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Purification and Characterization of a Staphylococcal Epidermolytic Toxin

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A staphylococcal exotoxin that causes epidermolysis when injected into the skin of the newborn mouse and man was highly purified by conventional biochemical techniques. With *Staphylococcus aureus* EV, the epidermolytic toxin was a major protein component of supernatant culture fluids. The initial step in purification was zone electrophoresis in Pevikon carried out at pH 9.0, the isoelectric point of alpha-hemolytic toxin, which remained near the origin. Fractions containing the epidermolytic toxin, but free of alpha-toxin, were then subjected to cation exchange chromatography on carboxymethyl-Sephadex C-50 to remove trace contaminants. A major highly purified epidermolytic toxin migrated as a single band in polyacrylamide gel electrophoresis, sedimented as a single component in the analytical ultracentrifuge, and elicited a single precipitating antibody after injection into rabbits. A smaller amount of a second epidermolytic toxin, identical in molecular weight and antigenicity but differing in electrophoretic behavior from the major molecular species, was also identified. The epidermolytic factor had a molecular weight of 28,600 ± 400 by sodium dodecyl sulfate-acrylamide electrophoresis and 32,500 ± 120 by approach to sedimentation equilibrium.

The molecular basis of the staphylococcal type of toxic epidermal necrolysis was singularly advanced by the development of a murine model of this disease by Melish and Glasgow (12). They have subsequently identified an exotoxin in the supernatants of cultures of *Staphylococcus aureus* strains obtained from patients with this syndrome (13). The specificity of this toxin in loosening keratinocytes to cause a rent within the epidermis suggested that this agent might serve as a probe to study the factors that normally participate in epidermal cell adhesion and might explain the epidermolysis that occurs in other bullous human disease states as well as in staphylococcal toxic epidermal necrolysis.

Culture fluids of staphylococcal strains that produce the epidermolytic toxin are usually contaminated by appreciable amounts of the staphylococcal alpha-hemolytic toxin. Since the alpha-toxin acts upon many different cell membranes (7), we preferred to free the epidermolytic toxin from the alpha-toxin as well as from any other contaminants before we began any biochemical and ultrastructural studies.

Accordingly, we purified this protein by conventional biochemical methods. Since the isoelectric point of the epidermolytic toxin differs from that of the alpha-toxin, the initial step in purification involved zone electrophoresis at the isoelectric point of alpha-toxin. This enabled us to separate them; trace proteins that contaminated the epidermolytic toxin were further removed by carboxymethyl (CM)-Sephadex chromatography. Because the action of the toxin is to produce epidermolysis, i.e., the loosening of cells from one another, we prefer to call it "epidermolysin" as we have done in this article.

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MATERIALS AND METHODS

Bacteria. Strain EV, a phage group 2, type 55/71 coagulase-positive *S. aureus*, isolated from a case of staphylococcal toxic epidermal necrolysis, was stored in frozen skim milk (Difco) until used. Trypticase soy broth and Trypticase soy agar plates (Difco) were prepared for propagating the strains, making dilutions, and doing colony counts.

Production of toxin. Seamless cellulose dialysis tubing sacs were filled with 100 ml of medium 199 (Grand Island Biological Co.) adjusted to pH 7.4

with $NaHCO_3$. They were inoculated with a wire loop and one or two sacs were introduced surgically into the peritoneal cavity of pentobarbital-anesthetized 3.5-kg rabbits. The sacs were removed at the time of the animals' death or after 48 h.

Sterilization of culture fluids. The contents of the sacs were centrifuged at 10,000 × *g* for 20 min at 4 C and the supernatant was sterilized by passage through a 0.45-μm membrane filter (Millipore Corp.). A sample was removed to confirm sterility by culturing, and sodium azide, 0.2 g/liter, was then added.

Concentration. Ultrafiltration in an ice bath in a Diaflo dialysis cell with a PM 10 membrane (Amicon) was performed to reduce the fluid volumes.

