Purification and Characterization of Staphylococcal Enterotoxin A*

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ABSTRACT: A method has been developed for the purification of enterotoxin A in yields of 35% from cultures of *Staphylococcus aureus*. The method involves chromatography on carboxymethylcellulose and gel filtration with Sephadex G-100 and G-75. The purified enterotoxin A is a simple protein and is essentially homogeneous as determined by ultracentrifugal analyses. Sedimentation and diffusion coefficients at zero protein concentration ($s^0_{20,w}$ and $D^0_{20,w}$) are 3.04 S and 7.94 × 10^{-7} cm^2 sec^{-1}, respectively. The intrinsic viscosity is 0.0407 dl/g. The molecular weight computed from sedimentation–diffusion data (34,500) is in good agreement with the values obtained from the modified Archibald method (35,200). A value of 2.236 × 10^6 for the Scheraga–Mandelkern [Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179] parameter β and a value of 1.236 for the frictional ratio (f/f_0) were obtained from physicochemical data. Paper electrophoresis at 0.1 ionic strength revealed that enterotoxin A has an isoelectric point near 6.8. The dose required to produce emesis in monkeys by oral feeding is approximately 5 μg.

The enterotoxins, common causes of food poisoning, are proteins produced in foods and culture media by the staphylococci. The classification of these proteins as enterotoxins A, B, C, etc. is based on their reactions with specific antibodies (Casman *et al.*, 1963). Enterotoxin A appears to be more frequently associated with food poisoning outbreaks than any of the other identified enterotoxins (Casman, 1960; Hall *et al.*, 1965; Casman, 1965). Although enterotoxin B is a cause of food poisoning (Fujiwara, 1961) it also is associated with staphylococcus strains isolated from humans afflicted with enteritis (Surgalla and Dack, 1955) and other staphylococcus infections. The limited information available concerning the incidence of food poisoning outbreaks involving enterotoxin C indicates this enterotoxin to be of increasing importance (Bergdoll *et al.*, 1965a).

Enterotoxin B has been purified (Bergdoll *et al.*, 1959; Schantz *et al.*, 1965) and its physicochemical properties studied in detail (Hibnick and Bergdoll, 1959; Spero *et al.*, 1965; Wagman *et al.*, 1965; Bergdoll *et al.*, 1965b). This enterotoxin was the first to be purified primarily because of the relatively large amounts of enterotoxin produced by the B-type strains (50–500 μg/ml of bacterial culture supernatant as compared to 1–5 μg/ml for enterotoxin A). The purification of enterotoxin A was accomplished after a staphylococcus strain was found that would produce relatively small amounts of the impurities that complicate the purification of the enterotoxin. This paper reports the purification and some of the physicochemical properties of enterotoxin A.

Materials and Methods

Staphylococcus aureus strains 196E, C-246-3, and 100 were used for the production of enterotoxin A for the purification studies. Strain 196E is a variant of a strain isolated in 1940 by G. G. Slocum of the U. S. Food and Drug Administration from cooked ham implicated in a food poisoning outbreak. It produces both α- and β-hemolysin. This strain was selected as the prototype strain for enterotoxin A. Strain C-246-3 was isolated in 1955 by E. P. Casman of the U. S. Food and Drug Administration from canned shrimp implicated in a food poisoning outbreak. It produces no α- and very little β-hemolysin. Strain 100 was isolated in 1932 by E. O. Jordan, University of Chicago, from a cake implicated in a food poisoning outbreak. It produces α- but very little β-hemolysin. This strain was used for production of the enterotoxin for most of the experiments reported.

The enterotoxin for the purification studies was produced by culturing in sterile medium (400 ml in 2-l. erlenmeyer flasks) consisting of 3% N-Z Amine NAK (Sheffield Chemical Co.), 3% Protein Hydrolysate Powder (Mead Johnson and Co.), 0.001% niacin and 0.00005% thiamin, adjusted to pH 7.6, and incubated for 24 hr at 37° on a Gyrotory shaker operated at approximately 275 rpm. The cells were sedimented in a

* From the Food Research Institute and Department of Biochemistry, University of Chicago, Chicago, Illinois. *Received June 27, 1966.* This investigation was supported by U. S. Public Health Service Grant AI-04511 from the National Institute of Allergy and Infectious Diseases and by contributions from various companies and associations of the food industries.

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bucket centrifuge at 4000 rpm for 45 min at 5°. Concentration of the bacterial culture supernatant containing the enterotoxin was accomplished by dialysis for 24–48 hr against an equal volume of 50% aqueous Carbowax 20 M (Union Carbide Corp.) followed by dialysis against running tap water for 48 hr. The dialyzed crude enterotoxin solution was adjusted to pH 5.7 with H_3PO_4 and centrifuged to remove any insoluble material.

The carboxymethylcellulose (CM-cellulose) used in the first and second steps in the purification was Selectacel ion-exchange cellulose, No. 77, Type 20 (Carl Schleicher and Schuell Co.). It was washed with 0.1 N NaOH, H_2O, 0.1 N HCl, and again with water before use (Peterson and Sober, 1956).

