$$\frac{d \ln \langle r \rangle}{dt} = \omega^2 s \qquad (34^*)$$

This approximate equation has been developed by Vinograd *et al.* for the radially shaped cell in a centrifugal field. It can be seen to be identical to our equation (28), which is derived without approximation for a cell of uniform cross-sectional area in a centrifugal field. An exact solution for the case of the radially shaped cell is given by our equation (20). However, errors involved in using equation (34*) are insignificant when narrow bands are studied.

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Purification of Staphylococcal Enterotoxin B*

Edward J. Schantz, William G. Roessler, Jack Wagman,† Leonard Spero, David A. Dunnery, and Merlin S. Bergdoll‡

ABSTRACT: A method has been developed for the isolation of enterotoxin B in highly purified form and in yields of 50–60% from cultures of *Staphylococcus aureus*. The method involves removal of the toxin from the culture and from the bulk of the impurities with CG-50 resin and purifying it to a high degree by chromatography on carboxymethyl-cellulose. Physical, chemical, and biological studies on the purified preparation show that it is a simple protein, molecular weight 35,300. The dose of purified protein required to produce emesis or diarrhea in monkeys is 0.1 μg by intravenous injection and 0.9 μg by oral feeding per kg of animal weight.

Staphylococcus aureus produces a variety of toxic substances. One of these is enterotoxin B, which causes emesis and diarrhea in experimental animals very similar to that caused by enterotoxin A, which is usually found in cases of food poisoning in humans (Casman *et al.*, 1963). The enterotoxins apparently are metabolic products of *S. aureus* formed at the surface of the cell and released into the medium (Friedman and White, 1965), and are chemically, physically, and biologically distinct from the endotoxins (Martin and Marcus, 1964). Bergdoll *et al.* (1959a, 1961) reported the first significant purification of this toxin by a combination of acid precipitation, adsorption on Amberlite IRC-50 (XE-64), ethanol precipitation, and starch-bed electrophoresis. Recently Frea *et al.* (1963) effected a partial purification of this toxin by a combination of ethanol precipitation, filtration on Sephadex, and electrophoresis on Sephadex. Only milligram quantities of toxin were obtained by these methods. This paper describes a method of purification on a larger scale based on chromatographic procedures employing carboxylic acid resins that has resulted in preparation of enterotoxin B of higher purity and in higher yields than that obtained by the cited methods.

* From the U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md. *Received December 31, 1964; revised March 15, 1965.*

† Present address: Robert A. Taft Sanitary Engineering Center, U.S. Public Health Service, Cincinnati, Ohio.

‡ Present address: Food Research Institute, University of Chicago, Chicago, Ill.

Methods

The toxin was produced by culturing *S. aureus* strain S-6 for 18 hours with aeration in a medium adjusted to

*p*H 6.5, containing 1% N-Z-Amine A (Sheffield Chemical Co.), 1% protein hydrolysate powder (Meade Johnson & Co.), 0.001% thiamin, and 0.001% nicotinic acid. The fermented culture usually assayed between 0.1 and 0.2 mg of toxin per ml. The culture was passed through a Sharples centrifuge to remove the bulk of the cells. The carboxylic acid exchange resin, used for the first and second steps in the purification, was Amberlite CG-50, 100–200 mesh (Mallinckrodt Chemical Works). This resin was exchanged with sodium at least once and equilibrated as specified in the purification procedure by suspending the resin in 3–4 volumes of 0.05 M monosodium phosphate and titrating to the desired *p*H with sodium hydroxide. Before use the partially neutralized resin was washed thoroughly with water to remove the buffer. The CM-cellulose used in the final step in the purification was Selectacel ion-exchange cellulose, No. 77, Type 20 (Carl Schleicher & Schuell Co.) and was used as purchased after washing with water and equilibrating with 0.01 M sodium phosphate buffer at *p*H 6.2. The equilibration was carried out most satisfactorily by suspending the CM-cellulose in 0.01 M monosodium phosphate and adding alkali until the *p*H reached 6.2 (see Peterson and Sober, 1956). The linear-gradient buffer system used to elute the toxin from the CM-cellulose was patterned after that described by Bock (1954).

The absorbance at 277 mμ was used as a rapid but nonspecific measure to follow the proteins through the chromatographic fractionations. These measurements were made with a Beckman Model DU spectrophotometer. The results are expressed as mg of protein per ml, using an extinction value of 1.4 for 1 mg/ml at 277 mμ in a 1-cm cell.

