Purification and Some Chemical and Physical Properties of Staphylococcal Enterotoxin A*

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ABSTRACT: Enterotoxin A from a selected high toxin producing strain (15–25 μg/ml in deep culture) of *Staphylococcus aureus*, designated 13N-2909, has been obtained in highly purified form as indicated by sedimentation velocity, sedimentation equilibrium, disc electrophoresis, and several immunological procedures. The effective dose, ED_{50}, by intravenous route to produce emesis and diarrhea in rhesus monkeys is 0.03 μg/kg of animal weight with 95% confidence limits of 0.017–0.065 μg/kg. Purification was accomplished by the following steps: (1) removal of the toxin from culture (diluted five times with water, and adjusted to pH 5.6) with CG-50 carboxylic acid resin; (2) chromatography on carboxymethylcellulose; (3) chromatography on hydroxylapatite; and (4) filtration on Sephadex G-75. Two major and two minor components were found by isoelectric focusing. This paucidispersity is attributed to differences in charge. The principal component has an isoionic point of 7.26 at 4°. The enterotoxin has a molecular weight of 27,800 as determined by sedimentation equilibrium. A value of 27,500 was obtained from polyacrylamide gel electrophoresis in the presence of denaturant. The molecule is a single polypeptide chain with one disulfide bridge and no free sulfhydryl groups. Serine was identified as the C-terminal residue but no free N terminal was found. A complete amino acid analysis is reported.

In 1965 we reported a procedure for purifying staphylococcal enterotoxin B, employing chromatographic procedures that enabled us to purify large quantities of the toxin (Schantz *et al.*, 1965). Chu *et al.* (1966) have described a method for the purification of enterotoxin A that employs dialysis against Carbowax to reduce the culture volume and remove some impurities as an initial step. While this technique is applicable to small volumes of culture, it is not practical for handling volumes necessary for the isolation of gram quantities of highly purified toxin for chemical, physical, and immunological studies. The present paper describes a method for purifying staphylococcal enterotoxin A that employs chromatographic methods that have enabled us to obtain good quantities of highly purified enterotoxin A. This report also presents a determination of the molecular weight, a complete amino acid analysis of the protein, and studies on the terminal amino acid residues. The implications of these findings on the structure of this toxin and a comparison to other types of enterotoxin are discussed.

Materials and Methods

Materials. The toxin was produced by culturing *Staphylococcus aureus*, strain 13N-2909 (Friedman and Howard, 1971), for 24 hr in a medium containing 3% protein hydrolysate powder (Mead Johnson), 3% NZ-amine, type NAK (Sheffield), 0.2% yeast extract (Difco), and 0.2% glucose added aseptically and adjusted to pH 6.8. The resins, CG-50 (Rohm and Haas), carboxymethylcellulose, type 20 (Schleicher & Schuell), hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories), and Sephadex G-75 were prepared and used as described in the stepwise procedure below. All solvents and chemicals were reagent grade. Hydrazine, benzaldehyde, and phenyl isothiocyanate were prepared as previously described (Spero *et al.*, 1965). The antisera used for immunological tests during the first part of the work were made from partially purified toxin.[1] As more highly purified toxin was obtained, antisera with higher specificity were prepared for the Oudin tests. Antisera for the Oakley (1953) tests were prepared from toxin in crude culture.

Amino Acid Analysis. The procedures for sample preparation, hydrolysis, calibration of the analyzer, the actual analyses, and calculations were the same as previously described (Spero *et al.*, 1965). The analyzer was increased in sensitivity through the incorporation of a 7- to 9-mV resistor card in the recorder. Since the logarithmic scale is almost linear in this range and there is a large variation in concentrations of the various amino acids, it became necessary to carry out repetitive runs with each hydrolysate, using different volumes of applied sample to ensure optimally sized peaks for each amino acid.

Cystine was determined as cysteic acid after performic acid oxidation (Moore, 1963). Free sulfhydryl was determined by the Ellman reagent by the modification of Janatova *et al.* (1968), in which a very high concentration of reagent is used in the presence of 6 M guanidine hydrochloride. Tryptophan was measured spectrophotometrically in 6 M guanidine hydrochloride (Edelhoch, 1967).

Amide nitrogen was done by the method of Stegemann (1958) except that the ammonia analysis was done on the amino acid analyzer restored to its original lower sensitivity.

End-Group Analysis. The FDNB[2] method of Sanger and

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[1] Produced at Food Research Institute, University of Wisconsin, Madison, Wis.