Sephadex G-100 (40–120 μ) (Pharmacia, Uppsala, Sweden), used in the third step in the purification, was suspended in 0.05 M sodium phosphate, pH 6.85 stirred for 1 hr, and allowed to stand for 24 hr. The supernatant was decanted and the Sephadex was resuspended in the buffer solution before packing in a column according to the method of Flodin (1961). Sephadex G-75 (medium, 100–270 mesh), used in the fourth step in the purification, was suspended in 0.005 M sodium phosphate, pH 6.85, and treated in the same manner as the Sephadex G-100.

The protein concentration was estimated by measuring the optical density at 280 mμ with a Beckman Model DU spectrophotometer. An extinction value of 1.3 for 1 mg/ml in a 1-cm cell was used to calculate the protein concentrations.

The toxicity of the various enterotoxin preparations was determined by intragastric administration of solutions of the enterotoxin (usually 50 ml) by catheter to young rhesus monkeys (*Macaca mulatta*, wt 2–3 kg) (Bergdoll *et al.*, 1959) or by intravenous injection in the saphenous vein. For intravenous injections the enterotoxin was dissolved in 2 ml of pyrogen-free saline (0.85%). Emesis within a 5-hr period was accepted as a positive reaction for enterotoxin.

The concentration of enterotoxin A in the various preparations was determined by a modification of the single-diffusion technique (Bergdoll, 1962). In this technique an agar column containing the enterotoxin antiserum is layered with the enterotoxin solution (10–200 μg/ml). The front of the enterotoxin–antienterotoxin precipitin band that is formed in the antiserum–agar column moves down the column at a rate corresponding to the concentration of the enterotoxin and the concentration of the antibody. The distance that the band moves in a given time is measured and the enterotoxin concentration is calculated from a standard curve obtained by plotting the log of the enterotoxin concentrations (micrograms per milliliter) against the distance that the enterotoxin–antienterotoxin band moved in millimeters into the agar column in a given time (usually 7 days).

The double-diffusion method of Oakley and Fulthorpe (1953) was used to follow the progress of the purification of the enterotoxin. The antisera used in these tests was obtained from rabbits that had been immunized (separately) with partially purified enterotoxin A from staphylococcus strains 196E and C-246-3. The antigen solutions used (without dilution) were the various fractions resulting from the purification procedures. The number and position of the precipitin lines in the agar layer between the antiserum and antigen layers were used as a guide to the progress of the purification. A modification of the double-diffusion method was used in estimating the concentration of impurities in the purified enterotoxin preparations (Bergdoll *et al.*, 1965a). The modification involved using a range of enterotoxin concentrations (2 mg–1 μg/ml of 0.02 M potassium phosphate, pH 7.4, 0.85% sodium chloride) against a range of antiserum concentrations (one-plus-one dilution to a dilution that resulted in a visible precipitate band with 1 μg or less of the enterotoxin). The minimal enterotoxin concentration, at which a precipitin band appeared resulting from the presence of an impurity, was compared to the minimal enterotoxin concentration which was required to produce a precipitin band to the enterotoxin. The relationship of the two concentrations gave a rough estimate of the impurity concentration. The antitoxins were prepared by injection of rabbits with partially purified enterotoxin A from staphylococcus strains 196E and C-246-3 and with purified enterotoxin A from strain 100 as described by Bergdoll *et al.* (1965a).

A modification of the casein hydrolysis method described by Laskowski (1955) was used to estimate the proteinase activity of different enterotoxin A preparations. The incubation mixture contained 5 ml of 1% casein (vitamin free, Nutritional Biochemicals) in appropriate phosphate buffer (pH 6.0–8.0) and 1 ml of exterotoxin A preparation at a concentration of 3–7 mg of protein/ml. The incubation was carried out at 37° for 50 min. Aliquots of 1.0 ml were withdrawn from the incubation mixture at 0, 10, 20, 30, and 50 min, and 2 ml of 5% trichloroacetic acid[1] was added to each aliquot to precipitate the protein. The absorbance at 275 mμ of TCA-soluble peptides was then determined. Tyrosine was used as a standard. The initial rate was determined by extrapolating the progress curve of the reaction to zero time. A proteinase unit is defined as the preparation which gives an absorbance at 275 mμ equivalent to 1 μg of tyrosine/min at 37°.

A Spinco Model E analytical ultracentrifuge equipped with a rotor temperature indicating and control unit was used throughout the ultracentrifugal analyses. Sedimentation velocity measurements were made according to Schachman (1957) with either a conventional 12-mm, 4°, single-sector cell or a rubber valve-type synthetic boundary cell. The ultracentrifuge is equipped with a schlieren optical system and was run at speeds of 59,780 or 42,040 rpm. Enterotoxin A (2–10 mg) in 1 ml of 0.1 M sodium phosphate, pH 6.85, was used in each experiment.

[1] Abbreviation used: TCA, trichloroacetic acid.

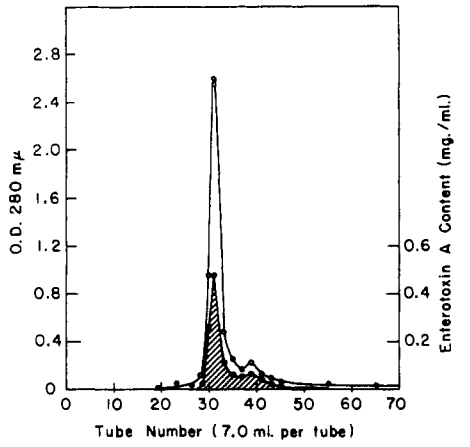

FIGURE 1: Chromatography of enterotoxin A on CM-cellulose. Elution pattern obtained when 500 ml of dialyzed, concentrated crude enterotoxin A containing 1.3 g of protein was chromatographed on CM-cellulose equilibrated with 0.01 M sodium phosphate, pH 5.7; column size, 2.2 × 50 cm; elution buffer, 0.2 M Na_2HPO_4; flow rate, 4.8 ml/min; —O—O—, optical density; —●—●—, enterotoxin content (single diffusion).

