Scatchard, G., and Black, E. S. (1949), *J. Phys. Colloid Chem. 53*, 88.
Scatchard, G., Scheinberg, I. H., and Armstrong, S. H., Jr. (1950), *J. Am. Chem. Soc. 72*, 540.
Singer, S. J. (1965), *Proteins 3*, 269.
Singer, S. J., and Doolittle, R. F. (1966), *Science 153*, 13.
Turner, E. W., and Boyer, P. D. (1952), *J. Immunol. 69*, 265.
von Hippel, P. H., and Wong, K. Y. (1964), *Science 145*, 577.
Warren, J. C., and Cheatum, S. G. (1966), *Biochemistry 5*, 1702.
Warren, J. C., and Peterson, D. M. (1966), *Science 152*, 1245.
Weliky, N., Weetall, H. H., Gilden, R. V., and Campbell, D. H. (1964), *Immunochem. 1*, 219.
Whitney, P. L., and Tanford, C. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 524.

Purification and Partial Characterization of Enterotoxin C Produced by *Staphylococcus aureus* Strain 137*

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ABSTRACT: Enterotoxin C elaborated by *Staphylococcus aureus* strain 137 was purified by chromatography on a CM-cellulose column and gel filtration through Sephadex G-75 and G-50. The purified enterotoxin exhibited a high degree of homogeneity as determined by synthetic boundary spreading and by electrophoresis. This was confirmed by estimation of purity by the double-diffusion tube technique. The highly purified toxin is a simple, colorless, and antigenic protein with a sedimentation coefficient of 3.0 S. A molecular weight of about 34,100 was obtained from sedimentation and diffusion measurements. The basic nature of the protein was revealed by its isoelectric point which is 8.6 in Veronal buffer of 0.1 ionic strength. It possesses a marked degree of toxicity; 5 μg produces emesis in rhesus monkeys (2–3 kg) within 2–5 hr after intragastric administration.

Data on intrinsic viscosity, frictional ratio, and axial ratio suggest compactness of the enterotoxin molecule.

Various strains of *Staphylococcus aureus* produce a group of toxic substances which are called enterotoxins because of their effects on the gastrointestinal tract. Vomiting and diarrhea occur 2–5 hr after ingestion of food contaminated with these substances. An amount as low as 5 μg causes typical food poisoning symptoms when administered intragastrically to rhesus monkeys.

In 1963, a systematic nomenclature using sequential lettering was established to classify these food poisoning agents as immunologically distinct entities (Casman *et al.*, 1963). The purification and physicochemical properties of enterotoxin A (Chu *et al.*, 1966) and exterotoxin B (Bergdoll *et al.*, 1959, 1961, 1965a; Frea *et al.*, 1963; Schantz *et al.*, 1965; Spero *et al.*, 1965; Wagman *et al.*, 1965) have been reported.

An intensive search for new immunological types of enterotoxin stemmed from the common occurrences of staphylococcal food poisoning. In 1965, the identification of a new enterotoxin as enterotoxin C was reported (Bergdoll *et al.*, 1965b) and *S. aureus* strain 137 was selected as the prototype strain. *S. aureus* strain 361 elaborates the same immunological type of enterotoxin as that produced by strain 137. Availability of the purified enterotoxins produced by strains 137 and 361 made it possible to study and compare the exterotoxins from the two strains. Dissimilarities in certain properties have been observed which are presented in the accompanying paper (Avena and Bergdoll, 1967).

This communication deals with the purification of exterotoxin C produced by *S. aureus* strain 137. It also describes some of the properties of the purified enterotoxin.

Experimental Section

Materials. The CM-cellulose used in this work is a Selectacel ion-exchange product, no. 77, type 20 (Carl Schleicher & Schuell Co.). Before use, it was treated with 0.1 N NaOH, washed with distilled water until the pH of the washings was 7.0–7.5, followed by treatment with 0.1 N HCl, and then washed thoroughly to remove excess acid. Before packing into the columns,

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the washed CM-cellulose was equilibrated with buffer (0.01 M sodium phosphate buffer, pH 5.5).

Sephadex gels (bead form) were purchased from Pharmacia Fine Chemicals, Inc., New York, N. Y. Separate suspensions of Sephadex G-75 and G-50 in 0.02 M sodium phosphate buffer (pH 6.8) were prepared and the fines were removed by decantation. The remaining gel was used in the purification of enterotoxin C. The Sephadex gels were allowed to swell in the appropriate buffer for at least 72 hr at 2–4° before the columns were prepared.