[2] Abbreviations used are: FDNB, 1-fluoro-2,4-dinitrobenzene; SDS, sodium dodecyl sulfate.

TABLE I: Steps in the Purification of Staphylococcal Enterotoxin A.

Prepn	Vol (ml)	*A*/ml	Total Protein (mg)	Oudin (mg/ml)	Total Toxin (mg)	Recov (%)	Purity (%)
Culture	60,000			0.015	900		
Pool from CG-50	240	22.9	3920	3.25	780	87	20
Pool from CM-Cellulose	1,640	0.84	984	0.30	492	63	50
Pool from hydroxylapatite	350	1.8	450	1.26	441	90	98
Pool from Sephadex G-75	36	6.9	177	4.9	176	40	>99
					Overall recovery about 20%		

hydrazinolysis were carried out as previously described (Spero *et al.*, 1965). The cyanate method of Stark and Smyth (1963) and the Eriksson and Sjöquist (1960) modification of the Edman method were also employed in attempts to identify an N-terminal residue.

Ultracentrifuge Studies. A Spinco Model E analytical ultracentrifuge equipped with absorption optics and an ultraviolet scanner was used. The sedimentation coefficient was measured with schlieren optics, using a capillary-type double-sector synthetic boundary cell. Equilibrium molecular weight was determined with absorption optics in a double-sector cell (Schachman and Edelstein, 1966). Volumes of 0.15 ml of solution and 0.16 ml of solvent were introduced with a microliter syringe. Scans were made with a chart speed of 5 mm/sec and a scan speed of 0.63 cm/min at a wavelength of 280 nm. The temperature controlling unit was not used in equilibrium runs. Appropriate temperatures were obtained by adjusting the refrigeration pressure. The baseline deflection was measured by accelerating the rotor to 40,000 rpm and continuing the run until the upper portion of the solution was completely depleted of solute, a period of about 4 hr. We could not distinguish between scans made at this time and after deceleration to the original speed.

Isoelectric Focusing. An LKB isoelectric focusing apparatus was used with a pH gradient between 3 and 10.

Gel Electrophoresis. Polyacrylamide disc electrophoresis was performed at pH 4.3 as described by Reisfeld *et al.* (1962). For the determination of molecular weight in SDS and β-mercaptoethanol the method of Weber and Osborn (1969) was followed. A Canalco Model 6 instrument was employed.

Diffusion Coefficient. The method of Schantz and Lauffer (1962) was employed. The protein diffuses into a column of agar gel which is subsequently extruded and sliced. A plot of the protein concentration of each slice *vs.* distance on arithmetic probability paper permits a ready determination of the diffusion coefficient.

Results

Purification Procedure. The method is outlined in the following steps.

STEP 1. The fermented culture usually assayed about 15–25 μg of toxin/ml. When the fermentation was complete, the culture was cooled and passed through a high-speed Sharples centrifuge to remove the cells. The supernatant solution was diluted with four volumes of water and adjusted to pH 5.6. The toxin was removed from the diluted culture with CG-50 resin, equilibrated in 0.005 M sodium phosphate buffer at pH 5.6. The equilibrated resin was stirred in the culture for about 1 hr at room temperature and allowed to settle. This resin (2–3 g) was sufficient to remove the toxin from 1 l. of original culture. Usually 60 l. of culture or 300 l. of diluted culture was processed at one time with 120 g of the resin. The settled resin, containing the toxin and impurities, was placed in a column to accommodate a volume of resin 3.5 × 60 cm, washed with about one column volume of water, and the toxin fractionally eluted with 0.5 M sodium phosphate at pH 6.2 in 0.5 M sodium chloride at a flow rate of 6 ml/min. The fractions, 15 ml each, containing 1 mg or more of toxin per ml (based on absorbance at 277 nm using an extinction of 14 and Oudin tests) were pooled for further purification. At this point the pooled fractions amounted to about 0.4% of the culture volume, 240 ml, and the yield was about 85–90% (3920 mg of total protein, 780 mg of toxin; see Table I).