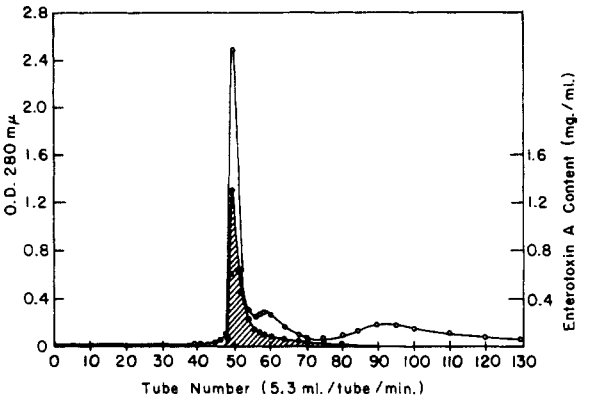

FIGURE 2: Rechromatography of enterotoxin A on CM-cellulose. Elution pattern obtained when 114 ml of dialyzed enterotoxin solution (tubes 29–40, Figure 1) containing 150 mg of protein was chromatographed on CM-cellulose equilibrated with 0.01 M sodium phosphate, pH 6.0; column size, 2.2 × 38 cm; elution gradient, 400 ml of 0.01 M sodium phosphate, pH 6.0 with 0.05 M, sodium phosphate, pH 6.6; flow rate, 4.8 ml/min; —O—O—, optical density; —●—●, enterotoxin content (single diffusion).

The samples used in experiments with the synthetic boundary cell were dialyzed against 0.1 M sodium phosphate, pH 6.85, for 24 hr. For determination of the diffusion coefficient, the valve-type synthetic boundary cell was used and the ultracentrifuge was operated at 20,420 rpm. In determining the molecular weight, during the approach to equilibrium, Archibald's method as modified by Ehrenberg (1957) was employed. A 12-mm rubber valve, synthetic boundary cell was used throughout the experiment. All the ultracentrifugal analyses were conducted at temperatures between 17 and 22° at 20,420 rpm. Measurements of photographic plates were made directly with a Nikon shadowgraph (Model 6C No. 5528). The area measurements in the diffusion coefficient studies and in the modified Archibald method were made by weighing the graph paper of the enlarged tracing. The sedimentation coefficient (s) and diffusion coefficient (D), were corrected to values for water at 20° to $s_{20,w}$ and $D_{20,w}$, respectively (Schachman, 1957). The least-squares method was used to obtain sedimentation and diffusion coefficients at zero protein concentration ($s^0_{20,w}$ and $D^0_{20,w}$) from $s_{20,w}$ and $D_{20,w}$ values at different concentrations. In the modified Archibald method, only the molecular weight at the meniscus was calculated. The molecular weights at different times of sedimentation were extrapolated to zero time.

Viscosity measurements were performed at 20° with a 0.5-ml capillary viscosimeter (Schachman, 1957). The outflow time of distilled water at 20° for the viscosimeter was 23.5 sec. Enterotoxin A (2–10 mg) in 1 ml of 0.1 M sodium phosphate, pH 6.85, was used in each experiment. The density of the solvent was determined by a 5-ml pycnometer at 20°. The viscosity of the enterotoxin A was calculated from the viscosity of water at 20°.

Paper electrophoresis was carried out using an E-C electrophoresis apparatus, Model 1331. The following buffers at 0.1 ionic strength were employed in the electrophoretic analyses: sodium acetate–acetic acid (pH 4.5), sodium phosphate (pH 6.0 and 7.0), Veronal acetate (pH 8.0, 8.3, and 9.0), and glycine–NaOH (pH 10.0). Electrophoresis was carried out at 800 v for 5 hr. Dextran was employed to determine electroendosmosis, and was detected by bromophenol blue (Block *et al.*, 1958). Bromophenol blue, 0.2% ninhydrin in acetone, and 0.83% amido black (Buffalo Black NBR, Allied Chemical, catalog no. 484) in a methanol–acetic acid–water mixture (5:2:5) were used to detect enterotoxin.

