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was equilibrated with phosphate buffer at the appropriate pH and then washed with distilled water. The CM-cellulose was stirred into the diluted crude toxin, allowed to settle, and then poured into a chromatography column. Elution characteristics are given under each purification scheme.

Isoelectric focusing. Isoelectric focusing in sucrose gradients was carried out as recommended by LKB (Stockholm, Sweden). Isoelectric focusing in gels containing pH 3 to 10 ampholines was done at 17 C according to the method of Wrigley (14).

Antiserum. Anti-SEC₁ was prepared by repeated intramuscular injections of the major component of purified SEC₁, isolated by electrofocusing. The preparation was mixed with complete Freund adjuvant (Difco) for injection into goats.

Ouchterlony (10) double diffusion. One percent ionagar (15 ml) dissolved in pH 8.3 borate buffer was layered onto glass plates (8 by 10 cm). Three-millimeter holes were punched at 5-mm intervals in a circular pattern with a well in the center; 5 μ l of antigen or antisera was placed in each well.

Purification of SEC₁. The culture supernatant from strain 137-H-2 was diluted 1:5 with distilled water and the pH was adjusted to 6.2 with phosphoric acid. CG-50 (350 g) equilibrated at pH 6.2 with 0.01 M phosphate was added to the diluted culture supernatant. The toxin was eluted from the column with 0.5 M phosphate buffer containing 0.25 M NaCl (pH 6.8). The toxin peak was dialyzed at 4 C against distilled water to reduce the salt concentration. The dialyzed toxin was diluted 1:10 with distilled water; 100 g of CG-50 equilibrated at pH 6.8 was added. The column was washed with distilled water and the toxin was eluted with 0.15 M Na₂HPO₄. The toxin peak was dialyzed against 0.01 M phosphate buffer (pH 6.2); 200 g of CM-cellulose equilibrated at pH 6.2 was added to the dialyzed toxin. The toxin was eluted from the column by a linear gradient (0.02 to 0.07 M phosphate buffer, pH 6.2 to 6.8). The toxin-containing fractions were combined, dialyzed against 0.01 M phosphate buffer (pH 7.0), and lyophilized.

Purification of SEC₂. The supernatant from a culture of strain 361 was prepared as for SEC₁, except that the pH was adjusted to 5.6. CG-50 (350 g) was equilibrated to pH 5.6 with 0.005 M phosphate. Elution of toxin was accomplished with 0.5 M phosphate buffer plus 0.5 M NaCl at pH 6.2. The toxin-containing eluate was dialyzed first against distilled water and then against 0.01 M phosphate buffer at pH 6.0. CM-cellulose (200 g) equilibrated at 0.01 M, pH 6.0, was added to the dialyzed toxin. The toxin was eluted from the column by using a linear gradient (0.01 to 0.05 M phosphate buffer, pH 6.0 to 6.8). The toxin-containing fractions were combined and dialyzed against 0.03 M phosphate buffer, pH 5.7. Hydroxylapatite (150 g) was swollen in 0.03 M phosphate buffer at 5.7. A column (5 by 60 cm) was poured at 4 C, and the column was further equilibrated with buffer at 4 C. The toxin was slowly passed through the column and then washed with the equilibrating buffer. Elution was accomplished by using a linear gradient (0.2 to 0.4 M phosphate, pH 5.7). The toxin peak was dialyzed against 0.01 M phosphate buffer (pH 7.0) and then lyophilized.

SEC₂ (72 h) purification. The supernatant culture fluid of a 72-h fermentation of strain 361 was concentrated to 4 liters by membrane filtration (Amicon TC1D with UM-10 membrane; Amicon Corp., Lexington, Mass.) at 4 C. The concentrated supernatant was dialyzed against distilled water and then centrifuged to remove any precipitate. CM-cellulose (Microgranular no. 52, Whatman; Reeve-Angel, Clifton, N.J.) was equilibrated with 0.01 M phosphate buffer at pH 5.5. A column (2.5 by 50 cm) was poured at 4 C. A 500-ml aliquot of toxin previously dialyzed against 0.01 M phosphate buffer, pH 5.5, was run slowly through the column. The column was washed with the equilibrating buffer and the toxin was eluted with a linear gradient (0.01 to 0.07 M phosphate buffer, pH 5.5 to 6.8). The toxin-containing peak was dialyzed against 0.01 M phosphate-buffered saline, pH 7.0. The toxin was further purified by molecular sieving on a Sephadex G-75 column equilibrated with phosphate-buffered saline. A symmetrical toxin peak was eluted, dialyzed against 0.01 M phosphate buffer, pH 7.0, and lyophilized.

