Beta Hemolysin: a Persistent Impurity in Preparations of Staphylococcal Nuclease and Enterotoxin

WILLIAM CHESBRO AND VERA KUCIC

Department of Microbiology, University of New Hampshire, Durham, New Hampshire 03824

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Purified staphylococcal nuclease and enterotoxin B from several sources contained beta hemolysin whose physicochemical resemblances to the other two proteins make its elimination difficult.

The development of a practical serological assay for the staphylococcal enterotoxins in 1958 (8) made it possible to devise methods for purifying these exoproteins (5, 36). Purified enterotoxins have usually been sought for one of three purposes: (i) investigation of their biological activity in experimental animals or model systems, (ii) analysis of their primary structure and physicochemical characteristics, or (iii) as immunogens with which to obtain specific antisera for detection and quantitation of these otherwise difficultly measured proteins. During the same interval, methods for purifying the nuclease secreted by Staphylococcus aureus were also developed (15, 16) primarily to obtain the enzyme in a homogeneous form for studies in which it was to be used as a model enzyme and protein. As one result of this methodological development, the enzyme has become commercially available.

Although the reported methods for purifying these exoproteins vary considerably in details, column chromatography on cation-exchanging resins or modified celluloses has been the technique used as the major step in obtaining their resolution. When we applied this technique to purification of enterotoxin B and nuclease, in conjunction with heat and salt fractionation and gel filtration, we found the final products still possessed hemolytic activity against sheep erythrocytes. Commercially available nuclease exhibited the same type of hemolytic activity, and, when purified enterotoxin B was obtained from another laboratory, it, too, was hemolytic.

We report here demonstrations that this hemolytic activity is not an intrinsic property of either protein, but is due to the persistence of the beta hemolysin through the purification procedures employed. The means by which this hemolysin has been detected as a contaminant and quantitated are described, as well as some of the physicochemical characteristics of the hemolysin contributing to its concurrent passage with the other two proteins through a variety of purification procedures.

MATERIALS AND METHODS

Cultures. Staphylococcus aureus UNH 15, originally isolated in this Department from a case of bovine mastitis, was used for the production of nuclease. The strain of Streptococcus agalactiae used was similarly obtained. S. aureus 243 was obtained originally from E. P. Casman and was used for the production of enterotoxin B and beta hemolysin. Lyophilized cultures of the organisms were revived by growth in Trypticase soy broth (BBL) for 18 hr at 35 C. Agar plates containing 5% whole sheep blood were streaked from the broth tubes and incubated for 24 hr at 35 C. Isolated colonies from the plates were used to seed the broth to be used as starter for the toxin production medium.

Production and purification of nuclease, enterotoxin B, and beta hemolysin. A dialysate medium for exoprotein production was prepared by taking 30 g of NZ Amine (Sheffield Chemical Corp., Norwich, N. Y.) and 30 g of yeast extract (Difco) up in 200 ml of distilled water. This solution was boiled 5 min, cooled, and dialyzed with constant stirring at 4 C for 5 days against 3 liters of distilled water containing 12 g of K2HPO4, 7.5 g of NaCl, and 75 g of KCl. The dialysate was adjusted to pH 7.2, dispensed, and autoclaved for 15 min.

A 10-ml amount of the production medium was seeded and incubated with shaking for 8 hr at 35 C. This in turn was used to seed 40 ml of the same medium which was similarly shaken for 16 hr, and the total volume then used to seed a thick-walled 4-liter flask containing 450 ml of production medium. The air in the flask was replaced with an 80% O2, 20% CO2 atmosphere, and the flask was shaken at 35 C. The atmosphere was replenished after 3.5 and 7 hr of shaking. After 10 hr of shaking, 5 ml of chloroform was added to the flask and it was placed at 5 C for 16 to 18 hr. The culture was then centrifuged, and
the supernatant fluid was retained as the starting material for exoprotein purification.

