2) have now shown that the beta hemolysin which they obtained in partially purified form was leukocidal for guinea pig macrophages and was able to liberate mucopolysaccharides from the rabbit erythrocyte stroma, polysaccharides and mucopeptides from staphylococcal cell walls, and rhamnose, glucose, *N*-acetyl glucosamine, and at least two polysaccharides from enterococcal cell walls. More recently, Maheswaran and Lindorfer (10), using beta hemolysin preparations of "demonstrated" purity, have shown the lysin to be a phospholipase, as it was able to degrade sphingomyelin. Similar findings were earlier reported by Doery et al. (4), who used beta hemolysin preparations of undefined purity.

Despite the multiplicity of activities that have now been ascribed to beta hemolysin, its role, if any, in human or bovine disease of staphylococcal origin as yet remains unknown, and is likely to remain so unless sufficient quantities of beta hemolysin are obtained in pure form. The work reported herein represents an attempt to develop a simple method of obtaining large quantities of purified beta hemolysin. Recently, attempts to purify beta hemolysin have also been made by Chesbro et al. (2) and by Maheswaran et al. (11). These investigators, whose procedures for purifying beta hemolysin differed from those reported here, have confirmed the major findings earlier reported by Haque (Ph.D. Thesis, Ohio State

[1] Portions of this study were presented at the 63rd Annual Meeting of the American Society for Microbiology, Cleveland, Ohio, May 1963. This paper is based on portions of a dissertation submitted by R. Haque to the Graduate School of The Ohio State University, Columbus, in partial fulfillment of the requirements for the Ph.D. degree.

University, 1963). Certain discrepancies were noted by these investigators, and these are discussed in the light of new evidence.

MATERIALS AND METHODS

Strain of S. aureus. *S. aureus* strain 681 used in this study was previously described (8, 9). It was routinely maintained on Trypticase Soy Agar (BBL) slants. Before use, it was streaked onto sheep blood-agar plates, and a single isolated colony showing a zone of beta hemolysin was picked and used in the production of beta hemolysin.

Production of beta hemolysin. Heart Infusion (HI) dialysate containing 0.3% agar (Difco), prepared by the method previously described (9), was used for the production of beta hemolysin. The medium, after sterilization by autoclaving and cooling to 45 C, was inoculated in bulk. The inoculum consisted of a 5-hr-old culture of the beta hemolysin-producing colony propagated in HI dialysate broth. The inoculated medium, while still liquid, was dispensed in 18- to 20-ml quantities in sterile petri plates, and the plates were incubated for 24 hr at 37 C in an atmosphere containing 20% carbon dioxide (13).

Harvesting of beta hemolysin. After incubation, the contents of the plates were pooled and the resulting slurry was centrifuged at 3,000 × *g* for 10 min. The supernatant fluid was collected, and the sedimented mass was washed twice with equal volumes of phosphate-buffered saline, *p*H 7.0. The washings were pooled with the supernatant fluid previously collected. The pooled material was aseptically filtered through a sterile 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.). The filtrate was tested for sterility and then was stored at −20 C. Upon thawing, the sterile filtrate was found to contain a white granular precipitate. This was removed by centrifugation, and the clear supernatant fluid obtained was designated as the crude beta hemolysin preparation.

Quantitation of beta hemolysin. The titration procedure previously described (9) was used. The highest dilution which lysed 50% of the sheep erythrocytes was recorded as the titer. The specific activity of the beta hemolysin preparation was determined by dividing the reciprocal of the titer of the preparation by the milligrams of protein per milliliter of the preparation. The efficiency of the purification procedure was determined by comparing the specific activity of the various preparations, the specific activity of the starting sample being taken as equal to one. Protein concentration was determined by the biuret method, as described by Gornall, Bardawill, and David (6).

Precipitation of beta hemolysin with ammonium sulfate. Initially, the procedure described by Cohn et al. (3) was adopted, and 50.0-ml quantities of crude beta hemolysin were equilibrated at 4 C against 450 ml of ammonium sulfate solutions of the desired molarity. The molarity of the solutions of the ammonium sulfate was then gradually increased, and the precipitates, if any, were recovered by centrifugation. These were dissolved in phosphate-buffered saline and then dialyzed, first against distilled water and then against phosphate-buffered saline, *p*H 7.0. The volume after dialysis was adjusted to 50.0 ml by the addition of saline, and the solution was tested for beta hemolysin and total protein. In subsequent experiments, the procedure used by Mahesawaran et al. (11) was employed.