Estimation of Protein and Ultraviolet Absorption Spectra. In the early stages of the purification, protein concentration was determined by the procedure of Lowry *et al.* (1951). Crystalline egg albumin was used as the standard. Later in the work, the absorbance at 280 mμ was used as a nonspecific measure of protein concentration, based on $E^{1\%}_{1\,cm}$ 12.1 which is the extinction value for purified enterotoxin B at 280 mμ (Bergdoll *et al.*, 1965a). After the highly purified enterotoxin C was prepared, the value of $E^{1\%}_{1\,cm}$ 11.3 which is the extinction value for enterotoxin C at 280 mμ was used in the calculation of protein concentration. Measurements of absorbance in the ultraviolet region were performed on a Beckman Model DU spectrophotometer.

Ultracentrifugal Analysis. The Spinco Model E analytical ultracentrifuge equipped with a phase plate as a schlieren diaphragm and with a rotor temperature-indicating and control unit was used in all analyses which were carried out in 0.05 M sodium phosphate buffer (pH 6.8) at 20°. Before the purified enterotoxin was studied in the ultracentrifuge, it was dialyzed thoroughly against 0.05 M sodium phosphate buffer (pH 6.8). Sedimentation velocity experiments were done on both the conventional 12-mm single 4° sector cell and valve-type synthetic boundary 12-mm cell with quartz windows. To determine diffusion coefficients at various protein concentrations, the latter type of cell was employed. Sedimentation coefficients at different protein concentrations were obtained from measurements of photographic plates made directly on a Model 6 Nikon shadowgraph. The maximum height–area method described by Schachman (1957), which involved determination of the second moments of the schlieren curves, was employed for the analysis of sedimenting boundaries to determine diffusion coefficients and polydispersity.

Viscosity. All viscosity measurements were made in a thermostated bath at 20° with the use of a capillary viscosimeter (Schachman, 1957). Enterotoxin (3–12 mg)/ml of 0.05 M sodium phosphate buffer (pH 6.8) was used in each experiment.

Paper Electrophoresis. All experiments were performed with equipment from E-C Apparatus Corp., Philadelphia, using Whatman No. 4 filter paper as the supporting material. The purified enterotoxin was dissolved in the appropriate buffer to give a concentration of 10 μg/ml. Five microliters was applied on the filter paper (15 × 46 cm) at a spot 20 cm from the anode end. Electrophoretic runs lasted for 6 hr at 600 v. Experiments were run in triplicate for each buffer. The buffers used (0.1 ionic strength) were the following: acetate (pH 4.4 and 5.1), phosphate (pH 5.9, 6.8, and 7.6), Veronal (pH 8.0, 8.3, 8.8, and 9.3), and glycine–NaOH (pH 9.6, 9.9, 10.5, and 11.0). Protein was detected with 0.1% bromophenol blue in 95% ethanol saturated with $HgCl_2$. To correct for endosmotic flow, dextrin (Nutritional Biochemicals Corp.) was run along with the enterotoxin and was detected with 0.5% alcoholic iodine.

Starch Gel Electrophoresis. The apparatus and preparation of starch gel as described by Smithies (1955) were employed with minor modifications. The starch gel, 14 g of hydrolyzed starch (Connaught Medical Research Laboratories, Toronto, Canada)/100 ml of buffer, was placed in a Plexiglass trough (0.3 mm deep, 25 mm wide, and 200 mm long) equipped with a Plexiglass cover to prevent evaporation. Contacts of the gel to the buffer solution (0.1 M Veronal buffer, pH 8.7) were made with three layers of Whatman No. 1 paper saturated with the buffer. Sponge bridges saturated with the buffer connected the buffer vessels to the electrode chamber. Direct current of 26 ma (145 v initially) was passed for 18 hr through the horizontal gel block at 5°. After completion of the electrophoretic run, the gel was stained with 0.1% Amido Black 10B and dissolved in the solvent, methanol–distilled water–glacial acetic acid (50:50:10, v/v). This solvent was also used for washing the gel.

Tests for α- and β-Hemolysins, Coagulase, and Hyaluronidase. The method of Kumar and Lindorfer (1962) was employed to test for α- and β-hemolysins with a slight modification. The sheep and rabbit red blood cells (Markham Laboratories, Chicago) were washed and suspended in 0.153 M NaCl containing 0.0128 M Tris, 0.0048 M KCl, and 0.0006 M $MgSO_4 \cdot 7H_2O$ instead of Veronal buffer.

To detect coagulase, 0.5 ml of reconstituted Bacto-Coagulase Plasma (Difco Laboratories, Detroit) was mixed with 0.5 ml of serial dilutions of each fraction in the purification procedure that was tested. The solutions were incubated at 37° for 1 hr and formation of a clot was considered positive. The mucin clot prevention test devised by McClean *et al.* (1943) was used for detecting hyaluronidase.

Enterotoxin Production. Enterotoxin was produced by growing *S. aureus* strain 137 in a culture medium as described by Bergdoll *et al.* (1965b).