To purify nuclease, a portion of the supernatant fluid was heated to 100 C for 10 min, and the insoluble material that resulted was removed by centrifugation. The supernatant fluid was brought to 50% saturation with (NH4)2SO4 at 4 C, and the precipitate forming after 24 hr was discarded. The solution was then brought to 80% saturation, and the precipitate that formed after 62 hr at 4 C was collected. This was dissolved in distilled water and reprecipitated by making the solution 0.3 M in trichloroacetic acid. This precipitate was redissolved and neutralized with 0.1 N NaOH to pH 7.0, and the solution was loaded onto a column containing 5 g of carboxymethylcellulose previously equilibrated with 0.05 M beta glycero-phosphate, pH 7.5. The nuclease-containing solution was washed into the column with the equilibrating solution and eluted by a nonlinear gradient of NaCl produced by a nine-chamber gradient maker (Vari-grad, Technicon Corp., Tarrytown, N.Y.). The eluting solutions were composed by dissolving the amount of NaCl necessary to give the desired conductance in 0.05 M beta glycero-phosphate, pH 7.5. The chambers were successively loaded with 100 ml each of solutions with the following conductances: 5, 10, 20, 30, 40, 50, and 60 millimhos. Five-milliliter fractions were collected, and the fractions containing peak activity were pooled and lyophilized.

The lyophilized fractions were dissolved in the least possible volume of beta glycero-phosphate buffer and passed through a column (35 by 2.5 cm) of Sephadex G-75 (Pharmacia Chemicals, Piscataway, N.J.). Peak fractions were pooled, lyophilized, and used in these studies.

Enterotoxin B and beta hemolysin were purified essentially as described previously (11), except that the eluting gradient was generated by loading the gradient maker with solutions constructed by dissolving the amount of NaCl necessary to produce the desired conductance in 0.01 M sodium phosphate buffer at pH 6.5. The chamber-loading sequence was 0.5, 1.0, 5.0, 10, 10, 20, 20, 40, and 60 millimhos.

**Measurement for hemolytic activities.** For tube assay of hemolytic activities, the 50% end point method (13) was used. Alpha hemolysin activity was determined by using rabbit erythrocytes suspended in isotonic saline. The method was modified for titrating beta hemolysin. Hemolysin dilutions were prepared in a magnesium-supplemented buffer consisting of 0.15 M NaCl, 0.075 M Na2HPO4, and 0.01 M MgCl2 adjusted to pH 6.8. The hemolysin so diluted was mixed in equal volume with a suspension of sheep erythrocytes and, after incubation at 37 C, was chilled to 0 C before centrifugation. The dilution yielding the 50% end point was taken as equal to one hemolytic unit.

To detect and measure beta hemolysin by plate assay, 5 ml of sheep erythrocytes washed in isotonic saline and resuspended to produce a 20% suspension was added to 95 ml of sterile Tryptose-blood-agar base (Difco). The mixture was added to plastic petri plates (100 by 15 cm) in 10-ml amounts. These were incubated for 48 hr at room temperature and then stored at 4 C. Similar plates were prepared for assay of alpha hemolysin, substituting rabbit for sheep erythrocytes.

Wells with an 8-mm inner diameter were cut in the agar, and 50 uliters of the sample to be tested was added to each well. Diffusion was allowed to proceed for 18 hr at 37 C; the plates then were chilled to 4 C, and the diameter of the hemolytic zones was measured after a further 4 hr.

**Production of antisera.** The antigens were incorporated in Freund's incomplete adjuvant, and a total of 1.5 mg in 3 ml of emulsion was injected: 1 ml intraperitoneally and 0.5 ml intramuscularly in each shoulder and flank. After 3 weeks, a further 0.5 mg in 1 ml of emulsion was injected intraperitoneally, and blood was drawn the following week.

**Serological methods.** Radial gel double diffusion was conducted in 50-mm petri dishes. These were cleaned with acetone and filled with 4 ml of 1.3% Noble agar (Difco) in half-strength Veronal (barbital) buffer (pH 8.6) to which 0.01% sodium azide was added as preservative. The plates were held at room temperature for 18 hr and then stored at 4 C. Wells were cut with an 8-mm inner diameter cork borer just before use. After addition of the reagents, the plates were held at room temperature.

When the precipitin pattern was established, the plates were successively rinsed with 0.85% saline and distilled water and then dried for 48 hr at 35 C. The precipitin bands were stained with 0.6% Amido Black 10B in methanol-acetic acid-water (45:10:45), and repeatedly rinsed in the same solution.