Precipitation of beta hemolysin with acetone. Unless mentioned otherwise, 2 volumes of acetone (chemically pure) chilled to −20 C were slowly added to a constant volume of crude beta hemolysin solution cooled to 4 C. The mixture was gently but continuously stirred for 1 min, and the resulting precipitate was collected by centrifugation. This was dissolved in phosphate-buffered saline, *p*H 7.0, and then was tested quantitatively for beta hemolysin and protein.

Concentrating dilute solution of beta hemolysin. Crude preparations of beta hemolysin were concentrated by dialysis against polyethylene glycol (20 M) followed by lyophilization. Acetone-precipitated preparations were concentrated by lyophilization. Fractions obtained by column chromatography were concentrated by pervaporation.

Column chromatography on ion-exchange celluloses. Both the carboxymethyl (CM) and the diethylaminoethyl (DEAE) celluloses (type 20) obtained from Carl Schleicher & Schuell Co., Keene, N.H., were used. These were converted to their hydroxyl and sodium forms, respectively, by the procedure described by Peterson and Sober (12) and then were equilibrated against the starting buffer until the filtrates showed the same *p*H and conductivity as the starting buffer. The ion-exchange material was then packed in glass columns [36 inches (91.4 cm) long; 0.75 inch (1.9 cm) inner diameter] to a height of 30 inches (76.2 cm). After the addition of the sample, the material absorbed onto the celluloses was eluted by the stepwise elution procedure, with the use of phosphate buffers of increasing molarities. In initial experiments, a total of 125.0 ml of the buffer of each concentration was employed, and the effluent was collected in a 50.0- and a 75.0-ml fraction. These were concentrated by pervaporation to 10.0 ml and then were analyzed quantitatively for their contents of beta hemolysin and protein. In subsequent experiments, fractions of 5.0 ml each were collected. These were first tested qualitatively for beta hemolysin by soaking a filter paper disc of 0.25 inch (0.64 cm) diameter (penicillin assay disc, Carl Schleicher and Schuell Co.) in the fraction and placing it on sheep blood-agar plate (phosphate buffer-saline-agar containing 3% washed sheep erythrocytes). The plates were refrigerated overnight when the typical zones of beta hemolysin were noted. Fractions containing beta hemolysin were then titrated while all of the fractions were tested quantitatively for protein.

Continuous-flow electrophoresis. Continuous-flow paper electrophoresis equipment was constructed, with few modifications, according to the description published by Blanco (1). A 10 × 17 inch (25.4 × 43.2 cm) filter paper curtain (Whatman no. 17) was employed. The sample was held 5 cm above the curtain and was applied to the curtain by capillary action with a filter paper strip which rested on the center of the curtain at a point 10 cm from the top of the curtain. The electrophoresis was conducted with a constant current of 40 ma. The procedure yielded 24 fractions

which were analyzed quantitatively for their contents of beta hemolysin and protein.

Production of anti-beta hemolysin. New Zealand white rabbits were immunized intravenously with crude preparations of beta hemolysin. The animals were given a series of injections at intervals of 2 days. The first four injections consisted of 0.1 ml of the crude beta hemolysin. The remaining five injections consisted of 0.3, 0.75, 0.75, 1.0, and 1.0 ml of the crude beta hemolysin. The rabbits were exsanguinated 1 week later. The antiserum so obtained had an anti-beta hemolysin titer of 1:25,600.

Immunoelectrophoresis. Gel for electrophoresis was prepared in phosphate buffer, *p*H 7.0, ionic strength 0.05, and contained 0.5% Ionagar No. 2 (Colab Laboratories, Inc., Chicago Heights, Ill.). The samples were electrophoresed for 2.5 hr at 5 v/cm. The antiserum prepared against the crude beta hemolysin was then added, and the plates were incubated at 4 C for 1 to 7 days. The gel was then washed, first with saline and then with distilled water, and finally was dried after it had been covered completely with a moist piece of filter paper. The precipitin lines were stained with nigrosine (nigrosine, 5%; acetic acid, 25.0 ml; water 1 liter). Excess stain (from the gel was removed) by washing it in a 2.5% solution of acetic acid.