Antisera to S. aureus Strain 137 Antigens. Antisera to crude enterotoxin concentrate and to the highly purified enterotoxin were prepared in rabbits following the procedure reported in the literature (Bergdoll *et al.*, 1965b).

Detection and Assay of Enterotoxin. Before enterotoxin of high purity was prepared, detection of the toxin in the fractions from the different purification steps was achieved by intragastric administration of 50 ml of the aqueous test solutions to young rhesus monkeys (2–3 kg). Food and water were provided *ad libitum* to the animals. A positive reaction for enterotoxin was indicated by emesis within a 5-hr

period after administration. Preparations of the highly purified enterotoxin were assayed for specific biological activity by intragastric and intravenous administration to monkeys. The calulated dosage of enterotoxin in 2 ml of 0.85% pyrogen-free saline was injected intravenously into the saphenous vein.

The identification of a specific antibody to the enterotoxin (Bergdoll *et al.*, 1965b) made possible the use of gel diffusion techniques to determine the presence of enterotoxin. A modification of the Ouchterlony double-diffusion technique (Bergdoll *et al.*, 1965b) was used to check the presence of enterotoxin in the various fractions in the purification procedure. The double-diffusion method of Oakley and Fulthorpe (1953) was used to detect impurities in the fractions and to estimate the purity of the enterotoxin preparation. When the purified enterotoxin became available, specific assay was carried out by a modification of the single-diffusion technique (Bergdoll, 1962; Hall *et al.*, 1963; Read *et al.*, 1965). Glass tubes of 4-mm i.d. and approximately 90 mm long were half-filled with buffered agar gel (pH 7.4, 0.3% agar) containing antiserum at a dilution sufficient to give a well-defined measurable precipitin band. In this work, the available antiserum gave sharp bands at a 1:40 to 1:60 dilution. Enterotoxin (0.5 ml) in standard gel diffusion buffer was layered above the gel and the tubes were capped with "Plasticine" (Harbutt's Plasticine Ltd., England). The standard gel diffusion buffer (0.02 M phosphate buffer, Na_2HPO_4-KH_2PO_4 (pH 7.4) containing 0.85% NaCl and 1:10,000 merthiolate) was used throughout. Migration of the antigen–antibody precipitate was measured with a vernier caliper after incubation of the tubes at 25° for 7 days. Log of the enterotoxin concentrations of 5–160 μg/ml gave a straight line when plotted against the distances of migration of the precipitin bands (Figure 1). Since the movement of the precipitin band is affected by pH and salt concentration, the different fractions that were assayed were dialyzed thoroughly against the standard gel diffusion buffer. The enterotoxin concentration in the different steps of purification was determined from the standard curve.

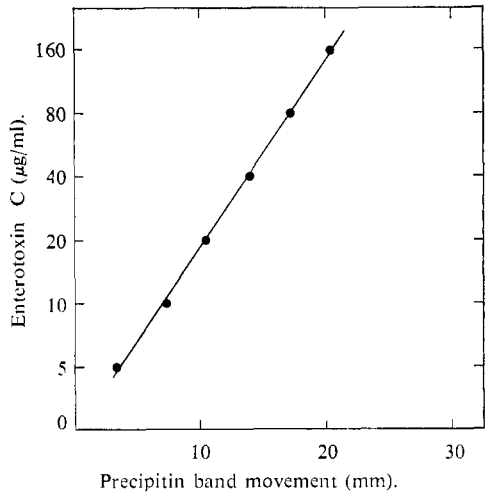

FIGURE 1: Standard curve used for the determination of enterotoxin C concentration (micrograms per milliliter) as shown in Table I. For the immunological assay (single-diffusion tube technique) a 1:60 dilution of specific antiserum was used. Precipitin band was developed for 7 days at 25°.

Results

Purification Procedure. All purification steps were carried out at 2–4°.

STEP I. CONCENTRATION OF BACTERIAL CULTURE SUPERNATANT WITH CARBOWAX. After sedimentation of the cells in a bucket centrifuge, the culture supernatant was concentrated by dialysis for 24–48 hr against Carbowax 20M (Carbowax–water, 1:1), followed by dialysis against deionized water. The solution was lyophilized and kept as a dry powder at room temperature until used for subsequent purification. The enterotoxin content of the bacterial culture supernatant varied from one batch to another, but the average was 60 μg/ml. After concentration with Carbowax, recovery of the enterotoxin was about 90%.