Analyses by the Preer (35) technique of quantitative linear double diffusion were done in 5-cm tubes with an inner diameter of 1.5 mm calibrated to contain 20 uliters between marked intervals. The tubes were filled with 0.1', Noble agar, drained, and air-dried. The tubes, in a 56 C water bath, were loaded with 20 uliters of antisera in 0.3'. Noble agar buffered with half-strength Veronal (pH 8.6) and then with 20 uliters of the same agar to constitute the neutral zone. The tubes were removed from the water bath, and 20 uliters of antigen in appropriately buffered solution was added after 20 min. The tubes were sealed with paraffin wax and incubated at 30 C. After 4 days, the distance from the antigen agar-neutral agar interface to the region of greatest density in the line of precipitation (P value) and the total length of the neutral agar zones were measured with hand calipers.

To quantitate beta hemolysin, a standard curve was constructed by using homologous antiserum diluted 1:2 in the bottom layer and 0.76, 0.38, 0.19, or 0.04 uliters of the hemolysin in the top layer. The P value was plotted against the logarithm of the antigen concentration. P values for unknown antigen solutions were compared with this standard curve.

**Electrophoresis.** For preparative fractionation of beta hemolysin, a vertical slab electrophoresis apparatus (EC Apparatus Corp., Philadelphia, Pa.) was employed. A slab of 1.3% agarose containing 0.1 M Na2HPO4, adjusted to pH 6.8, and 1.0% glycerol was used. Six slots were prepared in the top of the slab and filled with 20 uliters each of solution pre-
pared by condensation of the peak hemolysin fractions from column chromatography by osmiodialysis against a mixture of Aquacides I and II (Calbiochem). Buffer solutions in the electrode chambers were the same as that in the agar. One hundred and fifty volts were applied for 16 hr. The slab was removed, and the lateral edges were sliced off and stained with 0.002% Ponceau S in 3% trichloroacetic acid to reveal the location of the protein bands. These were eluted in the 0.1 M NaH₂PO₄ buffer and recentered by osmiodialysis.

Microimmunoelctrophoresis was conducted on the glass slides covered with 10 ml of 1.3% agarose containing half-strength Veronal buffer (pH 9.0) containing 0.01% sodium azide. A well with an inner diameter of 1.5 mm was cut in the center of the agar layer and filled with 10 μl of the antigen solution. The slide was held in a gradient of 11 v/cm for 30 min. Parallel troughs were cut on either side of the well and filled with antiserum, and the slides were incubated for 48 hr at room temperature in a humid chamber. They are then washed, rinsed, and stained in the same manner as the gel diffusion plates.

**Gel filtration and sedimentation studies.** Sedimentation constants were determined by the method of Martin and Ames (28). The sucrose density gradient of 5 to 20% was buffered with 0.05 M NaH₂PO₄ adjusted to pH 6.8. The sedimentation of the exoprotein was compared to that of horse heart cytochrome c, lysozyme, and ovalbumin as standards. Samples were centrifuged at 39,000 rev/min with a Spinco model L centrifuge and SW 39 rotor. The lustroid tubes were punctured and drained, two-drop samples being collected.

Gel filtration was performed by using Sephadex G-75 in a column 35 by 2.5 cm and at a flow rate of 20 ml/hr. The eluting solution was 0.05 M beta glycerophosphate adjusted to pH 6.8 and brought to a conductance of 10 millimhos with NaCl. The column was calibrated by the method of Andrews (2) with proteins with known molecular weights and Stokes radii.

**RESULTS**

**Separation of nuclease, enterotoxin B, alpha hemolysin, and beta hemolysins by column chromatography on phosphocellulose.** Figure 1 indicates the distribution of nuclease, enterotoxin B, alpha hemolysin, and beta hemolysins obtained when a concentrated culture supernatant fluid of *S. aureus* 243 is absorbed on a column of phosphocellulose, followed by elution with a NaCl gradient by methods described previously (10). The beta-hemolytic activity eluted in two distinct peaks, the B2 peak overlapping the nuclease peak and the B1 peak overlapping the enterotoxin peak. Electrophoretically distinguishable forms of the beta hemolysin have been previously reported (11, 24).

The central fractions of the B2 peak were pooled to be used in the generation of anti-beta hemolysin antisera without further purification since at the time at which the fractionation was performed it was only desired that the resultant antiserum be free of activity against the alpha hemolysin, enterotoxin B, and nuclease. It became apparent, as will be shown in a later section, that the antiserum obtained reacted with both beta hemolysins in immunodiffusion to produce separate precipitin lines, presumably due to the incomplete separation of B2 from B1 in the fractions used to immunize the rabbits.