RESULTS

Precipitation of beta hemolysin with ammonium sulfate. Dialysis of the crude beta hemolysin against increasing concentrations of ammonium sulfate yielded a precipitate when the concentration of ammonium sulfate had reached 1.5 M. This precipitate did not contain the beta hemolysin. No further precipitate appeared even when the concentration of ammonium sulfate was increased to 5.0 M. Beta hemolysin, therefore, did not precipitate by this procedure. Analysis of the supernatant fluid after removal of the ammonium sulfate by dialysis revealed that all the beta hemolysin was still present in this material.

Direct addition of 3.8 M ammonium sulfate (50.5 g per 100 ml) to the crude preparation (11) yielded a fairly heavy precipitate which contained all the beta hemolysin activity.

Precipitation of beta hemolysin with acetone. Addition of 10 volumes of chilled acetone (−20 C) to 1 volume of crude beta hemolysin (4 C) yielded a brown precipitate which appeared almost immediately and contained all the beta hemolysin activity. This precipitate also contained almost the entire amount of protein present in the crude material. In subsequent experiments, attempts were made to determine whether lesser amounts of acetone would selectively precipitate beta hemolysin. Quantities of 50 ml of crude preparation of beta hemolysin, previously dialyzed against two changes of phosphate buffer (0.05 M), *p*H 7.0, were mixed with various amounts of chilled acetone. The mixture was kept at 4 C until the precipitate appeared. This was recovered by centrifugation, dissolved in 50.0 ml of phosphate buffer, and dialyzed. Analyses of the dialyzed solutions showed that beta hemolysin was rapidly and fairly selectively precipitated when the ratio of acetone to crude beta hemolysin was 2:1 (Table 1). This ratio of acetone to crude beta hemolysin did not affect the hemolytic activity of beta hemolysin unless the precipitate remained in contact with acetone for as long as 4 hr at 4 C (Table 2). In later experiments, it was found that equilibration of the crude hemolysin preparations against the phosphate buffer prior to precipitation with acetone was not needed. Acetone was, therefore, added directly to the crude preparations of beta hemolysin, and the resulting precipitate, recovered by centrifugation, was dissolved in phosphate-buffered saline.

Concentration of crude beta hemolysin with polyethylene glycol. Prior to chromatography on ion-exchange celluloses, the crude preparations of beta hemolysin were concentrated fourfold by

TABLE 1. *Fractionation of crude preparation of beta hemolysin with various amounts of acetone*

Fraction no.	Ratio of acetone to crude beta hemolysin (by volume)	Time precipitate appeared (min)	Color of precipitate	Reciprocal of titer	Biuret-positive material (mg/ml)
Crude preparation	—	—	—	5,120	1.66
Dialyzed crude preparation	—	—	—	5,120	1.26
1	1:1	180	White	80	0.13
2	1.5:1	15–20	White	640	0.22
3	2:1	1–2	White	5,120	0.30
4	4:1	Immediately	Brown	5,120	0.54
5	6:1	Immediately	Brown	5,120	0.90
6	8:1	Immediately	Brown	5,120	1.08
7	10:1	Immediately	Brown	5,120	1.22

TABLE 2. *Effect of time of exposure of crude beta hemolysin to acetone*

Time mixtures refrigerated[a]	Reciprocal of titer of beta hemolysin
12 min[b]	5,120
12 min	5,120
20 min	5,120
40 min	5,120
60 min	5,120
2 hr	5,120
4 hr	2,560
6 hr	1,280
8 hr	1,280
12 hr	160

[a] Mixtures consisted of 10 ml of crude beta hemolysin and 20 ml of acetone.
[b] Control, no acetone.

dialysis against polyethylene glycol (20 M). This procedure resulted in a 64-fold increase in the titer of beta hemolysin (Table 3). Since this increase in the titer was unexpected, the crude preparations of beta hemolysin were titrated in the presence of 0.01% polyethylene glycol (20 M). The presence of polyethylene glycol resulted in an eightfold increase in the titer of beta hemolysin (Table 4).