Starch gel electrophoresis was carried out by a modification of the method of Smithies (1955) using an E-C pressure-plate electrophoresis cell. For electrophoresis, 0.5–1.0 mg of the sample dissolved in 0.1 ml of the appropriate buffer was applied to the gel. A voltage gradient of 20 v/cm was applied and electrophoresis was carried out for 6 hr with running tap water as the coolant. Buffalo Black NBR was used to locate the proteins. The enterotoxin was located as follows: a strip of the starch gel was transferred to a template column (Poulik, 1959) soaked with 0.02 M sodium phosphate, pH 7.0. After the agar was solidified antiserum to enterotoxin A was layered on top of the agar layer. The precipitate band to enterotoxin A developed in 12–24 hr. The nitrogen content was

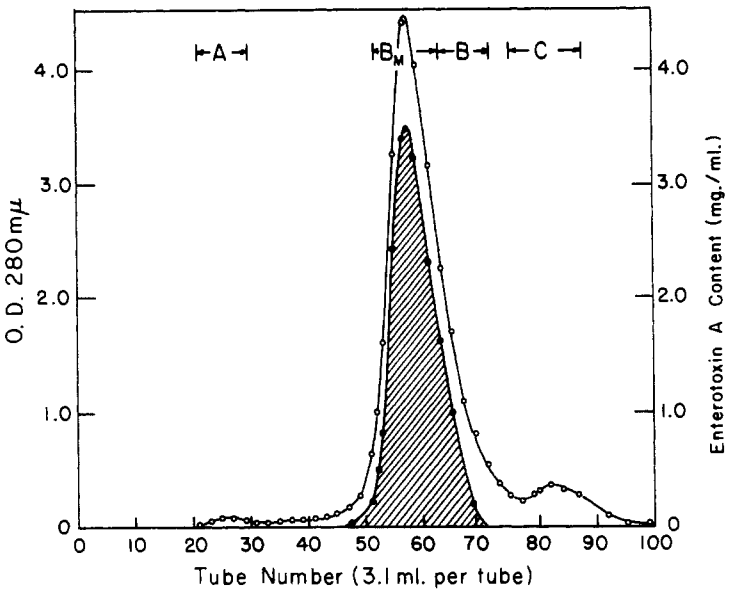

FIGURE 3: Sephadex G-100 gel filtration of partially purified enterotoxin A. Elution pattern obtained when 140 mg of partially purified enterotoxin (tubes 49–60, Figure 2) in 5 ml of distilled water was passed through a Sephadex G-100 column equilibrated with 0.05 M sodium phosphate, pH 6.85; column size, 2.2 × 73 cm; gel filtration buffer, same as equilibration buffer; flow rate, 18 ml/hr; temperature, 5°; —O—O—, optical density; —●—●—●, enterotoxin content (single diffusion).

determined with an F and M Scientific Model 185 carbon, hydrogen, nitrogen analyzer.

Purification Method. STEP 1. The concentrated bacterial culture supernatant (500 ml, adjusted to pH 5.7) containing approximately 1 g of protein was transferred to a column of CM-cellulose (2.2 × 50 cm) which had been equilibrated with 0.01 M sodium phosphate at pH 5.7. After washing the column with the equilibration buffer until the optical density of the percolate was near zero at 280 mμ the enterotoxin was eluted with 0.2 M Na_2HPO_4 (Figure 1). The fractions from the major peak were pooled, dialyzed for 4 hr at 5°, and lyophilized. Based on the amount of enterotoxin (determined by single diffusion) in the pooled fractions and the amount in the concentrated crude preparation the yield was about 80%. The purity of the enterotoxin in the pooled fractions was estimated from the total protein content (optical density at 280 mμ) and the enterotoxin content (single diffusion) to be 20%. Results from double-diffusion tests indicated the presence of four antigens.

STEP 2. The lyophilized enterotoxin from step 1 was dissolved in about 50 ml of distilled water and dialyzed against distilled water at 5° overnight. This solution was then adjusted to pH 6.0 and passed into a CM-cellulose column (2.2 × 38 cm) equilibrated with 0.01 M sodium phosphate, pH 6.0. After washing the column with the equilibration buffer until the optical density of the effluent was zero, the enterotoxin was removed by gradient elution in the following manner. Sodium phosphate (400 ml of 0.01 M), pH 6.0, was placed in a 1-l. bottle (with a sidearm attached to the top of the column) and stirred continuously with a magnetic stirrer. The second buffer, 0.05 M sodium phosphate, pH 6.6, was introduced dropwise into the top of the mixing chamber. A typical elution pattern is shown in Figure 2. The fractions composing the major peak were lyophilized and used in step 3. The yield was 55–60% with a purity of 70%.

STEP 3. The dialyzed, lyophilized material (140 mg) from step 2 dissolved in 5 ml of 0.05 M sodium phosphate pH 6.85, was passed into a column of Sephadex G-100 (2.2 × 73 cm) equilibrated with 0.05 M sodium phosphate, pH 6.85. The enterotoxin was eluted from the Sephadex with the same buffer at a flow rate of 18 ml/hr. The enterotoxin was present in peak B (Figure 3). Fractions (52–63) from the center portion of peak B (B_m) were combined and lyophilized without dialysis. The yield was about 40% with a purity of 85–90%. Double-diffusion results indicated the presence of small amounts of two contaminating antigens and starch gel electrophoresis results indicated the presence of two contaminating antigens.