**Identification of the hemolytic activity in nuclease and enterotoxin preparations as beta hemolysin.** Plates of washed sheep, bovine, horse, rabbit, and human erythrocytes were made, and both our preparations of nuclease and enterotoxin and those obtained as gifts or by purchase were tested for range of hemolytic activity by plate assay of 50-μlter volumes of solutions containing either 0.6 mg of nuclease or 0.5 mg of enterotoxin per ml diluted in 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-buffered saline, pH 7.5. Purified beta hemolysin at a concentration of 0.3 mg/ml was similarly added to blood-agar plates. The spectrum of hemolytic activity was the same for all preparations; sheep and bovine erythrocytes lysed completely after a hot-cold holding sequence, whereas rabbit, horse, and human erythrocytes were unaffected.

A test was made of the ability of the hemolytic activity in the nuclease and enterotoxin preparations to be synergized by the *S. agalactiae* factor [the basis of the CAMP test for this bacterium (32)]. Figure 2 shows a sheep blood-agar plate to which purified beta hemolysin, commercial nuclease, and culture supernatant fluid of *S. aureus* UNH 15 were added before the plate was incubated at 35 C for 18 hr. *S. agalactiae* was then streaked across the zones of partial hemolysis and allowed to grow. The area adjacent to the growth
streak was cleared in all of the zones of partial hemolysis in a manner typical of the CAMP reaction. The hemolytic activity of the enterotoxin preparations gave the same reaction when tested.

The various antisera generated against the purified exoproteins were tested for ability to neutralize the hemolysis-in-agar caused by the nuclease and enterotoxin preparations. Figure 3 shows that anti-beta hemolysin antiserum neutralized the activity of all three preparations, whereas anti-nuclease and anti-enterotoxin antiserum did not.

Attempts to identify a precipitin line yielded by the anti-beta hemolysin antiserum against the nuclease preparations were unsuccessful. When anti-beta hemolysin and anti-enterotoxin antisera were cross-tested against their respective antigens, however, the anti-beta hemolysin antiserum did react with a component of the enterotoxin preparation (Fig. 4). An excess of both antigens were employed in the tests shown in Fig. 4 to emphasize possible minor components in the preparations. Our beta hemolysin preparation at this point represented the pooled fractions from the center of the later eluting beta hemolysin peak (Fig. 1). It still contained a fraction contributed by tailing from the early eluting peak. So the preparation at this point contained a mixture of both beta hemolysins secreted by strain 243, and it is apparent that the cross-reacting component in the enterotoxin is related to only one of these. It can also be seen that the anti-enterotoxin antiserum, while reacting with its homologous antigen, produces no precipitin line against the beta hemolysin preparation, indicating that it is indeed beta hemolysin which is the cross-reacting component rather than enterotoxin.

**Quantitation of the beta hemolysin in nuclease and enterotoxin preparations.** An initial attempt to titrate the beta hemolysin activity in the exoprotein preparations by tube dilution methods failed to reveal the presence of any hemolytic activity. This was surprising in view of the steady demonstrability of such activity by using the plate technique. We, therefore, undertook development of a quantitative radial assay for beta hemolysin based on the hemolysis-in-gel phenomenon.

Purified beta hemolysin was diluted in Tris-buffered saline (pH 6.8); 50 μl of the diluted hemolysin was placed in the wells in sheep blood-agar plates, and the hemolysis zones ultimately resulting were measured. Plotting these diameters against hemolysin concentration yielded the curve shown in Fig. 5. Less than 1.0 ng of hemolysin was easily detectable, better than 100 times greater
sensitivity than was possible with the tube dilution method. Gow and Robinson (21) have reported detecting 2.0 to 4.0 pg of hemolysin by using a similar method.

The amount of hemolytic activity in the enterotoxin B and commercial nuclease was then assessed by the radial diffusion assay method. The beta hemolysin content of the nuclease was 0.02% and in the enterotoxin preparation was 0.16%.

Since the beta hemolysin in these preparations was not necessarily fully active, the figures represented a minimum content.