Ion-exchange chromatography. Chromatography of the partially concentrated preparations of crude beta hemolysin on ion-exchange celluloses at *p*H 7.5 revealed that they contained two types of beta hemolysin. One of these represented the major portion of the beta hemolysin activity of the crude preparations, and the other represented about 1/5,000 of the total activity. The major portion behaved as a cation and did not absorb onto DEAE cellulose. The minor portion behaved as an anion and did absorb on the DEAE cellulose from which it eluted when the column was developed with 0.08 M phosphate buffer, *p*H 7.5 (Fig. 1). On the CM cellulose too, the major and minor portions of beta hemolysin activities behaved as cations and anions, respectively (Fig. 2). The major portion absorbed onto the CM cellulose column, whereas the minor portion did not and appeared in the void volume. The cationic beta hemolysin absorbed on the CM cellulose was difficult to elute. Approximately 10-fold activity of the cationic beta hemolysin was lost during the elution process. Both the cationic and anionic beta hemolysin obtained by column chromatography caused hot-cold lysis of sheep erythrocytes.

Partially concentrated preparations of crude beta hemolysin were subsequently chromatographed on DEAE cellulose columns at *p*H 6.0, 6.5, 7.0, and 7.5. Best separation of beta hemolysin from the impurities was obtained at *p*H 6.0 (Fig. 3).

TABLE 3. *Concentration of crude beta hemolysin by dialysis, polyethylene glycol, and pervaporation*

Material	Vol (ml)	Titer of beta hemolysin	Biuret-positive material per ml (mg)
Crude beta hemolysin	2,620	2,560	1.76
Dialyzed crude beta hemolysin	2,750	5,120	0.70
Dialyzed crude beta hemolysin after concentration	600	327,680	2.80

TABLE 4. *Effect of polyethylene glycol on the activity of beta hemolysin*

Diluent	Titer of beta hemolysin
Phosphate-buffered saline containing 0.001 M magnesium sulfate	2,560
The above containing 0.01% polyethylene glycol, 20 M	20,480

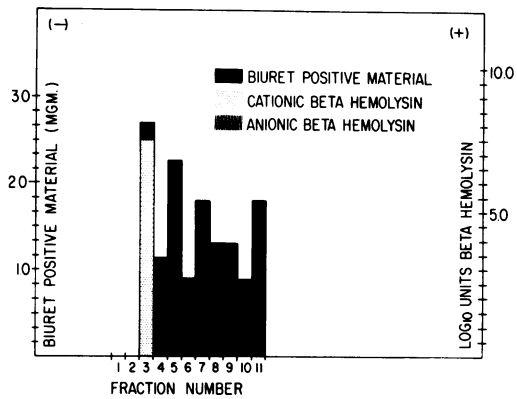

FIG. 1. *Fractionation of crude preparations of beta hemolysin on columns of DEAE cellulose at pH 7.5.*

Following this preliminary standardization, large quantities of partially concentrated crude preparations of beta hemolysin and acetone-precipitated preparations of beta hemolysin were separately chromatographed on DEAE cellulose columns at *p*H 6.0. Only the fractions containing cationic beta hemolysin which appeared in the void volume were collected. Prior to chromatography, however, these preparations were concentrated by lyophilization. Results of these experiments are summarized in Tables 5 and 6.

Lyophilization of the acetone-precipitated preparations afforded a 17-fold concentration of the sample and resulted in the removal of an appreciable amount of the biuret-positive material as an insoluble residue. This procedure, however, yielded an unexpected 256-fold increase in the titer of beta hemolysin. Lyophilization of the partially concentrated crude preparations of beta hemolysin did not yield any such increase in the titer of beta hemolysin (Table 6).

Continuous-flow electrophoresis. Results of the analysis of the various preparations of beta hemolysin on continuous-flow paper electrophoresis are summarized in Figs. 4–7. Again, the crude and the acetone-precipitated preparations of beta hemolysin showed the presence of two types of beta hemolysin. The major portion of beta hemolysin behaved as a cation and migrated slightly toward the cathode, whereas the minor portion of beta hemolysin behaved as an anion and migrated far toward the anode. The cationic beta hemolysin caused hot-cold lysis of sheep erythrocytes only. The anionic beta hemolysin exhibited a similar hot-cold lysis of sheep cells but also hemolyzed rabbit erythrocytes.