Zone electrophoresis. Electrophoresis in Pevikon (Mercer) was performed in a standard fashion (14) with pH 9.0, 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer. A 5- to 6-ml sample containing 5 to 10 mg/ml was applied and then subjected to electrophoresis at a constant voltage of 8 V/cm for 17 h. The block was cut into 1-cm sections and the fluid phase was recovered by filtration on a Buchner funnel.

Cation exchange chromatography. CM-Sephadex C-50 (Pharmacia) was hydrated and equilibrated with 0.01 M phosphate buffer, pH 6.0. A 1-ml sample containing 10 to 20 mg/ml was applied to a column of gel (0.9 by 10 cm) and washed. The rate of flow was maintained with a pump (Vari Perplex) at 15 ml/h. Proteins were eluted with a linear gradient that utilized 0.15 M sodium chloride buffered with 0.01 M phosphate in the terminating vessel; the volume of the starting and terminating buffers was 150 ml.

Protein determinations. Fractions were read at 230 nm in a Zeiss PMQ II spectrophotometer. Protein concentrations of solutions were measured by the method of Lowry et al. (11) with crystalline bovine albumin as the protein standard.

Assay for epidermolytic activity. Pregnant Webster/Swiss mice that arrived between days 14 and 16 of gestation were housed in individual cages and given food and water ad libitum. Newborn mice were kept with their mothers until just before use when they were randomly redistributed among the mothers. Groups of 1- to 3-day-old mice were injected subcutaneously in the nape of the neck with a 25-gauge needle with 0.02-ml samples of serial twofold dilutions of sample in 0.15 M NaCl in 0.01 M, pH 7.3 phosphate buffer (PBS). One unit of epidermolytic activity was taken as the reciprocal of the dilution that gave a positive Nikolsky sign, i.e., separation of the epidermis by finger torsion, in one animal in a group of animals in 3 h.

Assay for hemolytic toxins. To 0.4-ml amounts of serial twofold dilutions of sample in saline were added 0.2 ml of saline and 0.4 ml of a cold 1% suspension of washed rabbit or human erythrocytes. Tubes were maintained in an ice bath for 15 min and then transferred to a water bath at 37 C. Those with rabbit erythrocytes were incubated for 60 min; those with human erythrocytes were incubated for 15 min. After centrifugation, the supernatant fractions were quantitatively removed and diluted with 4.0 ml of 0.1% Na_2CO_3, and hemoglobin concentrations were determined spectrophotometrically at 430 nm. One hemolytic unit of alpha-toxin was defined as that quantity which release 50% of the hemoglobin from rabbit erythrocytes, and one hemolytic unit of delta-toxin was defined as that quantity which releases 50% of the hemoglobin from human erythrocytes under these conditions (8).

Disc gel electrophoresis. Polyacrylamide gel electrophoresis was carried out with the materials and buffers described by Davis (5) with 7.5% polyacrylamide. Gels were stained for protein with Coomassie brilliant blue in 10% trichloroacetic acid or placed on dry ice until firm and cut with a gel slicer into 1.3-mm segments; the segments were eluted overnight at 4 C in 0.3 ml of PBS.

SDS-acrylamide gel electrophoresis. For molecular weight determinations, polyacrylamide disc gel electrophoresis with 0.1% sodium dodecyl sulfate (SDS) was performed (15). The gels were stained with Coomassie brilliant blue (16) and molecular weight was estimated by comparison with standard proteins subjected to electrophoresis in the same fashion.

Ultracentrifugation. Epidermolysin, 0.3 mg/ml in 0.1 M NaCl, was centrifuged at 9,284 rpm at 20 C for 72 h in a Beckman model E analytical ultracentrifuge. The results obtained were used to calculate the molecular weight (6). A partial specific volume of 0.74 ml/g was assumed.

Production of antiserum to epidermolysin. Four 2.5-kg rabbits were injected in the footpads with 0.15 mg of purified epidermolysin emulsified with complete Freund adjuvant (Difco). Eleven days later, a booster injection of 0.05 mg of purified toxin emulsified in incomplete Freund adjuvant was given subcutaneously. Ten days later the animals were bled, given another booster with 0.05 mg of purified toxin in incomplete Freund adjuvant, and bled again after 14 more days. Antiepidermolysin and anti-alpha-toxin were assayed by the double immunodiffusion (Ouchterlony) technique in agar gel plates (Hyland). Commerical staphylococcal alpha-toxin and antiserum to alpha-toxin (Wellcome Reagents) served as controls.