To obtain an estimate that would include both hemolytically active and inert forms of the hemolysin, the Preer technique was employed. For this technique, it was necessary to have a beta hemolysin preparation that produced only one precipitin line in the standard. Consequently, the preparation used to this point was subjected to agar-gel electrophoresis. The separated hemolysins were extracted and reconcentrated by osmiodialysis. The more anionic form of the hemolysin was present in lower quantity and therefore was the form most correspondent to that in the enterotoxin (Fig. 4). The standard curve obtained against the hemolysin by the

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**Fig. 4.** Reaction of anti-beta hemolysin and anti-enterotoxin B antisera with purified beta hemolysin and enterotoxin B. The wells contained 50 μl of anti-beta hemolysin antiserum (1), 50 μl of purified beta hemolysin containing 10 μg of protein (2), 50 μl of anti-enterotoxin B antiserum (3), and 50 μl of purified enterotoxin B containing 25 μg of protein (4).

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**Fig. 5.** Relationship between concentration of beta hemolysin and the diameter of its hemolytic zone in sheep blood-agar. The specific activity of the hemolysin preparation was 30 hemolytic units per μg of protein. The well diameter was 8 mm.

**Fig. 6.** Relationship between concentration of beta hemolysin and the position of the precipitin line obtained with homologous antiserum by using the Preer double diffusion technique.

Preer technique is shown in Fig. 6. The lower limit of the method in our hands was about 50 ng of hemolysin.

Applied to the enterotoxin preparation, the technique indicated a minimum of 1.8% beta hemolysin to be present. Comparing this with the activity measurement indicated that the beta hemolysin was over 90% inactive.

Applied to the nuclease, using 200 μg of nuclease as the upper limit tested, no precipitin lines were formed. Since the minimum amount of beta hemolysin indicated as present by the activity assay was 0.02%, this would be 40 ng for the largest amount of nuclease tested, slightly below the limit of our Preer assay. The amount of hemo-
lysins present in the nuclease preparation was therefore between 0.020 and 0.025%.

Anti-beta hemolysin activity in antiserum prepared against nuclease and enterotoxin preparations. Although the beta hemolysin impurity in the nuclease preparation was unlikely to be enough to cause a response when the preparation was used as antigen, it seemed possible that it might be large enough to do so in the enterotoxin preparations. There was no evidence for anti-beta hemolysin activity in any of our anti-nuclease preparations when tested by gel double diffusion; nor did rabbit antiserum prepared against the enterotoxin by ourselves or others show any anti-beta hemolysin activity in the same tests. However, when burro anti-enterotoxin antiserum were tested (Fig. 7), anti-beta hemolysin activity was evident.

Characteristics of beta hemolysin making it difficult to separate from enterotoxin and nuclease. Purified beta hemolysin was subjected to coelectrophoresis with nuclease and enterotoxin B at pH 9.0 in Veronal buffer. The results are shown in Fig. 8 and 9.

The electrophoretic mobility of the hemolysin lies between those of the other two proteins so that it only slowly separates from either.

Fig. 7. Antibody to beta hemolysin in burro anti-enterotoxin antiserum. The wells contained 50 μl of anti-nuclease antiserum (1), 50 μl of commercial nuclease containing 0.3 mg of protein/ml (2), 50 μl of purified enterotoxin B containing 0.16 mg of protein/mg (3), 50 μl of purified beta hemolysin containing 0.1 mg of protein/ml (4), 25 μl of antihemolysin antiserum (5), and 25 μl of burro anti-enterotoxin B antiserum (6).

Fig. 8. Microimmunoelectrophoresis of a mixture of enterotoxin B and beta hemolysin. The center well (2) was loaded with 12.5 μg of enterotoxin B and 2.5 μg of beta hemolysin. Trough 1 contained anti-beta hemolysin antiserum; trough 3 contained anti-enterotoxin B antiserum. The anode was at the numbered end of the slide.

Fig. 9. Microimmunoelectrophoresis of a mixture of nuclease and beta hemolysin. The center well (2) was loaded with 12.5 μg of commercial nuclease and 2.5 μg of beta hemolysin. Trough 1 contained anti-nuclease antiserum. Trough 3 contained antihemolysin antiserum. The cathode was at the number end of the slide.

The molecular weights of the beta hemolysin fractions were then determined by density gradient ultracentrifugation, assuming a partial specific volume of 0.72 cm³/g, and by gel filtration. Table 1 contains a comparison of some of the physical characteristics of enterotoxin B, nuclease, and beta hemolysin obtained in this study or reported in the literature.