Continuous-flow electrophoresis of the preparations of beta hemolysin, obtained by chromatography of concentrated preparations of crude beta hemolysin and acetone-precipitated preparations of beta hemolysin on DEAE cellulose at *p*H 6.0, showed that these preparations contained the cationic beta lysin and were free from the anionic beta hemolysin. These preparations were also free from the major amount of protein material contained in the crude and the acetone-precipitated preparations of beta hemolysin.

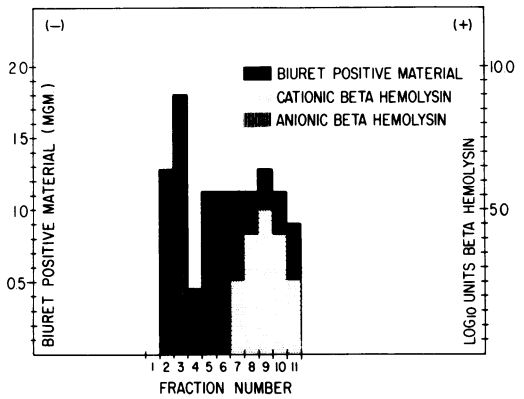

FIG. 2. *Fractionation of crude preparations of beta hemolysin on columns of CM cellulose at pH 7.5.*

Immunoelectrophoretic analysis. The arcs of specific precipitates which appeared after 1 to 7

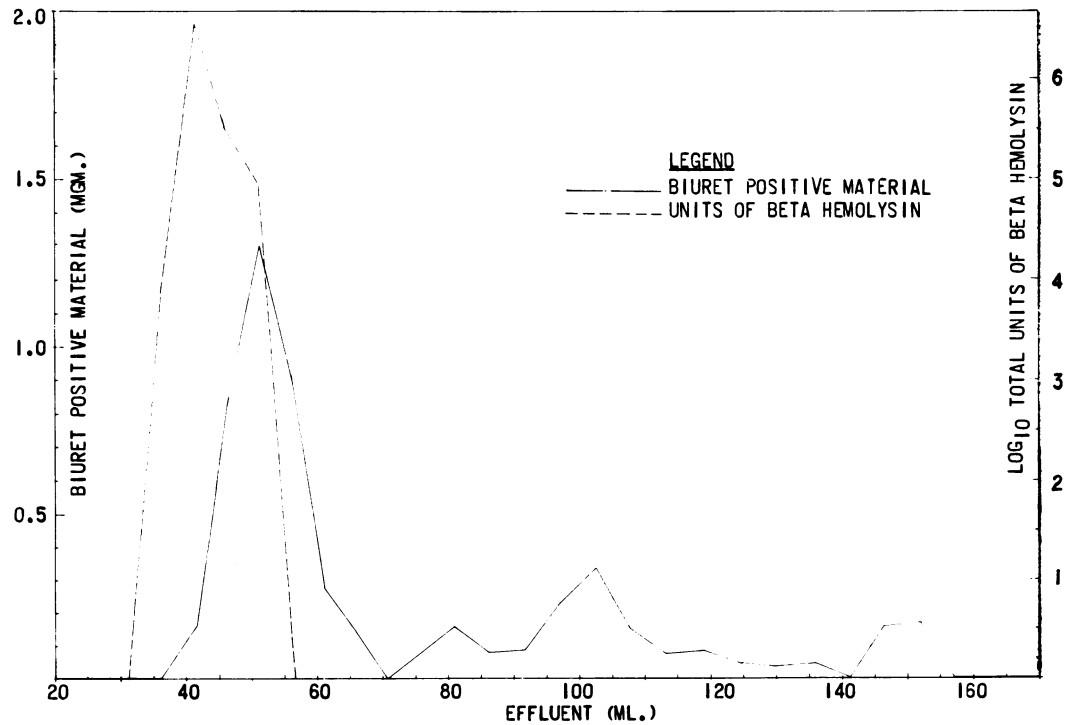

FIG. 3. *Chromatogram showing separation of cationic beta hemolysin on columns of DEAF cellulose at pH 6.0.*