Tritration of antiepidermolysin. To 0.1 ml of serial twofold dilutions of immunized rabbit serum in PBS was added 0.1 ml of a solution of epidermolysin containing 100 epidermolytic activity units/ml. The mixtures were incubated at 37 for 15 min and then at 4 C for 60 min and assayed for epidermolytic activity. Incubation of epidermolysin with unimmunized rabbit serum served as a control. The highest dilution of serum that prevented the development of a positive Nikolsky sign was defined as the titer of the serum.

RESULTS

Inoculation of mice with S. aureus. Serial 10-fold dilutions of 18- to 24-h Trypticase soy broth cultures of strain EV were prepared, and 0.1 ml was injected subcutaneously in the nape of the neck of 1- to 3-day-old mice. Dilutions were made in Trypticase soy broth and the number of organisms was quantified by colony

counts in Trypticase soy agar plates. When over 10^8 organisms/ml were injected, the mice generally died; when 10^7 organisms/ml were injected, a positive Nikolsky sign after 12 to 16 h with spontaneous peeling after 16 to 20 h demonstrated epidermolysis. An ecchymotic appearance developed at 6 to 12 h. Lesser dilutions, as well as the controls, produced no Nikolsky sign or peeling. This preliminary work confirmed that this strain produced an epidermolytic toxin. The appearance of the ecchymoses suggested that the strain produces alpha-toxin as well, since the subcutaneous injection of staphylococcal alpha-toxin into newborn mice produces ecchymoses, whereas purified epidermolysin does not (Fig. 1).

Production of epidermolysin. Incubation of the dialysis bags containing inoculated medium 199 for 48 h in the rabbits produced a white cloudy fluid that became clear after sterilization with the Millipore filter. In some cases, the rabbits died before 48 h; the fluid then was yellowish to brown with a putrid odor, but it still contained large amounts of epidermolysin that could be purified with the same scheme. After sterilization, assays for protein, epidermolysin, and alpha- and delta-toxins were performed. Typical results are shown in Table 1. Disc gel electrophoresis of this material showed many protein bands (Fig. 2), the most prominent of which were epidermolysin.

Purification of toxin. The fluid was then concentrated with the PM 10 ultrafiltration membrane and again assayed, with small quantities of delta-toxin detected. Pevikon block electrophoresis was then run and the entire block was assayed. With this step, epidermolysin and alpha-toxin were clearly separated (Fig. 2); no delta-toxin activity could be discerned. Disc gel electrophoresis showed three bands. The epidermolysin-containing fractions were pooled, concentrated by ultrafiltration, and run through the CM-Sephadex column with the sodium chloride gradient. The eluted