STEP II. CM-CELLULOSE CHROMATOGRAPHY. The lyophilized crude enterotoxin preparation was redissolved in the appropriate amount of 0.01 M sodium phosphate buffer (pH 5.5) (50 ml of buffer for 2–2.5 l. of bacterial culture supernatant). A large amount of the crude material did not dissolve and the resulting solution was turbid. To clarify the solution, it was centrifuged at 14,000 rpm for 15 min in a refrigerated Servall centrifuge. The pH was readjusted to 5.5 with either 0.01 M Na_2HPO_4 or 0.01 M H_3PO_4, before applying on a CM-cellulose column (2.2 × 61 cm) previously equilibrated with 0.01 M phosphate buffer (pH 5.5).

When all the sample had entered the column, elution with 0.01 M sodium phosphate buffer (pH 5.5) was started and elution with this buffer was continued until the "break-through" peak (A, Figure 2) consisting of unadsorbed material had returned to base line. A large portion of the yellowish-brown material which contaminated the enterotoxin was removed in the "break-through" peak. Elution of the adsorbed proteins was carried out by using a stepwise increase in concentration and pH of sodium phosphate buffer (Figure 2). A small amount of enterotoxin was detected by the Ouchterlony plate technique in fraction B. The bulk of the enterotoxin was in fractions C_1 and C_2.

Fractions C_2 (Figure 2) was discarded although it contained enterotoxin, since it was contaminated with a much larger amount of β-hemolysin (approximately six times) than fraction C_1. Other contaminants detected in fraction C_1 were hyaluronidase and traces of α-hemolysin and coagulase. The major portion of fraction D (Figure 2) consisted of β-hemolysin and contained only a very small amount of enterotoxin. The fractions (10 ml each) possessing an absorbance greater than 0.05 composing fraction C_1 were pooled

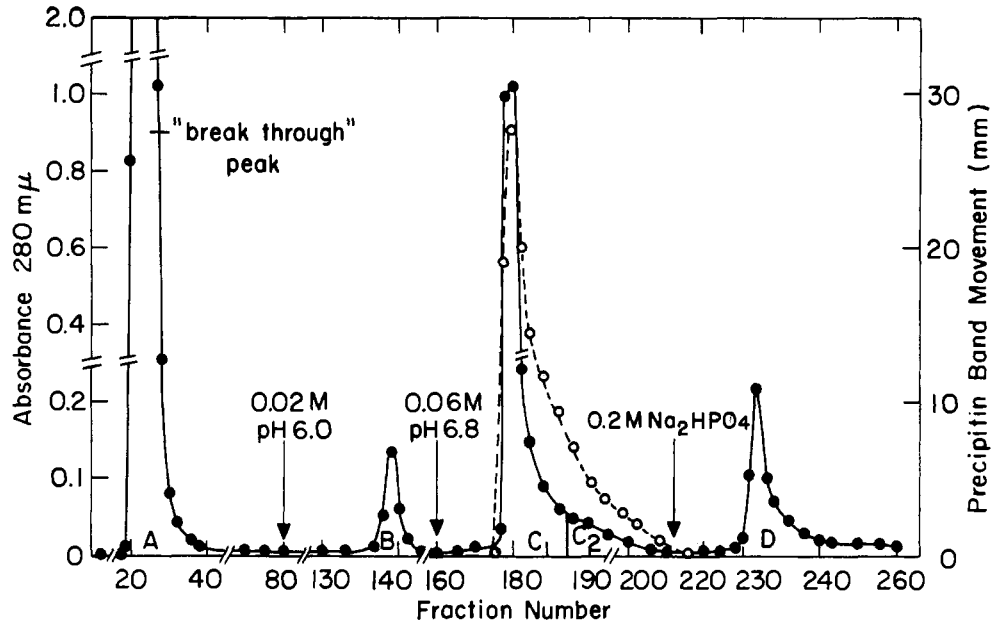

FIGURE 2: Chromatography of crude enterotoxin from step I on CM-cellulose column (2.2 × 61 cm) by stepwise elution with increasing concentration and pH of sodium phosphate buffer, starting with 0.01 M, pH 5.5. Fractions of 10 ml each were collected at a flow rate of 1 ml/2 min. (●——●) Absorbance at 280 mμ. (○- - -○) Precipitin band movement, determined by single-diffusion tube technique.

and lyophilized, followed by dialysis against distilled water to reduce the salts to about 0.005 M. After dialysis the solution was freeze dried. Based on the amount of enterotoxin in the pooled fractions and the amount in the bacterial culture supernatant (determined by single diffusion) the yield was about 30%. The purity of the enterotoxin in the pooled fractions was estimated from the total protein content (absorbance at 280 mμ) and the enterotoxin content (single diffusion) and found to be 80%. The low recovery was attributed to the formation of a precipitate after dialysis.