Specific assays for the toxin were carried out by a modification of the Oudin serological technique (Oudin, 1946; Bergdoll *et al.*, 1959b). Rabbits were injected subcutaneously with the purified toxin emulsified in Freund's complete adjuvant to provide hyperimmune sera as described by Silverman (1963). For the agar diffusion, glass tubes of 4 mm inside diameter and 100 mm long were half-filled with buffered agar gel (*p*H 7.3, 0.5% agar) containing antiserum at a 1:40 to 1:60 dilution. Approximately 0.5 ml of the toxin solution was layered above the gel, and the tubes were stoppered and incubated in a water bath at 30°. The migration of the precipitate was measured with a cathetometer at intervals of 24 hours over a 72-hour period. The distance of migration was plotted against the square root of the time in minutes. The slope of the line thus determined was plotted against the toxin concentration to provide a standard curve. This assay technique requires 72 hours for completion, but it is serologically specific for the toxic protein and served as a simple and reasonably accurate method (±10%) for the quantitative determination of the toxin throughout the purification procedure. The first antiserum used was prepared from a small amount of toxin (2H1725A) from Bergdoll *et al.* (1959b). As the availability and purity of the toxin increased new antiserum was made for this test so that the specificity for the toxin was also increased. The results of the Oudin tests are expressed as mg of toxin per ml or, with the Kjeldahl nitrogen value, as mg of toxin per mg of nitrogen. The theoretical value for the Oudin test therefore should be 6.22 mg of toxin per mg of nitrogen. Tests for impurities were carried out by the Ouchterlony technique (Ouchterlony, 1953), employing antisera from a relatively crude (purity about 20%) toxin preparation. Although the serological tests (Oudin and Ouchterlony) do not measure toxicity per se, they correlated well with toxicity tests in monkeys.

Final preparations of the toxin were assayed by intravenous injection or feeding to rhesus monkeys (*Macaca mulatta;* wt about 3 kg) to obtain the specific biological activity characterized by emesis or diarrhea. For the intravenous injections the toxin was dissolved in 0.02 M sodium phosphate buffer containing 0.85% sodium chloride, and the animals were injected in the saphenous vein, usually with 0.3 ml/kg of body weight. More than 200 animals were used for this study. Oral feeding was performed with the aid of an infant feeding tube that was inserted through the nasal passage to the stomach. Volumes of 5 ml or less were fed with the aid of a syringe. Food and water were provided ad libitum. About 100 animals were used to determine the oral dose. All animals were observed continuously for 5 hours after administration of the toxin. In conducting this research the animals were maintained in compliance with the principles established by the National Society for Medical Research.[1]

Sedimentation measurements on each of several preparations were carried out in a Spinco Model E ultracentrifuge and were routinely run at a concentration of 10 mg/ml in 0.05 M sodium phosphate at *p*H 6.8 (see Wagman *et al.*, 1965). Measurements in free electrophoresis were carried out in a Spinco Model H electrophoresis apparatus in various buffers at *p*H 4.0–9.0 with an ionic strength of 0.1 of which 0.08 was sodium chloride. Starch-gel electrophoresis was carried out as described by Smithies (1959) in 0.02 M sodium borate buffer at *p*H 8.6 for 16 hours at a temperature of 4° and at a current flow of 5 ma/cm^2 of cross section of the gel.

Purification Procedure

The method of purification is outlined in the following steps:

Step 1. The centrifuged culture containing up to 0.2 mg of toxin per ml (about 0.04 mg toxin per mg of nitrogen by Oudin test) was diluted with 2 volumes of water and adjusted to *p*H 6.4. The toxin was removed from the diluted culture with CG-50 resin (equilibrated with 0.05 M sodium phosphate at *p*H 6.4) by stirring for about 30 minutes at room temperature. Two g of this resin was sufficient to remove the toxin from 1 liter of diluted culture. Usually 15–20 liters of culture, or sufficient to isolate at least 1 g of purified toxin, was

[1] National Society for Medical Research Principles of Laboratory Animal Care. *Biomedical Purview 1,* 14 (1961).

processed at one time. The resin containing the toxin and impurities was filtered into a column (about 3 × 40 cm) and washed with one column volume of water, and the toxin was fractionally eluted with 0.5 M sodium phosphate at *p*H 6.8 in 0.25 M sodium chloride at a flow rate of 2–3 ml/min. The fractions (10 ml each) possessing an absorbance of 2 or more per ml were selected for Oudin tests and nitrogen analyses. The fractions possessing at least 3 mg of toxin per mg of nitrogen in these tests were selected for further purification. The peak of toxin usually followed close to an amber-colored band in the column but considerably ahead of a lemon-yellow band. At this point the pooled fractions amounted to about 1% of the culture volume; the Oudin test showed about 4 mg of toxin per mg of nitrogen, and the yield was about 80%.