STEP 2. The pool of fractions from step 1 was dialyzed to reduce the salts by dialyzing against a weaker buffer of 0.008 M sodium phosphate at pH 6.0 in 0.008 M sodium chloride. This process keeps the toxin in the presence of some of the buffer and increases its stability. The toxin was adsorbed on a column of CM-cellulose using 360 g (50–100 g/g of protein based on absorbance at 277 nm) prepared by soaking in distilled water 1 hr, decanting, and suspending it in 0.01 M monosodium phosphate solution. The pH was adjusted to 6.0 with NaOH, readjusted to 6.0 after 1 hr, and the cellulose placed in a column so that the length of the bed volume (1600 ml) is at least 15 times the diameter. The column was kept at 4° and washed with buffer (0.01 M, pH 6.0) at this temperature. The toxin solution was cooled to 4°, passed through the column at 2 ml/min, and the column was washed with one void volume (800 ml) of 0.008 M phosphate in 0.008 M sodium chloride at pH 6.0. The toxin was fractionally eluted at room temperature with a linear gradient of sodium phosphate, 0.01 M, pH 6.0 to 0.05 M, pH 6.8 (1800 ml of each buffer) at 3 ml/min. The fractions (14 ml each) containing the peak of toxin were selected and pooled for further purification. At this point the volume of the pooled fractions was 1640 ml and the purity was about 50%, based on Oudin tests. The recovery in this step was greater than 60%.

STEP 3. The combined fractions from step 2 were adjusted to pH 5.7 and centrifuged to remove suspended material if necessary. The toxin was then adsorbed on a column of hydroxylapatite. One gram was used for each 3.5 mg of protein, in this case 280 g (bed volume 750 ml; column 5 × 38 cm; length should be at least six times the diameter). Hydroxylapatite was prepared by suspending it in 0.008 M sodium phosphate at pH 5.7 with occasional stirring for 30 min. The suspension was allowed to settle so as to decant to a medium slurry. The column was then poured and equilibrated with 0.03 M sodium phosphate buffer at pH 5.7 for at least 24 hr at a flow rate of no greater than 0.5 ml/min. The sample was put

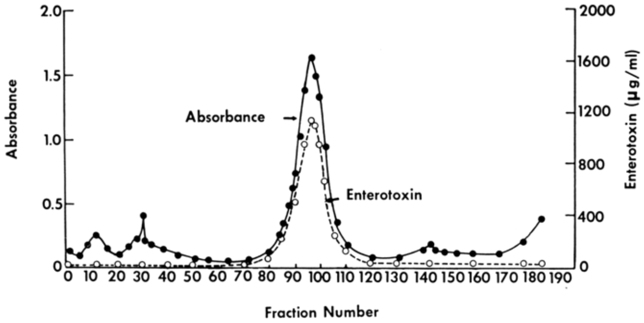


FIGURE 1: The elution pattern of partially purified enterotoxin A from hydroxylapatite. The combined fractions from step 2 were applied to a column equilibrated with 0.03 M sodium phosphate buffer at pH 5.7; 1 g of hydroxylapatite was used for each 3.5 mg of protein. Elution was by a linear phosphate buffer gradient from 0.2 to 0.4 M at pH 5.7 at a rate of 1 ml/min. The best fractions (tubes 85–110) contained 55% of the absorbance and 92% of the toxin. The purity of the pool was 98%.

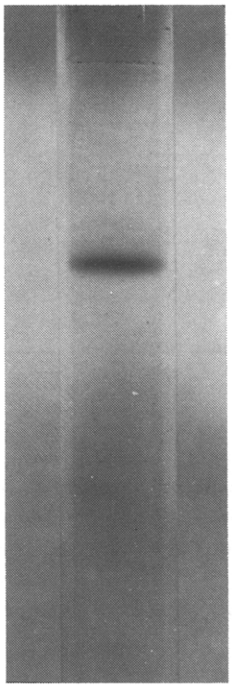


FIGURE 3: Disc electrophoresis of purified enterotoxin A in polyacrylamide gel (7.5%) in β-alanine–acetic acid buffer at pH 4.3. Approximately 20 µg of toxin was applied to the gel.

on the column with a flow rate of 0.5 ml/min. The column was then washed with one void volume (75% of bed volume) of 0.2 M sodium phosphate (pH 5.7). Elution was carried out with a linear phosphate buffer gradient from 0.2 M at pH 5.7 to 0.4 M at pH 5.7 at a flow rate of 1 ml/min. A volume of four times the bed volume for each buffer was used for this gradient. The peaks of protein emerging from the column (5-ml fractions) were located by the absorbance and the enterotoxin by additional Oudin tests. Figure 1 shows a typical elution pattern of toxin and impurities from hydroxylapatite. The fractions that showed a single antigen–antibody line, with only traces of impurity in the double-diffusion tests (Oakley tubes), were selected for chromatography on Sephadex. The volume of the pooled fractions was about 350 ml and contained 450 mg of protein by absorbance and 441 mg of toxin by Oudin tests. The purity at this point was approximately 98% and the recovery in this step was about 90% (Table I).