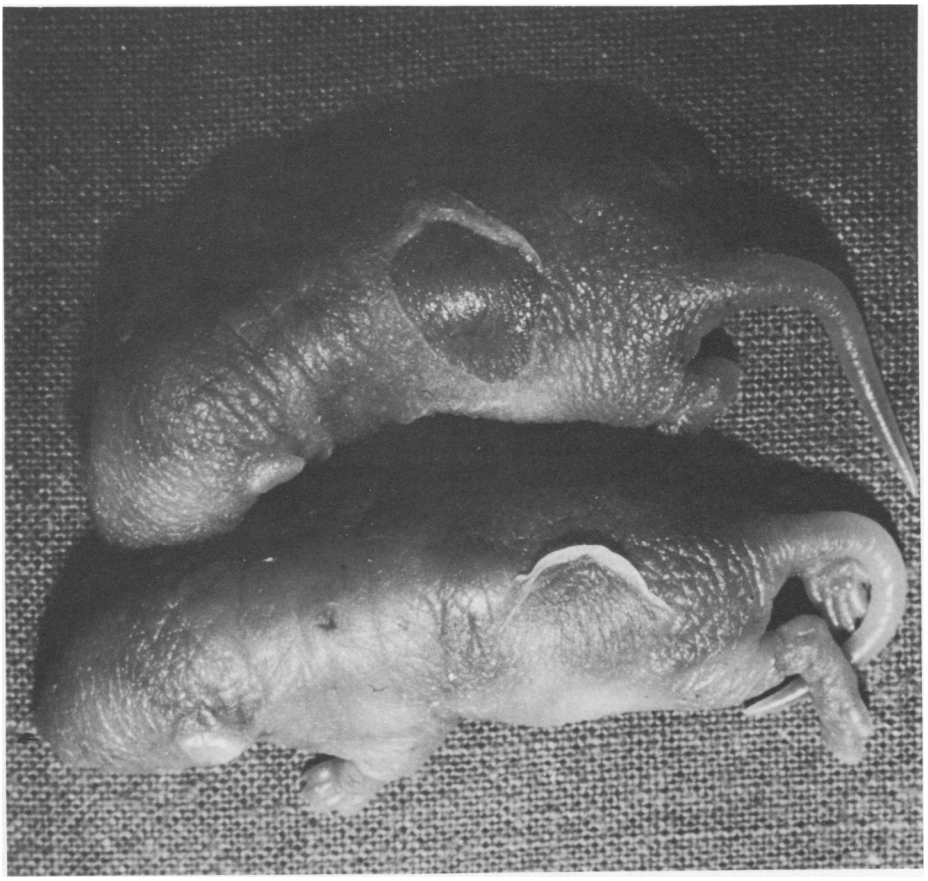

FIG. 1. *Two-day-old mice 45 min after injection of purified epidermolysin showing Nikolsky sign and peeling.*

TABLE 1. *Purification of epidermolysin*

Culture fraction	Alpha-toxin (HD_{50}/ml)[a]	Delta-toxin (HD_{50}/ml)	Epidermolysin (EU/ml)[b]	Protein (mg/ml)	Sp act of epidermolysin (EU/mg of protein)	Purification index of epidermolysin
Culture supernatant, strain EV	490	0	500	650	770	1
CM-Sephadex, pool	0	0	800	65	12,300	16

[a] Hemolytic units per milliliter.
[b] Epidermolytic activity units per milliliter.

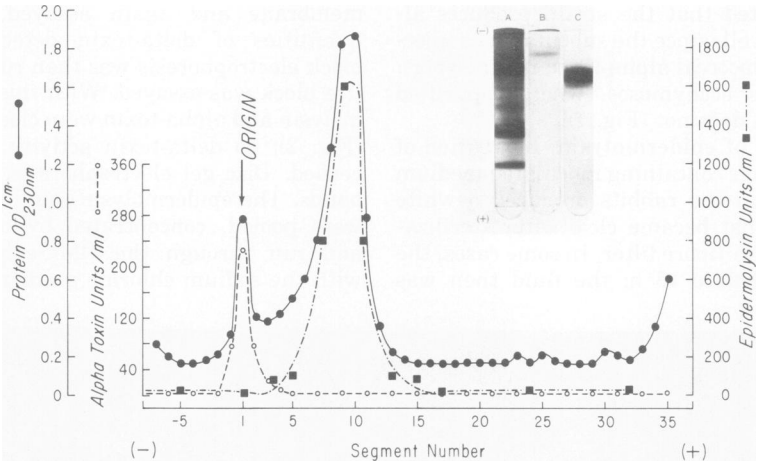

FIG. 2. *Pevikon block zone electrophoresis of staphylococcal culture supernatant in Tris-hydrochloride (pH 9.0) showing separation of alpha-toxin and epidermolysin. Insert shows acrylamide gel electrophoresis of (A) the crude supernatant, (B) fraction 1 containing alpha-toxin, and (C) pool of fractions 5 to 12 containing epidermolysin.*

fractions were assayed (Fig. 3). Disc gel electrophoresis showed two bands. As the epidermolysin-containing fractions came off the column, they first had only the faster-migrating band, which accounted for the left peak in Fig. 3; then both bands appeared and the later fractions contained only the slower-migrating band. A disc gel corresponding to a stained disc with both bands in equal amounts (tube 34 in Fig. 3) was sliced into 1.3-mm segments. These segments were then eluted with 0.3 ml of PBS and this eluent was assayed for epidermolysin. The fact that epidermolytic activity was demonstrated in slices that corresponded to each of the bands suggested two species of epidermolysin (Fig. 4). No alpha- or delta-toxin activity could be identified in any of the fractions.