Step 2. The pool of fractions from step 1 was dialyzed to reduce the salts to less than 0.01 M and the toxin was readsorbed on a column of CG-50. The CG-50 was equilibrated with 0.05 M sodium phosphate at *p*H 6.8 and washed with water to remove the buffer salts. Twenty g of resin were used per gram of protein. The equilibration of the resin at a higher *p*H for this column reduced the adsorption of the impurities but still allowed complete adsorption of the toxin. Thus the bulk of the impurities, including the soluble amber-colored pigments, passed through the resin. After adsorption of the toxin, the resin was washed with water and the toxin was fractionally eluted with 0.15 M disodium phosphate. The fractions containing the peak of toxin (about 5 mg or more of toxin per mg of nitrogen) were selected and pooled for further purification. At this point the pooled fractions showed an Oudin value between 5.5 and 6 with a purity of 85–90%. The overall yield was 70–80%.

Step 3. The combined fractions from step 2 were dialyzed to bring the salt below 0.01 M and the toxin was adsorbed and chromatographed on a column of CM-cellulose (about 3 × 30 cm). The cellulose was equilibrated with 0.01 M sodium phosphate at *p*H 6.2 and washed with water. Twenty g of this resin was used per gram of protein as indicated by the absorbance. After the toxin was adsorbed on the cellulose column, the column was washed with 0.01 M phosphate buffer at *p*H 6.2. Elution was carried out with a linear-gradient phosphate buffer from 0.02 M at *p*H 6.2 to 0.07 M at *p*H 6.8 at a flow rate of 2–3 ml/min. A volume of 300 ml of each buffer was used in this gradient. The purified toxin usually appeared in the fractions between 0.03 and 0.04 M at *p*H 6.5–6.7 but varied to some extent depending upon factors such as the amount of protein and sodium on the cellulose, the length of the column, and volume of buffers used to form the gradient. The exact peak, however, was located by measuring the absorbance at 277 mμ and more accurately evaluated with Oudin tests. The fractions that showed 6.2 mg of toxin or more per mg of nitrogen and that showed only a single antigen-antibody line in the Ouchterlony tests at a level of 0.5 mg of toxin per ml were selected as the purified toxin. A typical plot of the elution of the toxin from the cellulose is shown in Figure 1. These fractions were pooled, dialyzed to reduce the buffer salts to 2–3% of the protein concentration, centrifuged to remove any insoluble material, and freeze-dried. The overall yield of purified toxin usually amounted to 50–60% based on the Oudin tests on the original culture.

Characteristics of the Purified Toxin

Table I shows some of the chemical and physical properties of the purified toxin. The freeze-dried protein is a snow-white fluffy powder that is highly hygroscopic.

TABLE I: Some Properties of Purified Enterotoxin B.

Appearance (freeze-dried)	White fluffy powder
Solubility	Very soluble in water and salt solutions
Type of protein	Simple (contains amino acids only)
Nitrogen content	16.1%
Sedimentation coefficient ($s_{20,w}$)	2.89 S (single component)
Diffusion coefficient ($D_{20,w}$)	7.72×10^{-7} cm^2 sec^{-1}
Partial specific volume	0.743
Molecular weight	35,300
Electrophoresis (free)	Single component[a]
Isoelectric point	About 8.6[b]
Maximum absorption	277 mμ
Extinction ($E_{1\,cm}^{1\%}$)	14.
Toxicity, ED_{50}	0.1 μg/kg in rhesus monkeys[c]

[a] Carried out in a series of 0.02 M acetate, phosphate, and Veronal buffers, *p*H 4–9, in which the ionic strength was held at 0.1 by the addition of sodium chloride. [b] An exact determination was difficult because of the small change of mobility of the toxin with *p*H in this *p*H range. [c] Toxin administered by intravenous injection.