Alkaline treatment of purified toxins. Purified toxins were exposed to pH 9.0 at 37 C in 0.01 M tris(hydroxymethyl)aminomethane buffer (13).

RESULTS

SEC₁ purified by the described chromatographic method contained three components by electrofocusing (Fig. 1) in a sucrose gradient analytical instrument. The two major components had isoionic points of 9.19 and 8.83, which were determined at 4 C. Three bands were also demonstrated by gel electrofocusing (Fig. 2A). The most alkaline species by both procedures was present in only trace amounts, whereas the two major components were in a ratio of approx-

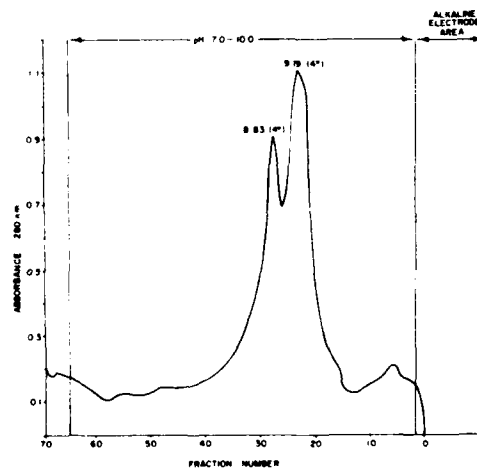


FIG. 1. Isoelectric focusing of staphylococcal enterotoxin C₁, using pH 7 to 10 ampholine-sucrose gradient. Electrofocusing was performed at 4 C. The pH values were determined at 4 C.



FIG. 2. Isoelectric focusing of enterotoxin preparations in polyacrylamide gel, using pH 3 to 10 ampholines. (A) SEC₁; (B) SEC₁ treated with alkali; (C) SEC₂; (D) SEC₂ treated with alkali; (E) SEC₂ (72 h).

imately 4:1, the pH 9.19 species being the major component.

Exposure of SEC₁ to pH 9.0 at 37 C for 10 days caused a marked increase in microheterogeneity and an almost complete loss of the two most alkaline species (Fig. 2B). There was also development of new, more acidic forms. The change in pattern was strikingly more drastic than that observed with SEB and indicated a much greater lability of SEC₁ to chemically induced deamidation. Significant alterations were even observed after dialysis against pH 9 buffer in the cold.

When purified SEC₂ was analyzed by a sucrose gradient analytical electrofocusing instrument, two sharp peaks with isoionic points of 8.40 and 7.10 were demonstrated (Fig. 3). In addition, there were at least three other components. Five major bands were seen by isoelectric focusing in gels (Fig. 2C). It is likely that the 8.40 component corresponds to the alkaline doublet of the gels and the broad shoulder on this peak is the major, next more acidic species seen

in the gel (estimated pI = 8.1). The former components were greatly diminished after exposure of the purified toxin to pH 9.0 at 37 C for 10 days (Fig. 2D).

SEC₂ (72 h) had two major components with only trace amounts in the more alkaline region of the gels. The densitometric scan illustrated how markedly the relative concentrations of the several components were altered. In addition, a definite new acidic species was demonstrable (Fig. 2E and 4).

All toxin preparations showed lines of complete identity when examined by the Ouchterlony technique using anti-SEC₁ antiserum. Composite immunoelectrophoresis (Fig. 5) of the five preparations reveals that SEC₁ treated with alkali had decreased cathodic movement

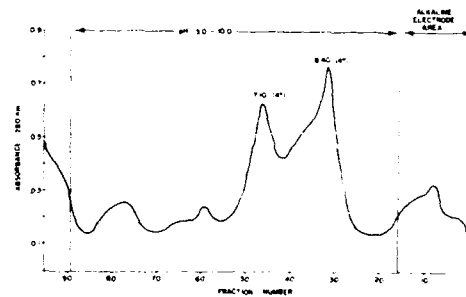


FIG. 3. Isoelectric focusing of staphylococcal enterotoxin C₂, using pH 3 to 10 ampholine-sucrose gradient. Electrofocusing was performed at 4 C. The pH values were determined at 4 C.