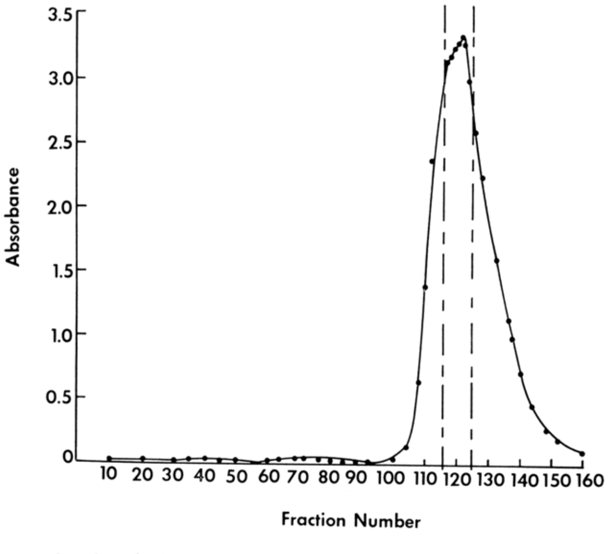
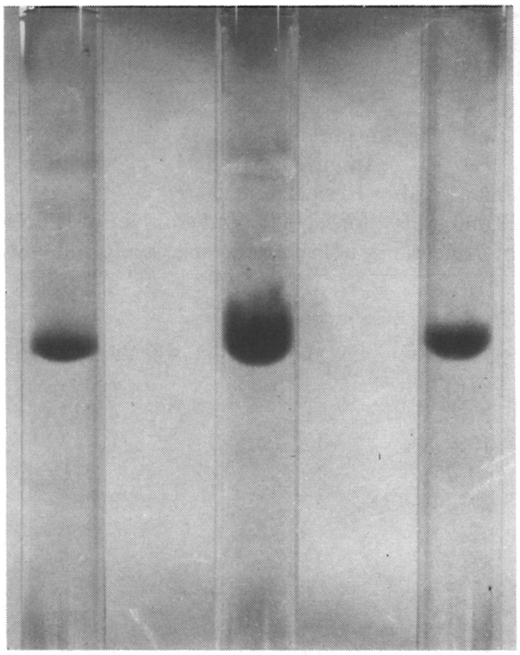


FIGURE 2: The elution of enterotoxin A from Sephadex G-75. The concentrated material from step 3 was dialyzed against 0.05 M sodium phosphate buffer at pH 6.8 in 1 M sodium chloride and chromatographed on a 5 × 85 cm column of Sephadex G-75, equilibrated with the same buffer; the flow rate was 0.6 ml/min at room temperature. Tubes 116–125 were selected as highly purified enterotoxin A, based on a single line in the Ouchterlony and Oakley tests.

STEP 4. The selected fractions from step 3 were pooled and concentrated by lyophilization to about one-tenth volume, dialyzed to remove most of the buffer salts, and lyophilized to dryness. The dried preparation was taken up in about $^1/_{30}$th the original volume of distilled water, dialyzed against 0.05 M sodium phosphate at pH 6.8 in 1 M sodium chloride, and passed through a column of Sephadex G-75 to remove a trace amount of high molecular weight material. The Sephadex column (5 × 85 cm, void volume 530 ml) was made up in the same buffer. This buffer was used to elute the toxin at a flow rate of 0.6 ml/min. The central fractions of the peak (3 ml each) of toxin (Figure 2) from this column were pooled and dialyzed against a sodium phosphate buffer at pH 6.8 at a concentration to bring the buffer salts to 3–5% of the protein concentration as determined by the absorbance. The volume of the pooled fractions in this case was 36 ml and contained 177 mg of protein by absorbance and virtually the same amount by Oudin tests, indicating a high degree of purity. Ouchterlony tests indicated a purity of better than 99%. Disc electrophoresis in polyacrylamide gel showed one component (Figure 3). The dialyzed solution of toxin was centrifuged to remove any insoluble material and freeze-dried. The overall yield of purified toxin usually amounted to 20%, based on the Oudin tests on the original culture. The results of each step are summarized in Table I.

Characteristics of the Purified Toxin. The freeze-dried toxin is a snow-white powder that is highly hygroscopic. It is very soluble in water and salt solutions. Usual tests for carbohydrate, lipid, and nucleic acids were negative. The Kjeldahl nitrogen content of the toxin is 16.2%. It has a maximum absorption at 277 nm, with an extinction ($E_{1\,\mathrm{cm}}^{1\%}$) of 14.6. The ratio of the absorption at 260 nm to that at 277 nm was 0.45–0.47, confirming that very little, if any, nucleic acid material is present in the preparation.