STEP 4. The lyophilized material from step 3 was dissolved in 5 ml of distilled water and passed into a column of Sephadex G-75 (2.2 × 70 cm) equilibrated with 0.005 M sodium phosphate, pH 6.85. The enterotoxin was eluted from the Sephadex with the same buffer at a flow rate of 15 ml/hr (Figure 4). The fractions composing the center portion of the peak were combined and lyophilized. The lyophilized toxin was dissolved in 5–10 of distilled water and dialyzed against 8 l. of

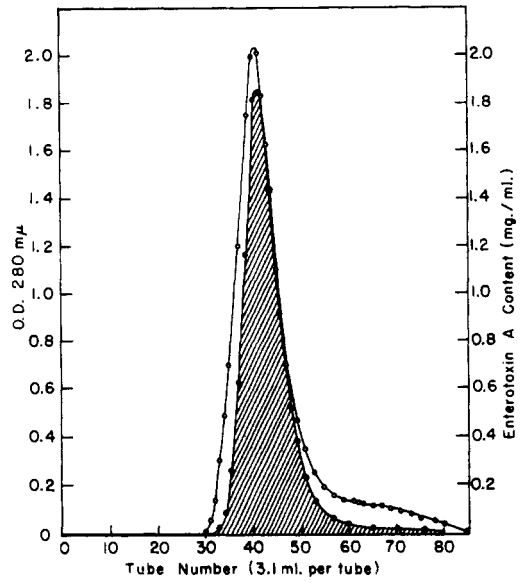

FIGURE 4: Purification of partially purified enterotoxin A on Sephadex G-75. Elution pattern obtained when 85 mg of partially purified enterotoxin (tubes 52–63, Figure 3) in 5 ml of distilled water was passed through a Sephadex G-75 column equilibrated with 0.005 M sodium phosphate, pH 6.85, column size; 2.2 × 70 cm; gel filtration buffer, same as equilibration buffer, flow rate, 15 ml/hr; temperature, 5°; —O—O—, optical density; —●—●—●, enterotoxin content (single diffusion).

distilled water (stirred magnetically) at 5° overnight. The yield was about 35% with a purity of at least 95%. Double-diffusion and starch gel electrophoresis results indicated the presence of trace amounts of two contaminating antigens. The amount of the two contaminants observed in the double-diffusion tests was calculated to represent less than 5% of the purified enterotoxin. The intensity of the precipitin lines formed with the two impurities in 100 μg of enterotoxin was equivalent to the intensity of the precipitin line formed with 1 μg of enterotoxin. Ultracentrifugal analysis showed no evidence of heterogeneity.

Characteristics of the Purified Enterotoxin. Some of the chemical and physical properties of the purified enterotoxin A are given in Table I. The freeze-dried protein is a fluffy, snow white material that is highly hygroscopic and very soluble in water and salt solutions. Aggregation has been observed after extensive dialysis. Tests for α- and β-hemolysins were negative. No proteinase activity has been found in the purified enterotoxin A. Less than 0.1 proteinase unit/mg of protein was found in the CM-cellulose preparation (purification step 2). At pH 6–8, the original bacterial culture filtrate contained 0.2, 0.3, and 0.3 proteinase unit/mg of protein, respectively. The active toxin is resistant to trypsin digestion. The nitrogen content of the dry protein (free of buffer salts) is 16.5%.

EFFECT OF HEAT ON ENTEROTOXIN A. Purified enterotoxin A is relatively heat labile as compared with enterotoxin B (Schantz *et al.*, 1965). A decrease of 50% in the reaction of enterotoxin A with its specific antibody (determined by simple diffusion) resulted when a solution containing 0.2 mg of enterotoxin A/ml of 0.05 M sodium phosphate, pH 6.85, was heated at 60° for 20 min. Heating a similar enterotoxin A solution at 70° for 3 min resulted in a 60% decrease in the antigen–antibody reaction. No antigen–antibody reaction was obtained after heating the enterotoxin at 80 and 100° for 3 and 1 min, respectively.

SPECTROPHOTOMETRIC ANALYSIS. Spectrophotometric analysis showed the enterotoxin to have a maximum absorption at 277 mμ with an extinction $E^{1\%}_{1cm}$ of 14.3.

TABLE I: Some Properties of Enterotoxins A and B.

Property	Enterotoxin A	Enterotoxin B
Nitrogen content (%)	16.5	16.1[a]
Sedimentation coefficient ($s^0_{20,w}$), S	3.04	2.89,[a] 2.78[b]
Diffusion coefficient ($D^0_{20,w}$), × 10^{-7} cm^2 sec^{-1}	7.94	7.72,[a] 8.22[b]
Reduced viscosity (dl/g)	0.0407	0.0392,[a] 0.0381[b]
Isoelectric point	6.8	8.6[a,c]
Partial specific volume	0.726	0.743,[a] 0.726[b]
Maximum absorption (mμ)	277	277[a,b]
Extinction ($E^{1\%}_{1cm}$)	14.3	14.0[a]
Molecular weight	34,700	35,300,[a] 30,000[b]
Toxicity (MED), μg	5[d]	5[d]

[a] Shantz *et al.* (1965). [b] Bergdoll *et al.* (1965ab). [c] Hibnick and Bergdoll (1959). [d] Toxin administered intragastrically; only emesis observed.

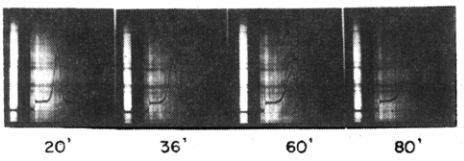

FIGURE 5: Sedimentation pattern of enterotoxin A in Spinco Model E analytical ultracentrifuge at concentration of 10.0 mg/ml in 0.05 M sodium phosphate buffer, pH 6.8. A synthetic boundary cell was used in this experiment. The pictures were taken at a bar angle of 60°. Time after reaching full speed of 42,040 rpm is indicated below each picture. The temperature was 20°.