It is very soluble in water and salt solutions. Usual tests for carbohydrate, lipid, and nucleic acids were negative. Tests for α- and β-lysins (blood agar plates), apyrase (potato starch as substrate), and dermonecrotic substances (intradermal injections in rabbits) were negative in the purified preparation. The Kjeldahl nitrogen content of the protein (free of buffer salts) is 16.1%. The amino acid analysis and identification and quantitation of the terminal amino acids are reported in an accompanying paper (Spero *et al.*, 1965).

In velocity ultracentrifugation, solutions of the purified enterotoxin exhibited only a single symmetrical sedimenting boundary. Detailed sedimentation analyses showed the toxin preparation to be homogeneous with respect to both molecular weight and density (Wagman

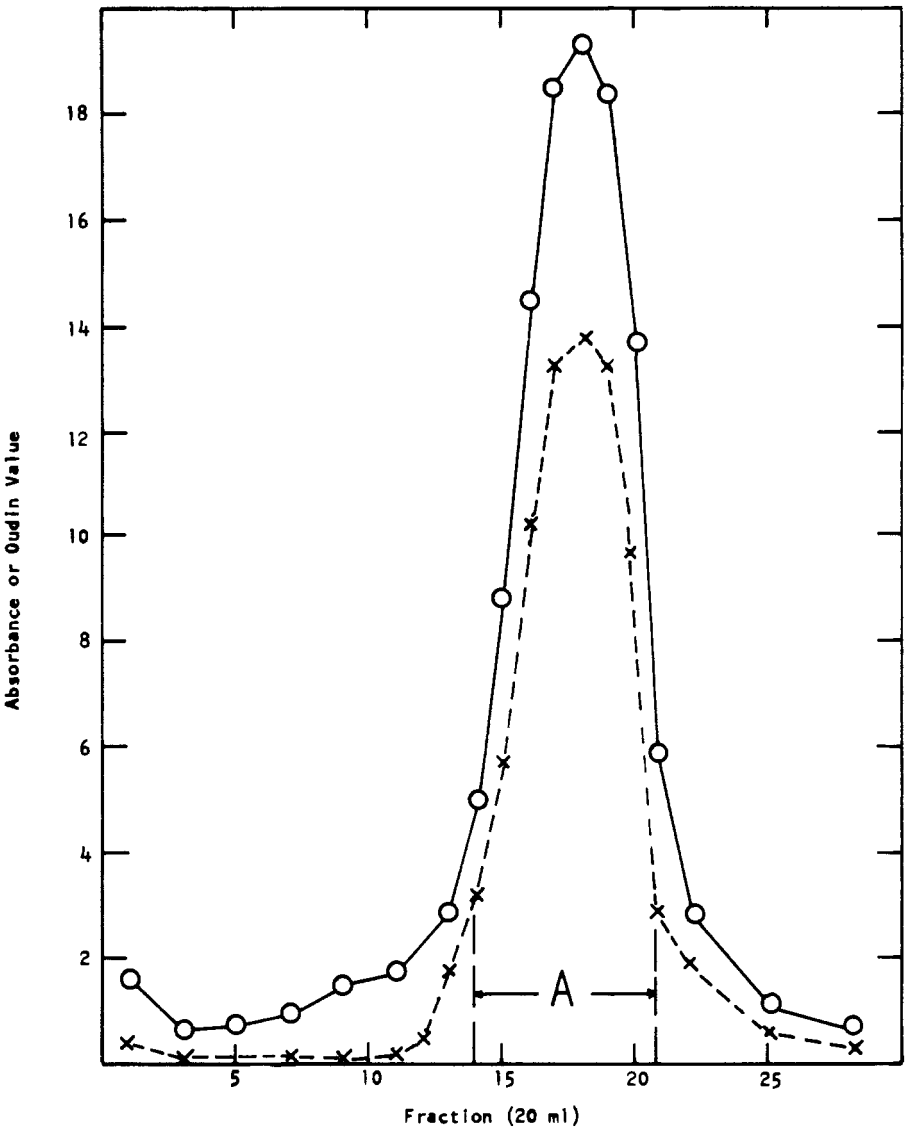

FIGURE 1: Typical elution pattern of enterotoxin B from CM-cellulose as indicated by absorbance (O—O) and Oudin value (X---X). The toxin was eluted with an increasing linear gradient of sodium phosphate buffer using 400 ml of 0.02 M at *p*H 6.2 and 400 ml of 0.07 M at *p*H 6.8. This particular column was made up with 30 g of CM-cellulose equilibrated at *p*H 6.2 in 0.01 M sodium phosphate. The fractions taken as purified toxin are shown under A.