**DISCUSSION**

Our results demonstrated that the hemolytic activity associated with a variety of enterotoxin
and nuclease preparations is due to unremoved beta hemolysin. The techniques most commonly used to purify enterotoxin and nuclease have been column chromatography on Amberlite IRC-50 (5), Amberlite CG-50 (36), and carboxymethyl cellulose (36), starch block electrophoresis (4), and gel filtration (19) for the former and column chromatography on diethylaminoethyl cellulose (31), carboxymethyl cellulose (22), and phosphorlated cellulose (10), gel filtration (39), and affinity chromatography (15) for the latter. Except for the last method mentioned, these techniques rely on the charge characteristics or molecular dimensions of the desired protein to effect its separation from the other staphylococcal exoproteins. Their resolving power is diminished in the case of beta hemolysin, by the overlap of the electrophoretic and dimensional characteristics with those of the other two proteins. The problem thus posed in purifying these three proteins is compounded by the existence of a variety of electrophoretically and dimensionally distinguishable forms of each of the three (9, 24, 41). Thus, the successful employment of any combination of the foregoing techniques upon the exoproducts of a given strain does not bring with it any assurance of success when applied to a second strain.

Presumably better methods of separating these proteins with broad molecular weight and charge resemblances would be those taking advantage of their other properties. A technique of this sort has been reported for purifying the nuclease (15). The enzyme is adsorbed to, and eluted from, 3'- (4-aminophenylphosphoryl) - deoxythymidine - 5'-phosphate, a compound with a high affinity for the active site of the enzyme which is conjugated to the gel in a Sepharose column. A comparable technique for utilizing intrinsic specificity for a substrate or inhibitor is not available for the enterotoxin, but there is some evidence (4; R. Bennett, personal communication) that the beta hemolysin is more strongly adsorbed to starch than is the enterotoxin, suggesting that electrophoresis on starch might be particularly useful for their separation.

The significance of the amount of hemolysin in preparations of the other two proteins will depend on the purpose for which they are purified.

The nuclease has been purified most often for studies of its structure and the basis of its enzymatic activity (14, 40). It has also been produced as a tool for studying nucleic acid sequences (33). Since the amount of beta hemolysin found to be present in nuclease preparations in our study did not exceed 0.02% (although this would represent a minimum level), it is unlikely to interfere significantly in research which uses nuclease for the foregoing purposes. Nor does the enzymatic activity of the hemolysin as a phospholipase (17) seem likely to interfere when the nuclease is studied, or utilized, as an enzyme.

However, although there are few studies reported on the possible role of the nuclease in staphylococcal disease, it is likely that more such studies will be undertaken in view of the high correlation of production of this enzyme with pathogenicity in S. aureus (7) and its commercial availability. In examining the effects of nuclease on host tissues or cells, the presence of even a small amount of residual beta hemolysin cannot

### Table 1. Comparison of some of the physicochemical characteristics of staphylococcal nuclease, beta hemolysin, and enterotoxin B

| Exoprotein         | Production strain | Isoelectric or isionic point | Amino acid analysis | Ultracentrifugation | Gel filtration | Reference no. |
|--------------------|-------------------|------------------------------|---------------------|---------------------|-----------------|---------------|
| Nuclease           | V8                | 7.0-7.5                      | 11,000-12,000       | 1.7                 | 17,650          | 3             |
|                    | V8                | 9.62                         | 16,850              | 1.7                 | 17,650          | 40            |
|                    | V8                | 10.11                        |                     |                     |                 | 41            |
|                    | Foggi             | 9.62                         | 16,600              | 1.8                 | 12,000          | 1             |
|                    | UNH-15            | 9.5                          | 16,500              | 1.74                | 17,650          | 25            |
| Beta hemolysin     | 243-B1            | 8                          | 15,000              |                     |                 | 21            |
|                    | 243-B2            | 9.5                          | 15,500              |                     |                 |               |
|                    | UNH-15            | 10                          | 13,600              |                     |                 |               |
|                    | MB-534            | 8.5                          | 15,500              |                     |                 |               |
| Enterotoxin B      | S6                | 8.6                          | 35,300              | 2.3-2.5             | 24,000-27,000  | 36            |
|                    | S6                | 8.6                          |                      | 2.89                |                 | 38            |

* W. Chesbro, unpublished data.