TABLE 5. *Isolation of beta hemolysin from crude preparations of beta hemolysin by chromatography on DEAE cellulose*

Material	Vol (ml)	Titer of beta hemolysin	Biuret-positive material per ml (mg)	Total units of beta hemolysin
Partially concentrated crude beta hemolysin	100	327,680	2.8	11,560,000
Crude beta hemolysin further concentrated by lyophilization	25 (4-fold)	2,621,440 (8-fold)	10.9 (4-fold)	6,012,450
Pooled and concentrated fraction containing beta hemolysin obtained by chromatography of concentrated crude beta hemolysin	25	5,243,280	2.0	65,541,000

TABLE 6. *Isolation of beta hemolysin from an acetone-precipitated preparation of beta hemolysin by chromatography on DEAE cellulose*

Material	Vol (ml)	Titer of beta hemolysin	Biuret-positive material per ml (mg)	Total units of beta hemolysin
Acetone-precipitated beta hemolysin	500	10,240	0.4	12,800,000
Acetone-precipitated beta hemolysin concentrated by lyophilization	30 (16-fold)	2,621,440 (128-fold)	1.23 (3-fold)	63,942,000
Pooled and concentrated fraction containing beta hemolysin obtained by chromatography of concentrated acetone-precipitated preparation of beta hemolysin	30	5,243,280 (256-fold)	0.8	196,623,000

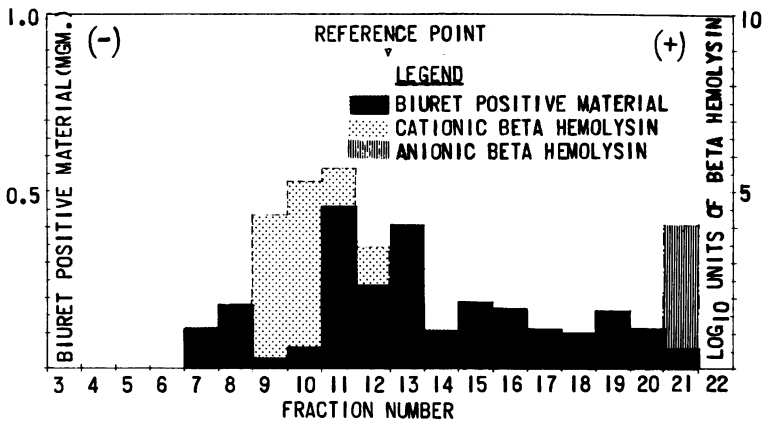

FIG. 4. *Continuous-flow electrophoresis of a crude preparation of beta hemolysin.*

days of incubation at 4 C were very faint and were not evident until after the gel was dried and stained. The crude preparations of beta hemolysin produced six arcs of specific precipitates. Two of these arcs were located toward the negative pole; the other four were located towards the positive pole. The acetone-precipitated preparations of beta hemolysin produced four arcs; two were located towards the negative pole and the other two towards the positive pole. The fractions containing the cationic beta hemolysin obtained by column chromatography of the crude and acetone-precipitated preparations of beta hemolysin on DEAE cellulose at *p*H 6.0 produced two arcs and one arc, respectively. These arcs were located towards the negative pole.

DISCUSSION

It is evident from the studies reported here that the beta hemolysin of *S. aureus* can be obtained in a purified form by a combination of methods of precipitation with chilled acetone followed by chromatography on DEAE cellulose at *p*H 6.0. The final preparation so obtained was immuno-