et al., 1965). Electrophoretic studies showed it to be a single component with an isoelectric point at about *p*H 8.6. Disk electrophoresis in polyacrylamide gel[2] showed one major toxic component accounting for about 95% or more of the total preparation. Solutions of the toxin deionized either on a column of mixed-bed resin, MB-1, or on a Dintzis (1952) column showed an isoionic point at *p*H 8.55. The toxin has a maximum absorption at 277 mμ with an extinction ($E_{1\,cm}^{1\%}$) of 14.0. The ratio of the absorption at 260 mμ to that at 277 mμ was 0.45–0.47, which confirms other tests showing that very little if any nucleic acid material is present in the preparation.

Electrophoresis in starch gel using 0.02 M borate buffer at *p*H 8.6 showed two major components that moved toward the cathode. In borate or Veronal buffer at *p*H 8.6, containing sodium chloride to bring the ionic strength to 0.1, the toxin remained at the origin in a single band. In 0.02 M phosphate buffer at *p*H 7.0, containing sodium chloride to bring the ionic strength to 0.1, the toxin moved between 1 and 2 cm toward the cathode in a single band. The faster-moving component in the 0.02 M borate buffer (4–5 cm from the origin) accounted for 60–70% and the slower-moving component (about 3 cm from the origin) accounted for 20–30% of the toxin placed in the gel. A rerun of the faster component produced two components in the same relative positions and proportions, whereas the slower component under the same conditions resulted in a single

[2] Test carried out by Dr. Harold Baier, U.S. Army Biological Laboratories, at *p*H 4.5 (β-alanine–acetic acid buffer).

component with unchanged mobility. Both components showed toxicity in monkeys and an Oudin value equal to that of the original purified toxin.

In free electrophoresis in 0.02 M borate buffer at *p*H 8.6 the toxin moved to the anode as a single component, indicating that the isoelectric point, under these conditions, was below 8.6. Results of studies carried out on the sedimentation of the toxin in 0.05 M borate buffer at *p*H 8.6 showed a single component only with a sedimentation rate equal to that obtained for the toxin at other *p*H values.

When the toxin was employed in the Ouchterlony test at concentrations of 0.5 mg or less per ml, only a single line of antigen-antibody formed. However, by using very high concentrations of the toxin (up to 15 mg/ml) in these tests, one or two faint lines appeared, indicating that small amounts of impurity might be present. A quantitative estimate of the impurities by serial dilution of the preparation showed that the faint lines disappeared completely on a 32-fold dilution of the toxin solution, but the enterotoxin line did not disappear until it had been diluted more than 8000-fold, indicating that the impurities should be less than one part in 200.

When the toxin was assayed by the intravenous route in rhesus monkeys, illness characterized by vomiting or diarrhea was observed in 50% of the animals (effective dose, ED_{50}) at 0.1 μg/kg body weight with 95% confidence limits of 0.05–0.2. The probit slope was 0.65 with 95% confidence limits of 0.4–0.9. By the oral route the ED_{50} was 0.9 μg/kg body weight (95% confidence limits: 0.2–5) with a probit slope of 1.0 (95% confidence limits of 0.2–2). The data were analyzed by the probit regression method suggested by Finney (1952). The toxin showed pyrogenic activity in rabbits, but the shape of the temperature-response curve is unlike that caused by endotoxin. Rabbits made tolerant to endotoxin still responded to enterotoxin in a characteristic manner (also see Clark and Borison, 1963).

The biological activities described were retained after heating a solution of the toxin at 60° and *p*H 7.3 for as long as 16 hours. At 100° for 5 minutes, less than 50% of the biological activity was destroyed although the toxin was coagulated at this temperature. At room temperature (22–25°) the toxin, in 0.05 M phosphate at *p*H 4–7.3, was stable for a week or more but on longer standing some insoluble material formed with loss in biological activity. Even at *p*H 10 no detectable loss in biological activity was observed for several days. The freeze-dried toxin stored at 4° for over 1 year showed no loss in biological activity or changes in its solubility in water, but when stored at room temperature for this length of time some insolubility and loss in biological activity was observed. The small amount of buffer (2–3% of the protein concn) left in the toxin solution from the CM-cellulose before drying helped to stabilize the toxin to drying and storage. The biological activity was not destroyed by the action of trypsin, chymotrypsin, rennin, or papain. However, it was destroyed by ficin and protease (crude, Nutritional Biochemicals Corp.). Pepsin destroyed the activity at a *p*H of about 2 but was ineffective at higher *p*H values.