F 4: Polyacrylamide gel electrophoresis of staphylococcal e....oxins A and B. The gels, 10% acrylamide, 0.27% cross-linker, contained 0.1% SDS. Proteins were incubated at 37° for 2 hr in 0.01 M phosphate buffer (pH 7.0), 1% in SDS and 1% in β-mercaptoethanol. Separations were carried out at 8 mA/gel for 4 hr. Gels were stained with buffalo black. Samples are from left to right: enterotoxin A, equal weights of enterotoxin A and B, enterotoxin B.

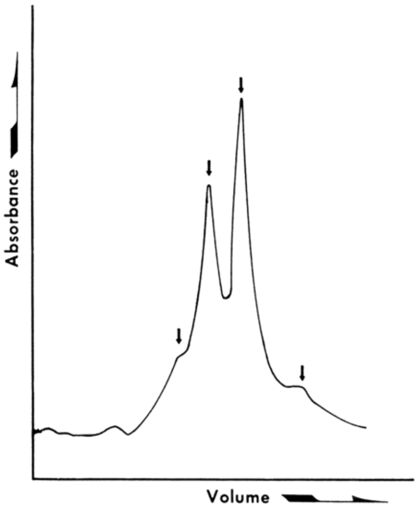


FIGURE 5: Separation by isoelectric focusing of staphylococcal enterotoxin A. The sample was equilibrated overnight and then electrophoretically separated for 24 hr at 4° in a pH gradient between 3 and 10 of synthetic carrier ampholytes and a linear density gradient of sucrose from 0 to 50%. The final voltage was 1000 V. The column was eluted by a peristaltic pump from the bottom at a rate of 1 ml/min.

Tests for α- and β-lysins and dermonecrotic substances were negative. Ouchterlony tests at 0.5 mg/ml and double-diffusion tubes (Oakley tubes) showed only a single line of antigen–antibody formed. Assays of the toxin in rhesus monkeys[3] by the intravenous route resulted in illness characterized by vomiting or diarrhea or both in 50% of the animals (ED_{50}) at 0.03 μg/kg of body weight. By the oral route the ED_{50} was 1 μg/kg of body weight.

The biological activities described were retained after heating a solution of the toxin at 60° and pH 7.3 for as long as 16 hr. At room temperature (22–25°) the toxin in 0.05 M phosphate buffer at pH 6.8 was stable for several days with no detectable change in biological activity. The freeze-dried toxin containing 3–5% of sodium phosphate buffer salts (solution before drying at pH 6.8) was found to be very stable.

Sedimentation Coefficient. Experiments were carried out in 0.05 M phosphate buffer at pH 6.8 at protein concentrations from 2.0 to 10.0 mg per ml. The slope of the concentration *vs.* sedimentation coefficient curve did not differ significantly from zero. The average $s_{20,w}$ was 3.03 S. This number is virtually identical with the figure reported by Chu *et al.* (1966), 3.04, who did, however, find a significant regression slope.

Molecular Weight. BY SEDIMENTATION EQUILIBRIUM. The molecular weight was determined in 0.075 M sodium phosphate buffer at pH 6.5. Experiments were carried out at 20–22° for about 30 hr at nominal speeds ranging from 14,000 to 18,000 rpm. All the data were used in the least squares analysis of the ln recorder deflection *vs.* r^2 plots. There was no curvature to the data. In a typical experiment at 14,000 rpm the slope was 0.3297 with a standard deviation of but 0.0012 and the correlation coefficient was 0.99987. The weight-average molecular weight calculated from these data, assuming a partial specific volume of 0.732 (*vide infra*), is 27,800. Several other determinations on different preparations all gave nearly identical values. In addition, one determination was carried out by the meniscus depletion technique at a nominal speed of 34,000 rpm. This method is probably less accurate than conventional equilibrium using absorption optics for, although it does give an immediate base line, the number of data points and the accuracy with which each one may be read are reduced. It was used here to insure that our values with the conventional technique were not low. Centrifugation at the higher speed should tend to remove a higher molecular weight component and thus lower the weight average figure obtained. Instead, a slightly increased value, 30,200, was obtained.

BY GEL ELECTROPHORESIS. Under the experimental conditions of Weber and Osborn (1969) a single line was obtained for staphylococcal enterotoxin A. Six separate determinations of the molecular weight were made with ovalbumin and myoglobin as the primary markers. The results ranged from 26,000 to 29,700, with an average value of 27,500. Staphylococcal enterotoxin B, whose molecular weight by amino acid sequence has recently been found to be 28,500 (Huang and Bergdoll, 1970), migrated a very slightly smaller distance but was indistinguishable from the A toxin when a mixture of the two was run (Figure 4). These results also demonstrate that enterotoxin A is a single polypeptide chain.