STEP III. GEL FILTRATION THROUGH SEPHADEX G-75. The lyophilized fraction C_1 from step II (35–40-mg sample/column) was redissolved in the appropriate amount of 0.02 M sodium phosphate buffer (pH 6.8) (approximately 1% of gel bed volume) and layered carefully on a column of Sephadex G-75 (2.0 × 91 cm) previously equilibrated with the buffer used for elution. The redissolved sample was slightly brownish. A typical elution pattern is illustrated in Figure 3. The major peak contained the enterotoxin as shown by gel diffusion tests. The brownish contaminant was eluted immediately after the toxin peak. Fractions (3 ml each) with an absorbance of 0.3 and higher were combined and freeze dried, followed by dialysis against distilled water to eliminate most of the salts. The dialyzed solution was lyophilized and the material was kept in the dry form until it was used in step IV. Filtration through Sephadex G-75 raised the enterotoxin purity from 80 (step II) to 95%. The yield at this stage of purification was 25%.

STEP IV. FIRST FILTRATION THROUGH SEPHADEX G-50. The freeze-dried material from step III (35–40-mg sample/column) was dissolved in 0.02 M sodium phosphate buffer (pH 6.8) (approximately 1% of the gel bed volume) and applied on a Sephadex G-50 column (2.0 × 95 cm) equilibrated with the sodium phosphate buffer, which was also used for elution. Although the enterotoxin started to emerge at tube number 34 as determined by single-diffusion test, only the fractions (3 ml each) giving an absorbance greater than 0.4 were pooled, freeze dried, and dialyzed against distilled water until almost salt free. After dialysis, the enterotoxin-containing solution was relyophilized. The yield was about 20% with a purity of approximately 98%.

STEP V. SECOND FILTRATION THROUGH SEPHADEX G-50. The lyophilized sample from step IV was dissolved in the proper amount of 0.02 M sodium phosphate buffer (pH 6.8) (approximately 1% of bed volume), refiltered through Sephadex G-50 (2.0 × 95 cm), and eluted as summarized in Figure 4. The enterotoxin content represented by precipitin-band movement in single-diffusion tubes is also shown. Pooled fractions with absorbance greater than 0.5 were concentrated by freeze drying, dialyzed against distilled water, and relyophilized. When monkeys were challenged with the purified material, an amount of 5 μg was adequate to produce food poisoning symptoms.

Test for Purity. Immunological assay with the double-diffusion tube technique indicated a high degree of purity of the enterotoxin preparation from step V. A precipitin band was formed with 1 μg of the toxin/ml,

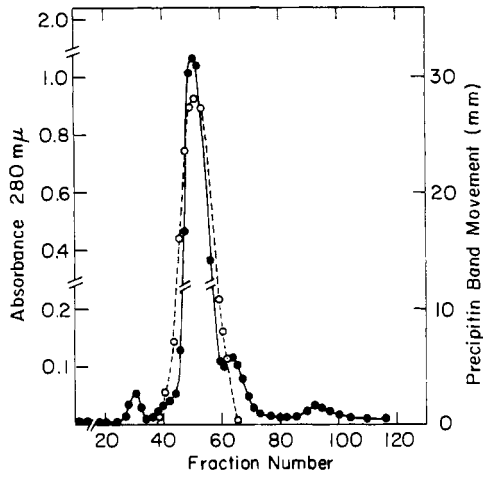

FIGURE 3: Elution pattern from a Sephadex G-75 column. Partially purified enterotoxin from step II, fraction C_1 (39 mg of protein) was placed on a Sephadex G-75 column (2.0 × 91 cm) and eluted with 0.02 M sodium phosphate buffer, pH 6.8. The flow rate was 3 ml (one fraction)/15 min. (O——O) Absorbance at 280 mμ; (O- - -O) Precipitin band movement.

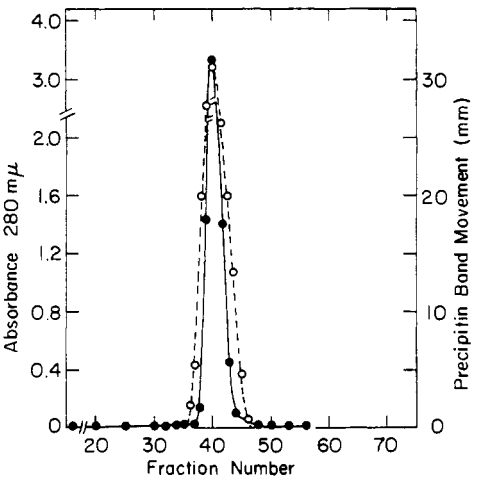

FIGURE 4: Second gel filtration through Sephadex G-50 column (2.0 × 95 cm) of the lyophilized fractions from step IV (fractions 39–44) and eluted with 0.02 M sodium phosphate buffer, pH 6.8. The flow rate was 3 ml (one fraction)/14 min. (O——O) Absorbance at 280 mμ. (O- - -O) Precipitin band movement.