Molecular weight determinations. SDS disc gels were done and showed only one band. Comparison with the SDS gels with known standards gave a molecular weight for epidermolysin of 28,600 ± 400. Molecular weight determination by ultracentrifugation gave a result of 32,500 ± 120.

Antibody production. Rabbits given injections and booster injections of purified toxin

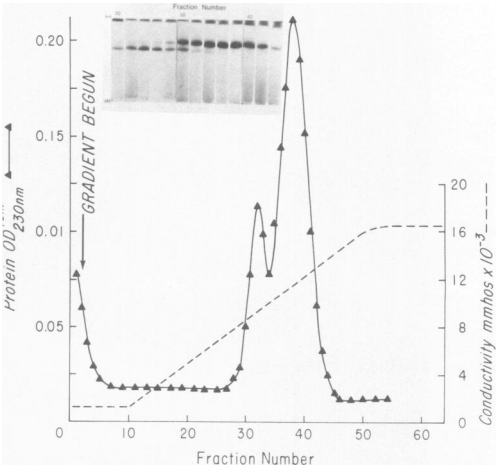

FIG. 3. *Chromatography on CM-Sephadex in phosphate, 0.01 mol/liter (pH 6.0), with a sodium chloride gradient of pooled epidermolysin fractions from Pevikon block electrophoresis. Insert shows acrylamide gel electrophoresis of fractions 30 to 42.*

(comparable to slower-migrating species from fractions 39–41 in Fig. 3) in Freund adjuvant produced detectable quantitites of antibody to

epidermolysin in 21 days and larger amounts after further booster injections. At no time could antibody to alpha-toxin be demonstrated as assayed in Ouchterlony plates (Fig. 5). The antibody reacted with the faster-migrating species of epidermolysin (from fraction 40 in Fig. 3) as well as with the slower-migrating species.

DISCUSSION

Several reports have already been published on the methods of purifying the epidermolytic toxin produced by strains of *S. aureus* that are responsible for cases of toxic epidermal necrolysis, Ritter von Rittershain dermatitis exfolia-

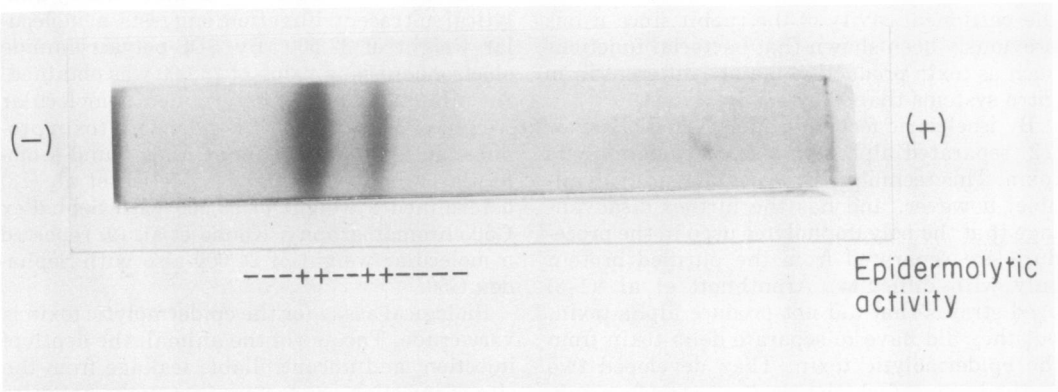

FIG. 4. *Polyacrylmide gel electrophoresis 7.5% (pH 8.3) of purified epidermolysin stained for protein with Coomassie brilliant blue in trichloroacetic acid. A parallel gel was cut in 1.3-mm segments, eluted, and assayed for epidermolytic activity.*