and nuclease preparations is due to unremoved beta hemolysin. The techniques most commonly used to purify enterotoxin and nuclease have been column chromatography on Amberlite IRC-50 (5), Amberlite CG-50 (36), and carboxymethyl cellulose (36), starch block electrophoresis (4), and gel filtration (19) for the former and column chromatography on diethylaminoethyl cellulose (31), carboxymethyl cellulose (22), and phosphorlated cellulose (10), gel filtration (39), and affinity chromatography (15) for the latter. Except for the last method mentioned, these techniques rely on the charge characteristics or molecular dimensions of the desired protein to effect its separation from the other staphylococcal exoproteins. Their resolving power is diminished in the case of beta hemolysin, by the overlap of the electrophoretic and dimensional characteristics with those of the other two proteins. The problem thus posed in purifying these three proteins is compounded by the existence of a variety of electrophoretically and dimensionally distinguishable forms of each of the three (9, 24, 41). Thus, the successful employment of any combination of the foregoing techniques upon the exoproducts of a given strain does not bring with it any assurance of success when applied to a second strain.

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be discounted as a possible course of ambiguity, exemplified by the effect of the hemolysin upon host cells (in this case sheep erythrocytes) which drew our attention to its presence in nuclease preparations used in this investigation in the first place.

Studies on the enterotoxins have usually had different primary aims than have studies on nuclease, being intended to elucidate the basis of its emetic activity or to develop methods for its detection in foodstuffs. A smaller number of studies have been concerned with its mode of synthesis and secretion by the bacterium (27, 30) or with the physicochemical description of the enterotoxin molecule (38, 42).

With regard to the emetic activity of enterotoxin, the presence of small amounts of beta hemolysin is unlikely to interfere since there is no evidence to suggest that the beta hemolysin is itself emetic when administered by the oral route to any of the test animals usually employed in enterotoxin testing; and mixing enterotoxin preparations with specific anti-beta hemolysin antisera prior to monkey feeding does not prevent the subsequent emesis (E. Schantz, personal communication). However, when administered by injection, the hemolysin is clearly damaging to tissue (11), so that tissue or systemic effects after injection of enterotoxin preparations might arise from the hemolysin, if present as a contaminant. For instance, the enterotoxin has been reported to be pyrogenic (29), even after boiling. Although the beta hemolysin is heat-labile, as we have shown here, there is demonstrable activity left even after boiling for 10 min.

In studies aimed at detecting enterotoxin in foodstuffs, four methods have generally been employed: (i) scoring test materials for their ability to produce emesis in monkeys (6), cats (12), or human volunteers (18) after injection or ingestion; (ii) use of single or double gel diffusion employing anti-enterotoxin antisera (8, 23, 43); (iii) hemagglutination inhibition (26) and reversed passive hemagglutination (37) in which the behavior of erythrocytes to which enterotoxin or anti-enterotoxin has been absorbed serves to indicate and quantitate the presence of enterotoxin in test materials; and (iv) immunofluorescence utilizing anti-enterotoxin antisem (20).

Detection of enterotoxin in foods by the response of susceptible animals after ingestion (see i above) is unlikely to be affected by the presence of beta hemolysin for the reasons noted above. Methods ii, iii, and iv, however, are subject in various degrees to interference by beta hemolysin contaminating the enterotoxin used either as the comparative standard or as the antigen in production of the specific antiserum.

Gel diffusion in two dimensions is least easily confused by the presence of beta hemolysin since the resultant multiplicity of precipitin lines is readily recognizable, and the identity of a given line can be established by comparison with standards. However, this is also the least sensitive of the gel diffusion methods (34).

Multiple precipitin lines are also readily evident in gel diffusion in one dimension, but this technique is somewhat more dependent on the monovalency of the antiserum for recognition of the protein being measured since simultaneous comparisons of several samples against a common antiserum are not intrinsic to the method.

The hemagglutination and immunofluorescence methods are the most susceptible to confusion by hemolysin in the enterotoxin, or antihemolysin antibody in the anti-enterotoxin antiserum, since their accuracy depends wholly on the monovalency of the antiserum and the purity of the antigen.

Studies on the synthesis and secretion of enterotoxin by S. aureus could be interfered with by the hemolysin contaminant by its effect on means employed to quantitate the enterotoxin, similar to the situation in detecting enterotoxin in food.

Physicochemical studies of the enterotoxin are likely to be least affected by contaminating beta hemolysin even at the level of 1 to 2% by weight, since the characterizing instruments and methods used, e.g., ultracentrifugation and amino acid analysis, are not seriously interfered with by this amount of impurity.

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