FROM SEDIMENTATION AND DIFFUSION. The diffusion coefficient at 20° was found to be 9.8×10^{-7} cm² sec⁻¹. From the Svedberg equation a molecular weight of 28,000 was calculated.

Isoelectric Point. Two major and two minor components are distinguished by the isoelectric focusing technique (Figure 5). At the maximum concentration of each component the pH values at 4° were 8.14, 7.68, 7.26, and 6.64 in order of elution. The component present in greatest concentration

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[3] In conducting the research reported herein, the investigators adhered to Guide for Laboratory Animal Facilities and Care established by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, NAS–NRC.

TABLE II: Amino Acid Composition and Integral Residues of Staphylococcal Enterotoxin A.

Amino Acid	Amino Acid Residues (g/100 g of Dry Protein)	Nitrogen (g/100 g of Protein)	Minimal[a] Mol Wt	Amino Acid/ 28,000 g of of Protein	Nearest Integral No. of Amino Acid Residues for 28,000 g of Protein	Integral No. of Residues × Respective Residue Mol Wt
Lysine	11.26	2.46	1,121	24.98	25	3,204
Histidine	3.16	0.97	4,274	6.55	7	960
Amide ammonia	1.80[b]	1.57		31.42	31[b]	(−30.66, +18.01)[c]
Arginine	4.02	1.44	3,824	7.37	7	1,093
Aspartic acid	15.53	1.89	729	38.41	38	4,373
Threonine	5.96	0.83	1,670	16.77	17	1,719
Serine	2.99	0.48	2,871	9.75	10	871
Glutamic acid	12.36	1.34	1,028	27.24	27	3,486
Proline	1.35	0.19	7,089	3.95	4	388
Glycine	2.96	0.73	1,896	14.77	15	856
Alanine	1.94	0.38	3,604	7.77	8	569
Half-cystine	0.66	0.09	15,361	1.82	2	204
Valine	4.93	0.70	1,980	14.14	14	1,388
Methionine	0.96	0.10	13,512	2.07	2	262
Isoleucine	4.11	0.51	2,708	10.34	10	1,132
Leucine	9.78	1.21	1,139	24.58	25	2,829
Tyrosine	10.63	0.91	1,512	18.52	19	3,110
Phenylalanine	4.31	0.41	3,365	8.32	8	1,177
Tryptophan	1.46	0.22	12,754	2.19	2	372
Total	98.37	16.43			240	27,970

[a] Based on values in the first column corrected to 100% recovery. [b] Omitted from the total. [c] To correct for the molecular weight difference between OH and NH_2, 0.989, is subtracted per amide residue. To correct for the mole of water on the terminal amino acids, the molecular weight of water is added.

has an isoelectric point of 7.26. (It should be noted that the isoelectric pH obtained in electrofocusing has been shown to be the isoionic point (Vesterberg and Svensson, 1966).) This value is appreciably higher than the value of 6.8 obtained by Chu *et al.* (1966) by means of paper electrophoresis. We feel that most of this difference is due to the difference in pK values for the titrating group in the isoelectric region at the temperatures of measurement. From the van't Hoff equation, assuming that ΔH for the imidazole ionization is 7 kcal (Cohn and Edsall, 1943), an increase of 0.39 in pH occurs in going from 25 to 4°.

Since the enterotoxin was demonstrated to be homogeneous in gel electrophoresis in SDS after unfolding, indicating that all the protein present was of the same molecular size, it is likely that the four components found by isoelectric focusing differ only in charge.

Amino Acid Analysis. Table II shows a complete amino acid analysis of staphylococcal enterotoxin A. The results are based on two analyses at three periods of hydrolysis: 24, 48, and 96 hr. Serine, threonine, and tyrosine were extrapolated to zero time according to zero-order kinetics. Only the 48- and 96-hr values for valine and isoleucine were averaged. Integral values are reported for all the amino acids but even a casual inspection of the column giving actual residue values makes it evident how small a change is required to change some of them. A relative error of but 0.25% in the aspartic acid value would increase the calculated number of residues from 38 to 39.

Two half-cystine residue were found. The titration for free sulfhydryl showed less than 0.05 mole/mole of protein. The tryptophan analysis gave 2.19 residues/molecule a significantly higher value than was found for enterotoxin B. This difference is reflected in the extinction coefficients, 14.6 for enterotoxin A and 14.0 for enterotoxin B.