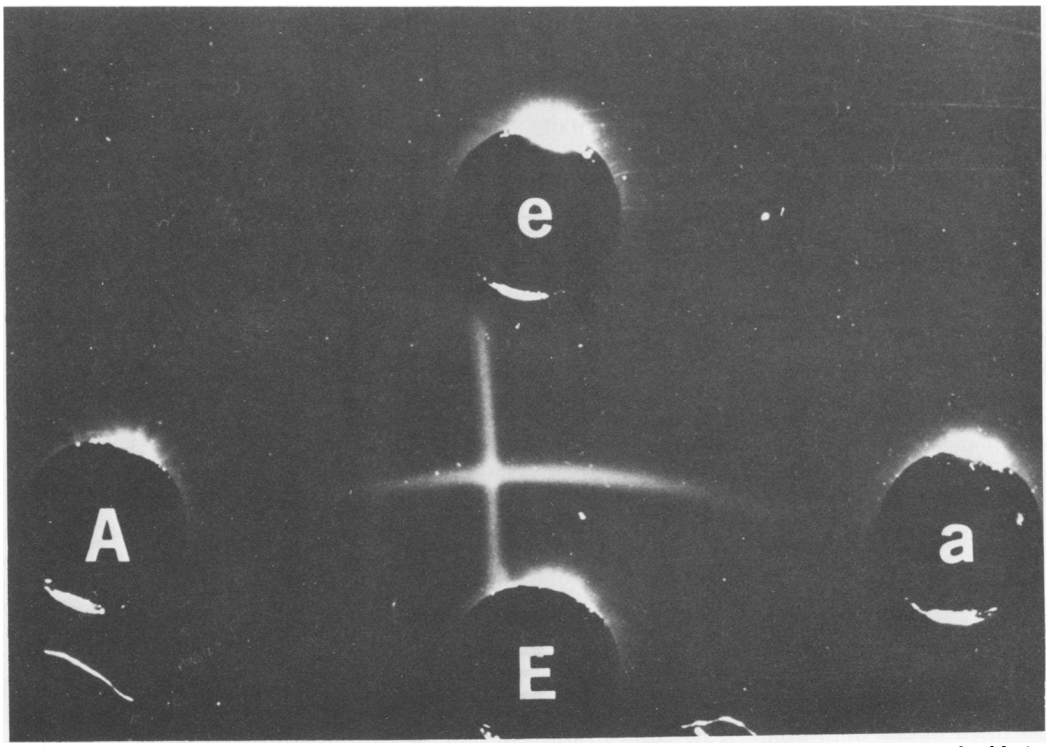

FIG. 5. *Antigenic distinction of staphylococcal alpha-toxin and epidermolysin by Ouchterlony double immunodiffusion in agar. Antigens were placed in wells of highly purified epidermolysin (E) and alpha-toxin (A); antisera were placed in wells of antibody to epidermolysin (e) and antibody to alpha-toxin (a).*

tiva neonatorum, bullous impetigo, and the staphylococcal scarlatiniform eruption. In vitro culture techniques with incubation in yeast-Trypticase soy broth in a 10% CO_2 atmosphere (8, 9), in a semisolid nutrient agar in 20% CO_2 (2, 3) and in a yeast diffusate medium in the absence of added CO_2 (1) have been reported. Like Melish et al. (13), we used an in vivo technique with medium 199 in a dialysis bag in the peritoneal cavity of the rabbit since it has previously been shown that bacterial functions such as toxin production may be different in in vitro systems than they are in vivo (4).