while more than 200 μg/ml was required to produce a precipitin line with any of the contaminants. The purified material traveled as a single spot by paper electrophoresis at various pH values ranging from 4.4 to 11.0. The enterotoxin which was kept as a dry powder at room temperature, however, yielded two components on starch gel electrophoresis in 0.1 Veronal buffer (pH 8.7). One component which appeared to remain very close to the point of sample application toward the anode accounted for 80–90% and the other which moved about 1 cm from the origin toward the cathode accounted for 10–20% of the toxin placed in the gel. Both components were found to be toxic in monkeys and gave a reaction of immunological identity.

Schlieren curves obtained in synthetic boundary experiments with solutions of enterotoxin in 0.05 M sodium phosphate buffer (pH 6.8) revealed only a single symmetrical boundary which, of course, is a qualitative statement to indicate purity. To test for homogeneity, the sedimentation-velocity data were analyzed for boundary spreading according to the treatment of Baldwin and Williams (1950). The boundary spreading expressed in terms of the second moment about the mean in the gradient-sedimentation curves is given by the equation $\sigma^2 = (\rho\omega^2 rt)^2(1 + \ldots) + 2Dt/(1 - \omega^2 st)$, where σ^2 = second moment of the curve which defines the sedimenting boundary, ρ = polydispersity coefficient, ω = angular velocity of ultracentrifuge rotor, r = distance from center of rotation to centroidal ordinate of boundary, t = time, and D = diffusion coefficient. If the material is homogeneous, a plot of $\sigma^2(1 - \omega^2 st)$ against t is a straight line with a slope equal to $2D$. At 52,640 and 21,740 rpm, the data indicated a linearity of the plots of $\sigma^2(1 - \omega^2 st)$ against t with slopes that are nearly equal. Apparent diffusion coefficients, $D_{20,w}$, of 7.25 and 7.35 × 10^{-7} cm^2/sec were obtained for speeds 52,640 and 21,740 rpm, respectively. There is very little effect of centrifugal field strength on apparent values of D and hence the boundary sharpening effect owing to concentration are negligible. The results indicate a high degree of enterotoxin purity and also yield apparent values of D which should not be significantly different from true values.

Sedimentation and Diffusion Coefficients and Molecular Weight. Sedimentation coefficients, $s_{20,w}$, obtained at different protein concentrations from the movement of the maximum ordinate of the schlieren curves are listed in Table I. Concentration dependence of $s_{20,w}$ was low since the measured value with a solution

TABLE I: Sedimentation and Diffusion Coefficients of Staphylococcal Enterotoxin C by Velocity Ultracentrifugation.[a]

Concn (mg/ml)	$s_{20,w}$ (S)	Concn (mg/ml)	$D_{20,w}$ (cm^2/sec) × 10^{-7}
12.0	2.81	10.0	7.35
9.0	2.93	7.0	7.51
7.0	2.89	5.0	7.73
6.0	3.01	2.5	7.92
3.0	2.98	0	8.10[b]
0	3.0[b]		

[a] In 0.05 M sodium phosphate buffer (pH 6.8) at 20°.
[b] By linear extrapolation.

containing 12 mg/ml was 6.3% below the value of 3.0 S for zero protein concentration.

Values for $D_{20,w}$ at different enterotoxin concentrations calculated from the rate of increase of the second moment about the mean in the gradient curves for synthetic boundary experiments at 21,740 rpm are shown in Table I. The values extrapolate linearly to a value of 8.1×10^{-7} cm²/sec at zero protein concentration. By combining the value of 0.732 ml/g for the partial specific volume, $\bar{V}$, as determined by pycnometry in 0.05 M sodium phosphate buffer (pH 6.8) with $s^0_{20,w}$ and $D^0_{20,w}$ (extrapolated values obtained at zero protein concentration) in the Svedberg equation (Svedberg and Pedersen, 1940), a molecular weight of 34,100 was obtained. These data gave a value of 1.19 for the frictional ratio, $f:f_0$.

Intrinsic Viscosity. The intrinsic viscosity, $[\eta]$, in 0.05 M sodium phosphate buffer (pH 6.8) was determined to be 3.4 ml/g. By coupling intrinsic viscosity with $s^0_{20,w}$, $\bar{V}$, and M (3.0 S, 0.732 ml/g, and 34,100, respectively) in the equation of Scheraga and Mandelkern (1953), a value of 2.17×10^6 was obtained for β, a parameter related to the axial ratio, p, of an effective hydrodynamic ellipsoid. Assuming that the enterotoxin molecules in solution do not depart significantly from rigid, impermeable spheres, the molecular weight calculated from sedimentation and viscosity data (Schachman, 1957) is 33,065 and that calculated from diffusion and viscosity data is 36,470. The average of the two values is 34,768 which agrees closely to 34,100.