The ratio of the absorption at 260/277 mμ was 0.48 which showed the presence of very little if any nucleic acid material.

BIOLOGICAL ASSAY OF ENTEROTOXIN A. A limited number of unused rhesus monkeys (2–3 kg) were used to determine the ED_{50} for enterotoxin A. ED_{50} is defined as the amount of enterotoxin which causes emesis in approximately 50% of the animals challenged. Results from administration both *per os* and intravenously are given in Table II. The ED_{50} dose *per os* was determined to be 5 μg.

TABLE II: Effect of Enterotoxin A on Rhesus Monkeys.

Route of Administration			
Intragastric		Intravenous	
Amt (μg/animal)	Result[a]	Amt (μg/kg)	Result[a]
5	4/10	0.017	2/6
10	16/30	0.035	2/9
20	9/12	0.070	4/9
		0.140	5/6

[a] Number vomiting *vs.* number challenged.

SEDIMENTATION AND DIFFUSION ANALYSIS. Figure 5 shows the sedimentation pattern of purified enterotoxin A. A single symmetrical boundary was obtained during the sedimentation. The boundary-spreading effect was further studied according to Baldwin (1957) and a plot of the second moment about the mean of the gradient curve after correction for centrifugal force, $\sigma^2 (1 - \omega^2 st)$ against time, t (sec), is shown in Figure 6. The apparent diffusion coefficient was obtained from the slope of the line divided by 2. The sedimentation coefficients ($s_{20,w}$) and apparent diffusion coefficients ($D_{20,w}$) obtained from different concentrations of enterotoxin A are given in Figures 7A and B. Extrapolation

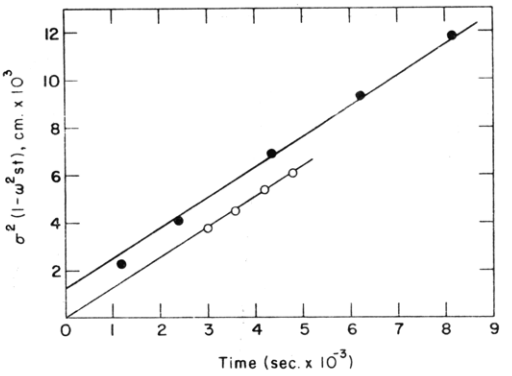

FIGURE 6: Boundary-spreading analysis for enterotoxin A. The experiments were carried out at 20,140 rpm (—●—●—) and at 59,780 rpm (—O—O—), respectively. Enterotoxin A, at a concentration of 5.0 mg/ml, was dialyzed against 0.05 M sodium phosphate buffer overnight. Both experiments were conducted at 17°.

to zero concentration gave an $s^0_{20,w}$ value of 3.04 S and a $D^0_{20,w}$ value of 7.94×10^{-7} cm² sec⁻¹.

VISCOSITY. Figure 8 shows the reduced viscosity and specific viscosity of enterotoxin A at different concentrations. The intrinsic viscosity was determined to be 0.0407 dl/g.

MOLECULAR WEIGHT FROM SEDIMENTATION VELOCITY ANALYSIS. The molecular weight of enterotoxin A was calculated from the $s^0_{20,w}$ value of 3.04 S and $D^0_{20,w}$ value of 7.94×10^{-7} cm² sec⁻¹ using the formula, $M = RTs/D(1 - \bar{V}_p)$. The apparent partial specific volume ($\bar{V}$) was calculated from the amino acid composition[2] according to Schachman (1957) to be 0.726 ml/g. A molecular weight of 34,486 was obtained.

MOLECULAR WEIGHT FROM THE MODIFIED ARCHIBALD METHOD. The sedimentation and diffusion coefficients (s/D) ratio was determined directly from the enlarged pictures obtained from the different times of sedimentation. Because of the piling up of material in the bottom of the cell, only the patterns at the meniscus were used. In the present work, experiments were carried out at enterotoxin A concentrations of 5.0 and 3.3 mg/ml in 0.1 M sodium phosphate, pH 6.85, at a temperature of 16.5 ± 0.5°. The ultracentrifuge was operated at a speed of 20,420 rpm. A value of 0.726 ml/g for the partial specific volume was used in the calculation of molecular weight. The molecular weights calculated from the schlieren patterns at different times of exposure are shown in Figure 9. Results obtained from the experiment at the concentration of 5.0 mg/ml were more consistent than those obtained from 3.3 mg/ml. The extrapolated molecular weights at the concentration of 5.0 and 3.3 mg/ml were 35,258 and 35,017, respectively.

ELECTROPHORETIC ANALYSES. Paper electrophoresis

[2] I. Huang, T. Shih, F. S. Chu, and M. S. Bergdoll, unpublished observation.

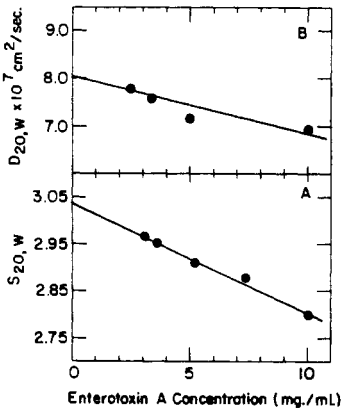

FIGURE 7: Dependence of sedimentation coefficient (A) and diffusion coefficient (B) of enterotoxin A on concentration. Sedimentation coefficients of various concentrations of enterotoxin A were obtained from the runs in 0.05 M sodium phosphate buffer, pH 6.85. Diffusion coefficients of various concentrations of enterotoxin A were obtained in the same buffer system using synthetic boundary cell at 20,140 rpm. The sedimentation and diffusion coefficients of enterotoxin A follow the equations $s_{20,w} = 3.035 - 0.022c$ and $D_{20,w} = 7.94 - 0.11c$, where c = concentration of enterotoxin A (mg/ml).