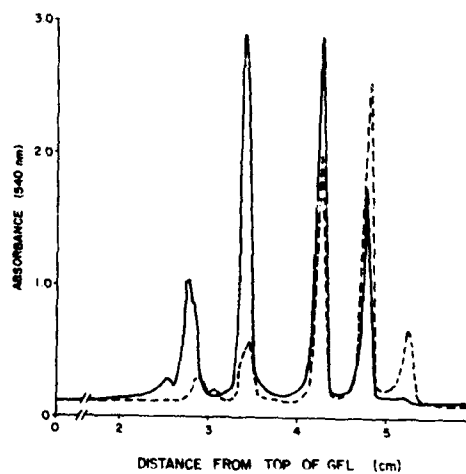


FIG. 4. Densitometric scan of electrofocused gels. SEC₁ (—) and SEC₂ (72 h) (---).

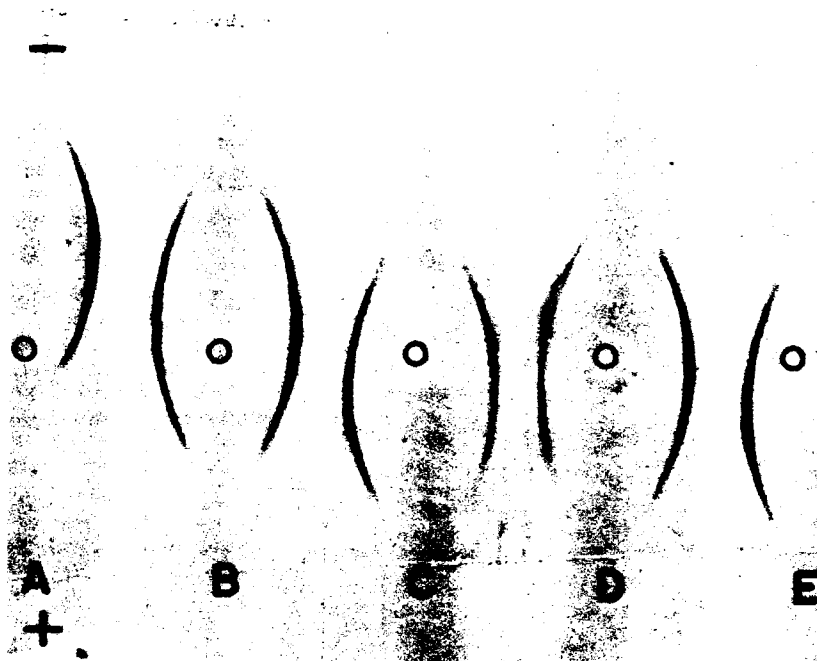


FIG. 5. Immunoelectrophoresis of staphylococcal enterotoxins. (A) SEC_1 ; (B) SEC_1 treated with alkali; (C) SEC_2 ; (D) SEC_2 treated with alkali; (E) SEC_2 (72 h). Antiserum to SEC_1 was used in all troughs.

compared with untreated SEC_1 . Alkaline treatment of SEC_2 did not appear to change the average charge. The more acidic nature of SEC_2 (72 h) was demonstrated by its slight anodic movement.

Sodium dodecyl sulfate-acrylamide electrophoresis revealed that all C-type enterotoxin preparations co-migrated with purified SEB; therefore, the molecular weight is between 28,000 and 29,000.

DISCUSSION

SEC_1 consists of three components and has comparable microheterogeneity to *S. aureus* enterotoxins A (12) and B (8). In addition, the two major components of SEC_1 have isoionic points approximately 0.4 pH units apart, similar to the difference in isoionic points seen with the major components of *S. aureus* enterotoxins A (12) and B (8) and consistent with a single charge difference between isoelectric species. The behavior of SEC_1 treated with alkali is similar to that reported for SEB (13), i.e., a sequential conversion from more to less alkaline forms and the appearance of new, more acidic species. It is noteworthy, however, that SEC_1 is considerably more altered than SEB.

SEC_2 demonstrates more isoelectric pauci-

dispersity than the other staphylococcal enterotoxins. Surprisingly, however, it is the most resistant variety to chemical deamidation induced by exposure to pH 9.0 and 37 C. Only the most alkaline species appeared to be affected, and the average charge, as evidenced by immunoelectrophoresis, was unchanged. A much greater change was brought about by prolonging the incubation of the SEC_2 culture to 72 h before isolation. The shorter time period suggests strongly that the change was produced enzymatically, presumably by a deamidase.