Isoelectric Point. Studies of enterotoxin C by paper electrophoresis indicated an isoelectric point of 8.6 in Veronal buffer of 0.1 ionic strength. Results from starch gel electrophoresis confirm this value.

Absorption Spectra. Spectra of the purified enterotoxin in 0.02 M sodium phosphate buffer at different pH values from 5.5 to 7.5 were found to be identical within experimental limits. There are protein maxima at 229 and 277 mμ. The tyrosine residue content estimated by measuring the absorbance at 294.4 and 280 mμ in 0.1 N NaOH solution with correction for spurious absorption as described by Goodwin and Morton (1946) was 9.75% which agrees with the value obtained from experiments on amino acid analyses, *i.e.*, 9.80% (Huang *et al.*, 1967). The tryptophan content estimated from the absorbance was 0.9%.

Potency of the Purified Enterotoxin. Intragastric administration of 5 μg of purified enterotoxin/monkey was sufficient to evoke vomiting in 50% of the 24 test animals. Symptoms of food poisoning were observed in seven of the nine experimental animals that received the toxin (0.5 μg/animal) by intravenous injection.

Some Characteristics of the Enterotoxin. The purified toxin which was dried by lyophilization is a white, fluffy material. It is very soluble in water and salt solutions. Test for carbohydrate using anthrone reagent proved negative. The chloroform–methanol extract of the purified preparation was found to be lipid free. Nucleic acid is apparently absent in the purified toxin as indicated by chemical tests, and confirmed by a low ratio of absorbance at 260:280 mμ. Tests for α- and β-hemolysins were negative. The nitrogen content of the protein (free of buffer salts) is 16.2% and the extinction, $E^{1\%}_{1\,cm}$ at 277 mμ is 12.1.

The toxic and antigenic properties of the enterotoxin were maintained even after storing the purified material in dry form at room temperature for a period of 4 months. Slight loss of antigenicity and formation of some insoluble material were observed when the enterotoxin in 0.05 M phosphate buffer (pH 5.5–7.5) was stored at 4° for about 1 month. Incubation in a water bath of solutions containing 120–140 μg of toxin/ml in standard gel diffusion buffer for 1 hr at 40–55° did not diminish the antigenicity nor the protein content of the solution as measured by the absorbance at 280 mμ. There was also no loss of protein and antigenicity when the solutions were incubated for 0.5 hr at 60°. Incubation at 60° for 1 hr, however, resulted in the formation of a turbid solution.

Discussion

Ion-exchange chromatography on CM-cellulose column and gel filtration through Sephadex have provided means to isolate and purify staphylococcal enterotoxin C produced by strain 137. The highly purified enterotoxin has been shown to possess a high degree of homogeneity. Immunological assay is a powerful tool for the investigation of protein purity and results from the double-diffusion tube technique indicated a high degree of purity. In 0.05 M phosphate buffer (pH 6.8) it gave no indication of more than one component by ultracentrifugal analyses and by paper electrophoresis at a wide range of pH (4.4–11.0).

The toxin is devoid of lipid, carbohydrate, or nucleic acid. Detailed analyses of the amino acid composition and terminal amino acids of the highly purified preparation (Huang *et al.*, 1967) show that it is a simple protein. No chemical component other than the amino acids is detectable in the molecule. Only one N-terminal and one C-terminal amino acid was found per molecule of 34,100 mol wt. This indicates that the enterotoxin is not contaminated with an appreciable amount of another protein having different terminal amino acids.

Enterotoxin C is basic and antigenic in nature. Its basic behavior under the influence of an electrical field is made evident by a value of 8.6 for its isoelectric point in Veronal buffer of 0.1 ionic strength. A similar electrophoretic behavior has been reported for enterotoxin B (Hibnick and Bergdoll, 1959; Schantz *et al.*, 1965). The purified protein is a potent toxic substance. Toxicity levels of enterotoxin C are comparable to those found for enterotoxin A (Chu *et al.*, 1966) and those reported for enterotoxin B (Bergdoll *et al.*, 1959; Schantz *et al.*, 1965).