Partial Specific Volume. The partial specific volume of enterotoxin A was calculated from the weight percentages and partial specific volume of each amino acid (Cohn and Edsall, 1943). A value of 0.732 ml/g was obtained.

Identification of Terminal Residues. A free N-terminal residue was not found by either the Edman method or the cyanate method. A dinitrophenol-containing spot was found with the Sanger technique. It was located in the DNP-serine-DNP-threonine area in the two-dimensional system of Levy (1955). The absorbance of the spot amounted to about 20% of theoretical. However, when the bicarbonate solution containing the extracted spot was acidified and extracted with ether, the material appeared to decompose. In the Blackburn–Lowther (1951) *tert*-amyl alcohol system, only an extremely faint spot was found where DNP-threonine should be located and most of the color was at the R_F of dinitrophenol. Authentic samples of DNP-serine and DNP-threonine were run through the identical series of operations with no significant loss. Recovery from the *tert*-amyl alcohol system papers was over 70%. It appears that the spot found initially is an artifact. It has not been identified.

The C-terminal residue was easily identified by the hydrazinolysis technique. An excellent yield of serine was obtained. No other amino acid was found in significant yield.

Discussion

The big problems in the purification of any bacterial product from the culture involve (a) the increase of the quantities in the culture by the selection of higher producing strains or the improvement of the environmental conditions of culturing; and (b) the development of a technique to remove the product, usually present in trace quantities. Our first attempts at culturing (strain 100) produced only 3–5 μg of enterotoxin A/ml, but selection of higher producing colonies brought the production up to 15–25 μg/ml (Friedman and Howard, 1971). No change in the immunological properties of the toxin were observed in material elaborated by the higher producing strains.

The key to the isolation procedure was the establishment of conditions for the removal of the enterotoxin from culture by adsorption onto CG-50 and its subsequent elution by strong buffer and neutral salt. Virtually no enterotoxin was detectable in the supernatant culture after the adsorption. Minor losses did occur upon elution from the ion-exchange resin but the overall yield for this step was highly satisfactory.

Ultimate purification was difficult. The peak from Sephadex G-75 was nearly symmetrical and showed constant immunological activity to protein ratios in Oudin assay. However, only the central portion of the peak gave a single line in Oakley double diffusion and it was necessary to eliminate the edge fractions which gave multiple lines.

In general the properties of the purified enterotoxin A were in line with the results of Chu *et al.* (1966) except that theirs contained minor amounts of impurities as indicated by Ouchterlony and Oakley tests. The properties of the enterotoxin are summarized in Table III.

Enterotoxin A is a simple protein composed of a single polypeptide chain, containing one disulfide bridge and no free sulfhydryl groups. It is in these respects identical with enterotoxin B. This is not at all surprising since the two proteins have the same biological action and are differentiated primarily on the basis of their immunological reactivity. It is not known whether a three dimensionally intact configuration is required for activity or only a relatively short linear section, but it would be expected that changes in amino acid composition in either case would be conservative. Indeed, after the enterotoxin A has been sequenced, it might be possible to eliminate parts of the molecule from active-site involvement by comparison of enterotoxins A and B and identifying those regions where drastic changes have occurred.

Essentially all of the material in the purified preparation is of the same molecular size and has identical immunological reactivity. There is, however, a paucidispersity found by isoelectric focusing. We believe this does not reflect the presence of extraneous protein but rather several different forms of enterotoxin A. The existence of multiple components in many highly purified proteins is well documented, *e.g.*, ribonuclease, myoglobin. Our initial report on the isolation of enterotoxin B (Schantz *et al.*, 1965), noted a heterogeneity in starch gel electrophoresis. Recently Chang and Dickie (1971) confirmed the finding of multiple electrophoretic forms of enterotoxin B using isoelectric focusing. They observed two major and at least one minor component. Both major components were toxic to monkeys and gave identical immunological reactions. Furthermore, in refocusing experiments, the more basic component gave rise to some of the more acidic component while the reverse conversion did not occur. They suggest that hydrolysis of labile amide groups is the cause of both the heterogeneity and the conversion from one electrophoretic form to another.

TABLE III: Some Properties of Purified Staphylococcal Enterotoxin A.