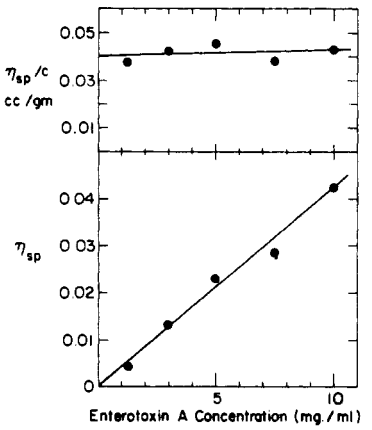

FIGURE 8: Plot of reduced viscosity (η_{sp}/c) and specific viscosity (η_{sp}) *vs.* concentration. The viscosity measurements of different enterotoxin A concentrations were made in 0.05 M sodium phosphate, pH 6.85, at 20°.

has been used for estimating the isoelectric point of enterotoxin A. For comparison, an enterotoxin B sample was also subjected to electrophoresis in the same paper. The mobilities of both toxins at seven pH values are compared in Figure 10. An isoelectric point near 6.8 (sodium phosphate, 0.1 ionic strength) was obtained for enterotoxin A as compared to a value of 8.7 (Veronal acetate, 0.1 ionic strength) for the isoelectric point of enterotoxin B. An enterotoxin A sample after dialysis for 72 hr gave an isoionic point of 6.5. Starch gel electrophoresis experiments with the purified enterotoxin showed a separation of the enterotoxin into two zones at pH 8.6 while only one zone with two faint bands was obtained when the experiments were conducted at pH 4.6. Similar phenomena for enterotoxin B have been observed by Joseph and Baird-Parker (1965) and Schantz *et al.* (1965). After electrophoresis the starch gel (pH 4.6 run) was subjected to immunodiffusion and only one precipitin band was observed.

Discussion

Three strains of staphylococcus, namely 196E, C-246-3, and 100, were used to produce enterotoxin A for the purification studies. Strains 196E and C-246-3 produced impurities which were difficult to separate from the enterotoxin by any of the methods used. The crude enterotoxin prepared from strain 100 was more amenable to purification than the crude enterotoxin prepared from the other strains and therefore was used for final development of the procedures for the purification of enterotoxin A.

The enterotoxin produced by strains 196E and C-246-3 was obtained originally in the crude state from the bacterial culture supernatants by precipitation with TCA at pH 2.9–3.0. This method was abandoned when it appeared that the precipitated enterotoxin was readily denatured during the purification and the impurities in the precipitated material were difficult to separate from the enterotoxin. The latter observation may have resulted from the association of the impurities with the enterotoxin during the precipitation with trichloroacetic acid. Carbowax has been shown to be a very effective agent in the concentration of protein solutions without undue effect on the proteins (Howe *et al.*, 1964). The ratio of carbowax to the crude enterotoxin solution was selected to give a 10- to 20-fold concentration in 24–48 hr. This method is more cumbersome than precipitation and is limited to smaller volumes, but it is the best method available for concentrating dilute solutions of enterotoxin A (1–5 μg/ml) without any damage to the toxin. It has not been practicable to date to remove enterotoxin A from the bacterial culture supernatants by adsorption on ion-exchange materials as is done with enterotoxin B because of the low concentration of enterotoxin A in culture supernatants (1–5 μg/ml *vs.* 100–500 μg/ml for enterotoxin B) and the lower capacity of the resins for this enterotoxin. The adsorption of enterotoxin from culture supernatants is affected by the composition of the media used in production of the enterotoxin (Bergdoll *et al.*, 1961) and in the case of low adsorption this might prohibit the use of direct adsorption even though the concentration of enterotoxin was increased. However, the adsorption of the enterotoxin from bacterial culture supernatants is under study and initial results indicate that these problems can be surmounted. Studies are in progess to increase the produc-

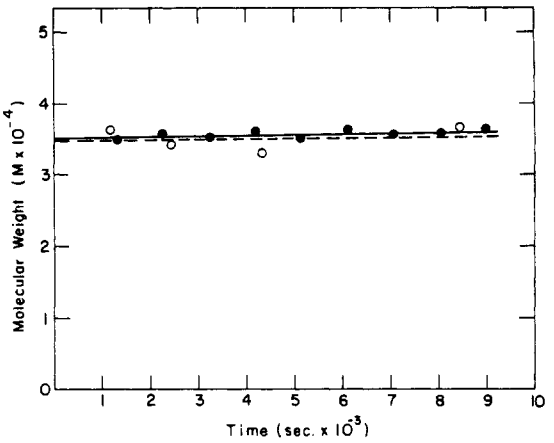

FIGURE 9: Dependence of molecular weight of enterotoxin A on time of ultracentrifugation as evaluated from modified Archibald method. The experiments were carried out at enterotoxin A concentrations of 5.0 mg/ml (—●—●—) and 3.3 mg/ml (---O---O--), in 0.05 M sodium phosphate buffer, pH 6.85, at 20,140 rpm; temperature, 17°. The extrapolated molecular weights for these two concentrations were 35,258 (5.0 mg/ml) and 35,017 (3.3 mg/ml), respectively.