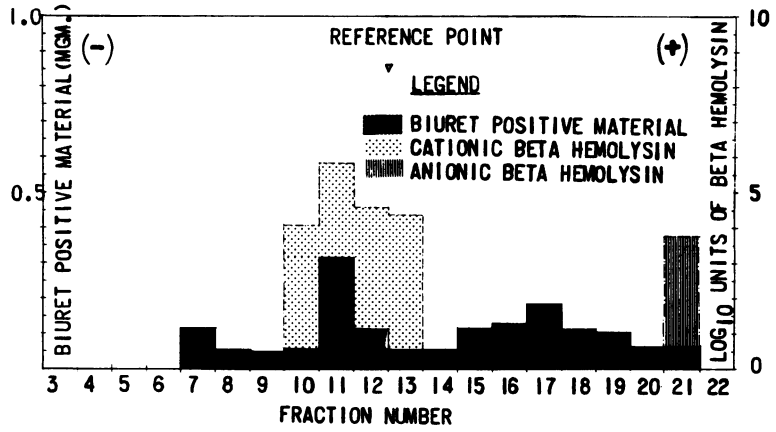

FIG. 5. *Continuous-flow electrophoresis of an acetone-precipitated preparation of beta hemolysin.*

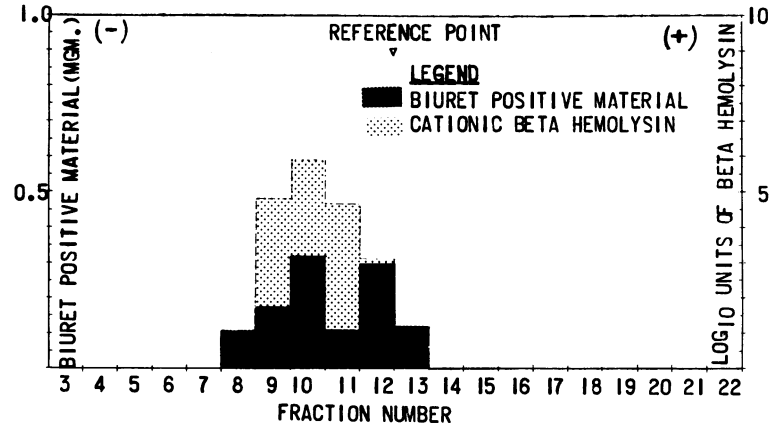

FIG. 6. *Continuous-flow electrophoresis of a preparation of beta hemolysin obtained by chromatography of crude beta hemolysin on DEAE cellulose.*

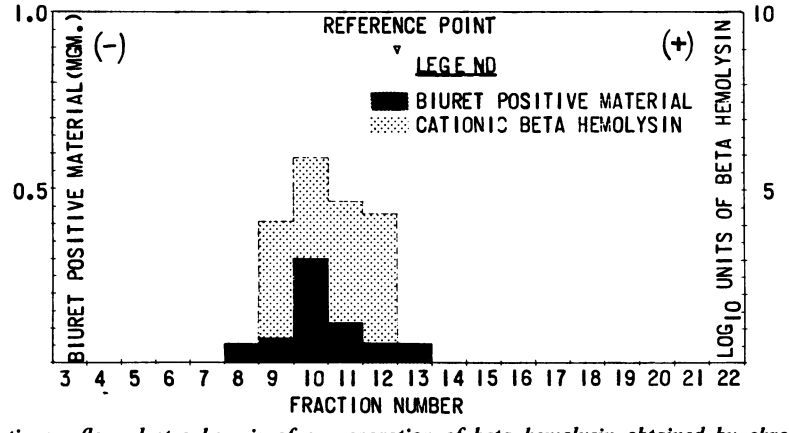

FIG. 7. *Continuous-flow electrophoresis of a preparation of beta hemolysin obtained by chromatography of acetone-precipitated beta hemolysin on DEAE cellulose.*

electrophoretically pure when tested with the antiserum produced against the crude cell filtrates.

The acetone-fractionation procedure is rapid and fairly selective for the precipitation of beta hemolysin and should find application in large-scale purification of beta hemolysin. The procedure did not show any deleterious effect on the hemolytic activity of beta hemolysin until the precipitate was allowed to remain in contact with the acetone for at least 4 hr.

Another method which would have similar applicability is the method of precipitation of beta hemolysin with ammonium sulfate recently reported by Maheswaran et al. (11). Our initial attempts to precipitate beta hemolysin with ammonium sulfate were unsuccessful. The failure was probably due to the slow addition of the ammonium sulfate by dialysis. The method of Maheswaran et al. (11) requires direct addition of large quantities of ammonium sulfate, and as such results in the precipitation of beta hemolysin. It is not yet known whether the 65% saturation of the crude preparations of beta hemolysin with ammonium sulfate selectively precipitates beta hemolysin. The method as reported, however, appears to be very useful and it will be advantageous to explore the possibility of precipitating beta hemolysin with small concentrations of ammonium sulfate. This has, however, not been attempted at present.