Discussion

Purification of enterotoxin B by chromatography on CM-cellulose has resulted in a higher yield of a product with higher purity and higher emetic potency than that prepared by other known methods. The simplicity of the chromatographic procedure enables considerable amounts of toxin to be purified at one time. One g or more of purified toxin was readily obtained from 15 liters of culture. The method for the removal of the toxin from the culture as described by Bergdoll *et al.* (1961) was modified as described in steps 1 and 2 of the purification procedure. This process removed the bulk of the impurities and allowed effective separation of the remaining impurities from the toxin on CM-cellulose. Because of the great variety of substances in the culture, the toxin cannot be satisfactorily isolated directly from the culture with CM-cellulose. With careful fractionation, high-purity enterotoxin usually was obtained in one pass through the CM-cellulose, and subsequent chromatography on the cellulose did not increase the purity significantly, as indicated by ultracentrifugal and electrophoretic studies and by serological tests on Ouchterlony plates.

Detailed studies on ultracentrifugation and free electrophoresis gave no indication of more than one component in the toxin preparations. These data normally should indicate a purity of 95% or better unless a contaminating component was present that was similar to the toxin in size, shape, and electrical properties. The results on electrophoresis in polyacrylamide gel showing a recovery of over 95% of the toxin in a single component are in line with these results. Serological studies using serial dilutions of the purified preparation with the Ouchterlony technique indicated a homogeneity or purity of more than 99%. Another important point relative to the homogeneity of the toxin is the fact that only one N-terminal and one C-terminal acid was found per molecule of 35,000 molecular weight, indicating that no appreciable amount of another protein with different terminal acids is present (Spero *et al.*, 1965).

Each of the foregoing techniques, within the limits of its sensitivity, indicates that the enterotoxin preparation is homogeneous. Therefore it is difficult for us to explain the separation of the toxin into two major components in starch-gel electrophoresis. All data indicate that the separation is dependent upon a low ionic strength within the gel during electrophoresis. It is uncertain, however, whether the effect is owing to a true heterogeneity of the protein molecules or to an interacting system of the type described by Cann and Goad (1964). This problem is under active investigation in our laboratory. Baird-Parker and Joseph (1964)[3] and Pert[4]

[3] The absence of emetic activity of one component reported by these workers may have been caused by denaturation owing to the high current density. Experience in our laboratory has shown that strong currents will destroy the emetic activity.

[4] Dr. J. H. Pert, National Red Cross Research Laboratory, Washington, D. C. Personal communication.

have found a similar separation in starch gel with samples of our purified enterotoxin B.

Comparisons of the purified enterotoxin B described in this paper with other preparations are difficult because of variation in techniques used in the evaluations. From our experience, differences between laboratories of 2-fold or less on the bioassay in monkeys cannot be considered significant. Differences of $\pm 10\%$ are apparent in the Oudin test but other investigators have not reported results of this test on a quantitative basis. A comparison of the sedimentation diagrams with those of the toxin presented originally by Hibnick and Bergdoll (1959) showed the preparation from CM-cellulose to be more homogeneous. The oral emetic dose given by these workers was 1 μg toxin nitrogen for a 3-kg monkey. This amounted to about 2 μg of toxin per kg or about two times the dose of our toxin. It is doubtful, however, if this difference can be considered significant. Frea *et al.* (1963) gave no data on sedimentation, but their quantitative data from electrophoresis in polyacrylamide gel do not indicate homogeneity. Their Ouchterlony tests show homogeneity but no data are given on the amount of toxin preparation or the nature of the antisera used in these tests. It is important in the latter tests that sufficient amounts of the preparation be used to bring the impurities to a detectable level and that the antiserum be prepared against crude enterotoxin so that the impurities can be detected. These investigators reported that symptoms of enterotoxin poisoning occurred in rhesus monkeys when 0.26 μg of protein nitrogen was injected intravenously per kg of animal weight. This dose amounts to 1.6 μg of protein per kg of animal weight and is five to ten times that required to produce the same symptoms with the purified toxin described here. Also, these investigators assumed the extinction value of the toxin to be the same as that for bovine serum albumin (6.5) for their calculations. The actual extinction value, subsequently determined, is 14, and it appears that their toxin preparation may have been contaminated with a nitrogenous substance possessing a lower extinction value than that of the toxin.

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