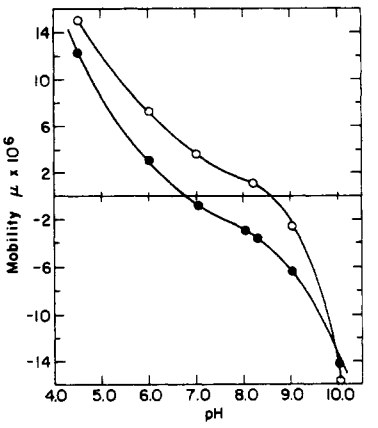

FIGURE 10: Mobilities of enterotoxin A on filter paper at different pH values. The data were obtained from experiments after correction of the electroendosmosis. The migration of enterotoxin A is compared to that of enterotoxin B. The line —●—●— represents the mobility of enterotoxin A. The line —O—O— represents the mobility of enterotoxin B.

tion of the enterotoxin by the various enterotoxin A producing strains.

Schantz *et al.* (1965) used CM-cellulose chromatography and Frea *et al.* (1963) used Sephadex G-100 and Sephadex column electrophoresis in the purification of enterotoxin B. The combination of CM-cellulose chromatography and Sephadex gel filtration in the present study proved to be an effective method for purifying the enterotoxin. Step 2 was initially accomplished by stepwise elution with (a) 0.03 M sodium phosphate, pH 6.35, and (b) 0.05 M sodium phosphate, pH 6.6. Enterotoxin was eluted with both buffers and in early experiments more enterotoxin of higher purity was present in the second eluate than in the first. Unexplained variability in the distribution of enterotoxin in the two eluates in later experiments led to the replacement of the stepwise elution with gradient elution. Enterotoxin of high purity can be obtained by employing the first three steps of the purification method, particularly if only the three or four fractions composing the center portion of the peak obtained in step 3 (Figure 3) are selected. Small amounts of impurities were removed with Sephadex G-75 by using a lower ionic strength phosphate buffer.

Studies in this laboratory showed that enterotoxin A is resistant to proteolytic enzymes. Resistance of enterotoxin B to proteolytic enzymes has also been reported (Bergdoll *et al.*, 1959; Schantz *et al.*, 1965). Although proteinase activity is present in the bacterial culture supernatants no adverse effect on the enterotoxins is observed as long as precautions are taken to avoid denaturation of the enterotoxins. The enterotoxin-containing preparations are kept under refrigeration or in the dry state to avoid denaturation and to reduce enzyme action. Essentially no proteinase activity is detectable in the enterotoxin fraction from step 2 of the purification procedure.

The complete removal of salts from the purified enterotoxin A results in some of the toxin becoming insoluble, the amount being dependent on the length of time the solution is allowed to stand at 5°. With the method used for dialysis of the purified enterotoxin (step 4) most of the buffer salts could be removed in a few hours without adverse effect on the enterotoxin.

Although small amounts of contaminating substances have been demonstrated to be present in the purified enterotoxin A from the starch gel electrophoretic analysis, the analysis of boundary spreading and the approach to equilibrium studies give no indication of heterogeneity. The plot of $\sigma^2(1 - \omega^2 st)$, the second moment about the mean of the gradient curve after correction for the centrifugal force at two different speeds, against time gives a straight line (Figure 6). Since the slope of these two lines is identical, the self-sharpening effect is therefore negligible (Schachman, 1959). In the approach to equilibrium studies, the molecular weight was almost independent of time during sedimentation (Figure 9). Unless the contaminating substances possess the same molecular shape and size, these data should indicate a purity of 95% or better. Paper electrophoresis showed only one spot.

The mol wt 34,500, obtained from the sedimentation velocity studies, is in good agreement with the results from the approach to equilibrium studies, 35,200. A value of 34,700 ± 1000 for the molecular weight of enterotoxin A is within the experimental error of the different analyses. From the values of s, D, and $\bar{V}$, the frictional ratio, f/f_0, was computed to be 1.236

(Oncley, 1941). By coupling the intrinsic viscosity data with sedimentation coefficient (3.04 S), partial specific volume (0.726), and molecular weight (34,700), a value of 2.236×10^6 is obtained for β according to the treatment of Scheraga and Mandelkern (1953). Although the partial specific volume of enterotoxin A used throughout all the calculations has not been experimentally determined, the apparent value obtained from amino acid composition should give quite a reliable estimate as is the case with many other proteins (Schachman, 1957). The β value for enterotoxin A seems to rule out the possibility of an oblate ellipsoid, and more likely corresponds to a prolate ellipsoid having an axial ratio, b/a, of approximately 5 (Scheraga and Mandelkern, 1953). The values for β and axial ratio are higher than those for enterotoxin B (Wagman *et al.*, 1965). No attempt has been made to calculate the effective hydrodynamic volume, V_e.

Acknowledgments

The authors wish to acknowledge the technical assistance of Matthew Laboda, Ljubica Davidovich, Lubinka Kujovich, James Lindsay, Donald Hughes, and Mahmood Khan.

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