Appearance (freeze-dried)	White fluffy powder
Solubility	Very soluble in water and salt solutions
Type of protein	Simple (contains amino acids only)
Nitrogen content (%)	16.2
Sedimentation coefficient ($s_{20,w}$) (S)	3.03
Diffusion coefficient (D)[a] (cm^2 sec^{-1})	9.8×10^{-7}
Molecular weight (sedimentation equilibrium)	27,800
Molecular weight (gel electrophoresis)	27,500
Molecular weight (s, D)	28,000
Electrophoresis[b]	Single component
Isoelectric point (isoelectric focusing)	7.26 (major component)
Maximum absorption (nm)	277
Extinction ($E_{1\,\mathrm{cm}}^{1\%}$)	14.6
Toxicity, ED$_{50}$ intravenous (μg/kg)	0.03 (0.017–0.065)[c]

[a] Determined in agar gel (Schantz and Lauffer, 1962). [b] Run in polyacrylamide gel; β-alanine–acetic acid buffer. [c] Rhesus monkeys weighing approximately 3 kg. Numbers in parentheses refer to 95% confidence limits.

The similarities between the two enterotoxins is striking and we believe that charge differences due to differing numbers of amide groups is the most likely explanation for the observed behavior in electrofocusing. The difference in net charge may also be due to noncovalently bonded amino acids. An analogous explanation, conjugation with RNA or some other acidic material, has been advanced to explain the chromatographic behavior of ribonuclease (Anfinsen and White, 1961). Finally one must entertain the possibility that these components are conformational isomers.

In the amino acid analysis, changes in almost every residue were observed between A and B toxins but the distribution of ionizable and nonionizable residues is not drastically altered. There is a loss of four basic residues and an increase of one acidic group, consistent with the lowered isoelectric point. Quantitatively the most striking change is the decrease in the number of methionines from eight to two.

The tryptophan value found by the Edelhoch spectrophotometric method indicates two residues per molecule. We previously reported the same number for enterotoxin B. Huang and Bergdoll (1970), however, in determining the sequence of enterotoxin B, found only one residue. Our value was based on a molecular weight of 35,000 which when recalculated to the correct figure of 28,500 is reduced to 1.60 moles/mole. We have repeated this determination with the Edelhoch method on a sample of the original material and obtained a value of 1.56 and believe this to be a real and accurate estimate. However, when the determination was carried out on a newly isolated preparation, we obtained a value of 1.92. We suggest that the explanation lies in the organism used for the fermentation. The original material was produced from a mixed population but the recent preparation was produced from a cloned isolate. We suggest allotypic heterogeneity in the original bacterial stock as the cause of this apparent analytical anomaly. This type of situation is well known in mammalian proteins, *e.g.*, β-lactoglobulin and carboxypeptidase.

The failure to find a free N-terminal residue is surprising since glutamic acid was found for enterotoxin B (Spero *et al.*, 1965) and two forms of enterotoxin C (Huang *et al.*, 1967). This situation is fairly common. Acylation and cyclization are both well documented but the possibility of a masked residue at the amino terminus must be considered.

The molecular weight value obtained for this material by sedimentation equilibrium is 27,800. Confirmation of the range was found in polyacrylamide gel electrophoresis of the reduced protein in sodium dodecyl sulfate. This is significantly lower than the figures reported by Chu *et al.* (1966) of 34,500 obtained from sedimentation-diffusion data and 35,200 by the Archibald method. It is interesting to note that the same techniques gave the erroneously high values of 35,300 and 35,100, respectively, for type B enterotoxin (Wagman *et al.*, 1965). We have, moreover, determined the molecular weight of enterotoxin B by sedimentation equilibrium and obtained values within 3% of that found by sequence determination, 28,500 (Huang and Bergdoll, 1970).

The same discrepancy appears to exist for enterotoxin C. Two forms (isoelectric points of 7.0 and 8.6) of this immunological type have been isolated and their molecular weights determined from sedimentation and diffusion (Borja and Bergdoll, 1967; Avena and Bergdoll, 1967). Values of 34,000 and 34,100 were obtained. A sample of the high isoelectric point material, purified by ion-exchange chromatography and isoelectric focusing, had a molecular weight by sedimentation equilibrium of 26,000 in our hands. It seems clear that the diffusion coefficients determined by the centrifuge method of Baldwin and Williams (1950) gave significantly low values with all of the staphylococcal enterotoxins. All of the types of staphylococcal enterotoxin thus far examined have a molecular weight in the 26,000–30,000 range. This is a much more satisfying situation than would be presented if the molecular weights covered a wide range. The constancy of size observed during evolution, *e.g.*, cytochrome *c* and hemoglobin (Eck and Dayhoff, 1966), would imply even smaller change in immunological types of the same microorganism.

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