Results from starch gel electrophoresis apparently seem to disagree with indications of a high degree of homogeneity of the enterotoxin preparation by ultracentrifugal analyses and double-diffusion tube tech-

nique. The discrepancy is difficult for us to explain, since the two components as found by starch gel electrophoresis possess identical toxic and immunological properties. One possibility can be raised, that is aggregation of the enterotoxin molecule (probably formation of a dimer) which cannot be detected by ultracentrifugal methods, relevant to the idea of Frieden (1962) that consecutive dimerizations could only yield one peak. The observation on molecular aggregation when solutions are subjected to lyophilization or pervaporation was reported for ovine prolactin (Sluyser and Li, 1964). Starch gel electrophoretic studies of Schantz *et al.* (1965) on enterotoxin B also indicated two components in borate buffer of low ionic strength (pH 8.6). These investigators suggested the possibility of the protein interacting with components of the solvent medium resulting in a mixture of two constituents that migrate like two stable, noninteracting molecules, a theory resulting from computations of Cann and Goad (1964) for zone electrophoresis. Recently, Cann (1966) provided experimental evidence for multiple electrophoretic zones given by bovine serum albumin arising from protein–buffer interaction. It is possible that enterotoxin C, under the conditions of the experiment as described earlier, interacts with a constituent of the buffer giving rise to two zones. This possibility is under further investigation in our laboratory.

Theoretical speculation on the effective sizes and shapes of proteins in solution is provided by a β function given by the equation of Scheraga and Mandelkern (1953). According to the table presented by them, a value of $\beta > 2.15 \times 10^6$ rules out the possibility of an oblate ellipsoid. Since the β value obtained for enterotoxin C is 2.17×10^6, it is reasonable to assume the shape of the molecule to be a prolate ellipsoid. The hydrodynamic data suggest the enterotoxin to be a relatively compact molecule.

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References

Avena, R. M., and Bergdoll, M. S. (1967), *Biochemistry 6*, 1474 (this issue; following paper).

Baldwin, R. L., and Williams, J. W. (1950), *J. Am. Chem. Soc. 72*, 4325.

Bergdoll, M. S. (1962), Proceedings of 14th Research Conference, American Meat Institute Foundation, University of Chicago, Chicago, Ill., Circular No. 70, p 47.

Bergdoll, M. S., Borja, C. R., and Avena, R. M. (1965b), *J. Bacteriol. 90*, 1481.

Bergdoll, M. S., Chu, F. S., Huang, L.-Y., Rowe, C., and Shih, T. (1965a), *Arch. Biochem. Biophys. 112*, 104.

Bergdoll, M. S., Sugiyama, H., and Dack, G. M. (1959), *Arch. Biochem. Biophys. 85*, 62.

Bergdoll, M. S., Sugiyama, H., and Dack, G. M. (1961), *J. Biochem. Microbiol. Technol. Eng. 3*, 41.

Cann, J. (1966), *Biochemistry 5*, 1108.

Cann, J. R., and Goad, W. B. (1964), *Arch. Biochem. Biophys. 108*, 171.

Casman, E. P., Bergdoll, M. S., and Robinson J. (1963), *J. Bacteriol. 85*, 715.

Chu, F. S., Thadhani, K., Schantz, E. J. and Bergdoll, M. S. (1966), *Biochemistry 5*, 3281.

Frea, J. I., McCoy, E., and Strong, F. M. (1963), *J. Bacteriol. 86*, 1308.

Frieden, C. (1962), *J. Biol. Chem. 237*, 2396.

Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.

Hall, H. E., Angelotti, R., and Lewis, K. H. (1963), *Public Health Rept. (U. S.) 78*, 1089.

Hibnick, H. E., and Bergdoll, M S. (1959), *Arch. Biochem. Biophys. 85*, 70.

Huang, I.-Y., Shih, T., Borja, C. R., Avena, R. M., and Bergdoll, M. S. (1967), *Biochemistry 6*, 1480 (this issue; paper after the following paper).

Kumar, S., and Lindorfer, R. K. (1962), *J. Exptl. Med. 115*, 1095.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

McClean, D., Rogers, H. J., and Williams, B. W. (1943), *Lancet 1*, 315.

Oakley, C. L., and Fulthorpe, A. J. (1953), *J. Pathol. Bacteriol. 65*, 49.

Read, R. B., Jr., Pritchard, W. L., Bradshaw, J., and Black, L. A. (1965), *J. Dairy Sci. 48*, 411.

Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.

Schantz, E. J., Roessler, W. G., Wagman, J., Spero, L., Dunnery, D. A., and Bergdoll, M. S. (1965), *Biochemistry 4*, 1011.

Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179.

Sluyser, M., and Li, C. H. (1964), *Arch. Biochem. Biophys. 104*, 50.

Smithies, O. (1955), *Biochem. J. 61*, 629.

Spero, L., Stefanye, D., Brecher, P. I., Jacoby, H. M., Dalidowicz, J. E., and Schantz, E. J. (1965), *Biochemistry 4*, 1024.

Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, New York, N. Y., Oxford University.

Wagman, J., Edwards, R. C., and Schantz, E. J. (1965), *Biochemistry 4*, 1017.