The results of column chromatography and continuous-flow electrophoresis of the crude and acetone-precipitated beta hemolysin revealed the presence of a major and a minor beta hemolysin component. These were originally defined as cationic and anionic beta hemolysins (R. Haque, Ph.D. Thesis, Ohio State Univ., Columbus, 1963). The preparations of anionic beta hemolysin obtained from the columns were active against sheep cells only, whereas the preparations of the anionic beta hemolysin obtained from the continuous-flow electrophoresis were active against sheep and rabbit erythrocytes. These findings had originally led to the conclusion that the anionic beta hemolysin was probably also active on rabbit erythrocytes (Haque, Ph.D. Thesis, 1963).

Recently, Maheswaran et al. (11) and Chesbro et al. (2) have confirmed the existence of cationic and anionic beta hemolysin. They, however, have reported that the anionic beta hemolysin was active only against sheep cells which were lysed in the hot-cold fashion. Rabbit cells were not affected. It is very likely that these discrepancies are due to the different strains of *S. aureus* used by the various investigators. The strain 681 used in the present investigation was recently examined by the electrophoretic localization technique (8). The beta hemolysin present in the cell-free filtrates of this strain was reported to migrate 36 mm towards the cathode and showed considerable tailing. Since beta hemolysin produced by certain other strains of *S. aureus* migrated as a well-defined peak, it was suggested that strain 681 produced at least two types of beta hemolysin. This strain also produced another hemolysin which lysed rabbit cells and migrated similarly to the anionic beta hemolysin. It is likely that the lysis of rabbit cells previously ascribed to the preparations of anionic beta hemolysin obtained by continuous-flow electrophoresis was due to this hemolysin.

In view of the failure of the anionic beta hemolysin preparations obtained from column chromatography to lyse rabbit cells, and in view of the findings reported by Maheswaran et al. (10, 11) and Chesbro et al. (2), it appears that the anionic beta hemolysin of *S. aureus* has hot-cold activity against sheep cells only. It shall thus be so identified. The relation of the anionic beta hemolysin to the cationic beta hemolysin is not yet known. It could represent fragments or aggregates of the cationic beta hemolysin.

During the course of the purification of beta hemolysin, certain unexpected increases in the titer of the beta hemolysin preparations were noted. The 64-fold increase in the titer of the partially concentrated crude preparations of beta hemolysin was probably due to the use of polyethylene glycol (20 M). This possibility is supported by the finding that the addition of the polyethylene glycol to the diluent for the titration of beta hemolysin resulted in an eightfold increase in the titer of beta hemolysin. Although it is not known whether the polyethylene glycol affected the erythrocytes or the beta hemolysin, and attempts to ascertain this were not made at present, these findings contraindicate the use of polyethylene glycol for concentrating beta hemolysin.

Another unexpected increase in the titer of beta hemolysin occurred during the lyophilization of the acetone-precipitated preparations of beta hemolysin. It is possible that this phenomenon is in some way related to the reported instability of the purified preparations of beta hemolysin (2, 10). The acetone-precipitated material in the present experiments represented partially purified preparations of beta hemolysin, and as such the beta hemolysin contained in them may have undergone fragmentation during lyophilization due either to freezing or to subsequent desiccation, or to both. Another explanation of this unexpected increase in the titer may be hidden in the large amount of material which did not go into solution when the lyophilized material was reconstituted. It is possible that some unknown inhibitor of the beta hemolysin had failed to go

into solution during the reconstitution of the lyophilized material. The presence of an unknown inhibitor of beta hemolysin has also been suspected by Gow and Robinson (7).

Whatever the cause, such unexpected increases in the titer of beta hemolysin prevent the evaluation of the various procedures of purifying beta hemolysin. This phenomenon may be in some way related to the molecular configuration and aggregation of the beta hemolysin molecule. It warrants further study, as its understanding will eventually aid in the purification of beta hemolysin in its native state.

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