By isoelectric focusing, Melish and Glasgow (12) separated alpha-toxin from epidermolytic toxin. This technique may not be readily available, however, and has the further disadvantage that the polyampholytes used in the procedure are separated from the purified protein only with difficulty. Arbuthnott et al. (1-3) used strains that did not produce alpha-toxin, but they did have to separate delta-toxin from the epidermolytic toxin. They developed two methods, one of which uses isoelectric focusing, and the other, treatment with hydroxylapatite. Kapral and Miller (8) used diethylaminoethyl-Sephadex column chromatography with a 0.1 M ammonium acetate buffer at pH 9.0 to separate alpha-toxin; however, their final product contained sufficient alpha-toxin to produce appreciable amounts of neutralizing antibody to it when injected into rabbits. Kondo et al. (9) partially separated alpha-toxin from the epidermolytic toxin using Sephadex G-75 with 0.01 M Tris at pH 7.5 followed by diethylaminoethyl-cellulose chromatography with the same Tris buffer and a linear sodium chloride gradient. With this method, they identified four species of epidermolytic toxin, two of which were completely separated from the alpha-toxin; the other two species had to be further purified by eluting the toxin from segments cut from acrylamide disc gels.

How the epidermolytic toxin separates the cells of the epidermis from one another in the mouse model and in man remains unknown. Since it may act on epidermal cell membranes, we believed that it ought to be purified in such a way as to eliminate the possibility that contaminant amounts of the alpha-toxin would produce spurious results when further electron microscopy and biochemical investigations were undertaken. Our method seems to have accomplished this because no alpha-toxin activity could be identified in the final product, no band in the polyacrylamide disc gels could be identified in the final product, no band in the polyacrylamide disc gels could be identified at the level where alpha-toxin migrates, no reaction occurred when immunodiffusion was carried out with anti-alpha-toxin, and no antibody to alpha-toxin was produced by immunization of rabbits with the final product. Furthermore, our method uses conventional biochemical techniques that are readily available and does not require the use of non-alpha-toxinogenic strains.

Our molecular weight determination by analytical ultracentrifugation suggests a molecular weight of 32,500. By SDS-polyacrylamide electrophoresis, a value of 28,600 was obtained. Arbuthnott et al. (3) determined a molecular weight of 33,000 with SDS gels; with toxin produced in a different manner, they found a molecular weight of 25,000 (1). Melish et al. (13) determined a weight of 23,500 with Sephadex G50 chromatography; Kondo et al. (9) reported a molecular weight of 24,000 also with Sephadex G50.

Biological assay for the epidermolytic toxin is very crude. The age of the animal, the depth of injection, and uncontrollable leakage from the site of injection are factors that make accurate quantitative results difficult. Moreover, every author used different standards to define units of activity. For any meaningful comparison for quantitative results, a more precise assay is needed. Our purification procedure, which starts with medium 199, results in a purification index of 16, which is comparable to the results of the other groups who have started with more complex culture fluids. With our final product, injection into 1-day-old mice allowed demonstration of a positive Nikolsky sign within 10 min.

Kondo et al. (9) analyzed their product with disc gel electrophoresis and found four different bands with epidermolytic activity. On SDS gels, all of these had the same molecular weight, but each band had a different specific activity.

We also have analyzed our product with disc gels and find two bands, both with epidermolytic activity and the same molecular weight. Antibody produced against one of them precipitates with the other in Ouchterlony immunodiffusion in a single band. Arbuthnott et al. (1) have also demonstrated two forms of the toxin with similar molecular weights but differing in electrophoretic migration. When gel electrofocusing was done in the presence of urea, they found up to seven components within the major form of the toxin. The antibody produced against their dominant form of toxin showed two distinct antigenic components. It should also be noted that Kondo et al. (10) have purified from non-phage group II staphylococci a toxin with epidermolytic properties that differs

in antigenicity from that produced by group II staphylococci but has a similar molecular weight.

Arbuthnott et al. (3) termed this toxic factor "epidermolytic toxin," Melish et al. (13) called it an "exfoliating toxin," and Kapral and Miller (8) called it "exfoliatin," the term then used by Kondo et al. (9). In dermatological parlance, "exfoliatin" suggests desquamation or scaling, but since separation of layers of the epidermis from one another is the clinical and histological effect of the toxin, it is actually an epidermolytic toxin, and we suggest the alternate name, "epidermolysin."

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