Two preparations of SEC_2 have been examined isoelectrically by Dickie and co-workers (5, 6). Both were isolated after 72 h of incubation and by a procedure involving a preliminary concentration by dialysis against polyethylene glycol. In one instance the component present in highest concentration had a pI of 7.35 and in the other a pI of 6.50 (Their pI values were obtained by measurement of pH of the samples at 25 C. The values cited here were corrected to 4 C, our temperature of measurement, by the van't Hoff equation, assuming that the amino groups of the ampholines have ΔH of 10,000 cal/mol.) The 7.35 component probably corresponds to our 7.1 component, and the composition of the preparation, lacking our 8.4 component, is comparable

to our SEC₂ (72 h) material. The other preparation was considerably more deamidated and contained as its most alkaline species a fraction with a pI of 7.25, again comparable to our 7.1 component. It was thus devoid of both the 8.1 and 8.4 isoelectric species found in our preparations. It is apparent that considerable care must be taken in the isolation of the enterotoxins if one is to avoid degrading the proteins, and it would be well to characterize individual preparations by their isoelectric focusing patterns. It was found that when the initial steps of the isolation were not carried out promptly, SEC preparations were badly nicked. The extent of nicking is readily determined by sodium dodecyl sulfate-polyacrylamide electrophoresis in the presence and absence of β -mercaptoethanol.

The data support the report of Avena and Bergdoll (1) that SEC₁ and SEC₂ are different enterotoxins with identical immunological reactions. The gel isoelectric focusing patterns obtained after prolonged exposure of SEC₁ at pH 9.0 bore little resemblance to those of SEC₂.

LITERATURE CITED

1. Avena, R. M., and M. S. Bergdoll. 1967. Purification and some physicochemical properties of enterotoxin C. *Staphylococcus aureus* strain 361. *Biochemistry* **6**: 1474-1480.
2. Baird-Parker, A. C. and R. L. Joseph. 1964. Fractionation of staphylococcal enterotoxin B. *Nature (London)* **202**:570-571.
3. Borja, C. R., and M. S. Bergdoll. 1967. Purification and partial characterization of enterotoxin C produced by *Staphylococcus aureus* strain 137. *Biochemistry* **6**:1467-1473.
4. Chang, P.-C., and N. Dickie. 1971. Fractionation of staphylococcal enterotoxin B by isoelectric focusing. *Biochim. Biophys. Acta* **236**:367-375.
5. Chang, P.-C., Y. Yano, M. Dighton, and N. Dickie. 1971. Fractionation of staphylococcal enterotoxin C₂ by isoelectric focusing. *Can. J. Microbiol.* **17**:1367-1372.
6. Dickie, N., Y. Yano, H. Roborn, and S. Stavric. 1972. On the heterogeneity of staphylococcal enterotoxin C₂. *Can. J. Microbiol.* **18**:801-804.
7. Jarvis, A. W., R. C. Lawrence, and G. G. Pritchard. 1973. Production of staphylococcal enterotoxins A, B, and C under conditions of controlled pH and aeration. *Infect. Immun.* **7**:847-854.
8. Metzger, J. F., A. D. Johnson, and W. S. Collins II. 1972. Fractionation and purification of *Staphylococcus aureus* enterotoxin B by electrofocusing. *Biochim. Biophys. Acta* **257**:183-186.
9. Metzger, J. F., A. D. Johnson, W. S. Collins II, and V. McGann. 1973. *Staphylococcus aureus* enterotoxin B release (excretion) under controlled conditions of fermentation. *Appl. Microbiol.* **25**:770-773.
10. Ouchterlony, O. 1953. Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion. *Acta Pathol. Microbiol. Scand.* **32**:231-240.
11. Schantz, E. J., W. G. Roessler, J. Wagman, L. Spero, D. A. Dunnerly, and M. S. Bergdoll. 1965. Purification of staphylococcal enterotoxin B. *Biochemistry* **4**:1011-1016.
12. Schantz, E. J., W. G. Roessler, M. J. Woodburn, J. M. Lynch, H. M. Jacoby, S. J. Silverman, J. C. Gorman, and L. Spero. 1972. Purification and some chemical and physical properties of staphylococcal enterotoxin A. *Biochemistry* **11**:360-366.
13. Spero, L., J. R. Warren, and J. F. Metzger. 1974. Microheterogeneity of staphylococcal enterotoxin B. *Biochim. Biophys. Acta* **336**:79-85.
14. Wrigley, C. W. 1968. Gel electrofocusing—a technique for analyzing multiple protein samples by isoelectric focusing. *Sci. Tools* **15**:17-23.