Purification and Some Physicochemical Properties of Enterotoxin C, *Staphylococcus aureus* Strain 361*

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ABSTRACT: Enterotoxin C elaborated by *Staphylococcus aureus* strain 361 has been purified by ion-exchange chromatography on CM-cellulose (stepwise and gradient elution) and molecular sieving through Sephadex G-75 and G-50 gels. Several lines of evidence are presented to demonstrate that the purified enterotoxin possesses a high degree of homogeneity. The highly purified antigenic preparation is a simple protein which bears significant emetic activity. Some of the determined physicochemical properties are sedimentation coefficient, 2.9 S; molecular weight, about 34,000; frictional ratio, 1.19; and isoelectric pH, approximately 7.0 in sodium phosphate buffer of 0.1 ionic strength. This neutral isoelectric point indicates that enterotoxin C produced by strain 361 is chemically distinct from an immunologically similar one isolated from strain 137.

The identification of a third enterotoxin as enterotoxin C has been reported and its importance as a food poisoning agent is discussed (Bergdoll *et al.*, 1965). Two strains of *Staphyloccocus aureus*, namely, 137 and 361, were involved in the identification of this enterotoxin. Since the enterotoxins produced by these two strains reacted with the same antibody, both toxins were labeled enterotoxin C. This nomenclature is in accordance with the classification of the enterotoxins based on their reactions with specific antibodies (Casman *et al.*, 1963). No investigation has been made to find whether or not enterotoxins which possess similar immunological properties have identical molecular structures. Purification and partial characterization studies of enterotoxin C from strain 361 were undertaken to uncover differences, if any, between this enterotoxin and enterotoxin C from strain 137 (Borja and Bergdoll, 1967). Some of the biological and physicochemical properties of purified enterotoxin C from strain 361 and studies concerning its homogeneity are discussed in this paper. Comparison between the enterotoxins from strains 137 and 361, with emphasis on their physicochemical behavior, is presented.

Experimental Section

Materials. The CM-cellulose and the Sephadex G-50 and G-75 were treated similarly as reported in the accompanying paper (Borja and Bergdoll, 1967).

Methods. The production of the enterotoxin, the methods of assay by ultraviolet absorption, gel diffusion techniques, and monkey feedings are described in the accompanying paper (Borja and Bergdoll, 1967). Ultracentrifugal analysis, diffusion coefficient and viscosity measurements, and paper electrophoresis experiments were carried out as described in the preceding paper (Borja and Bergdoll, 1967). In the paper electrophoresis experiments the buffers used were sodium acetate (pH 4.6), sodium phosphate (6.0, 6.5, 7.0, and 7.5), Veronal–acetate (pH 8.5), and glycine–NaOH (pH 9.6).

Partial Specific Volume. The partial specific volume was determined by density measurements with a 10-ml pycnometer. The buffer used for these determinations and the ultracentrifuge experiments was 0.05 M sodium phosphate (pH 6.8).

Agar Gel Electrophoresis. Bacto-Agar (Difco Laboratories) was made into a gel according to the method described by Wunderly (1960). Buffalo black was used for staining. For immunoelectrophoresis, antiserum mixed with agar gel was layered into the antiserum trough.

Density Gradient Centrifugation. An apparatus which was patterned after the design of Britten and Roberts (1960) was used for producing sucrose gradients. Sucrose solutions (5 and 20%, w/v), were prepared by dissolving U.S.P. grade sucrose (Fisher Scientific Co.) in 0.05 M Tris buffer (pH 7.5). Gradients were prepared and tested for linearity according to the method described by Martin and Ames (1961). After the gradients were stabilized for 24–48 hr at 3°, a 100-μl sample was layered gently into the gradient tube and centrifuged for 20 hr at 3°. A swinging bucket rotor (Spinco SW-39) operated at 39,000 rpm in a Spinco model L ultracentrifuge was used. At the end of the run, the gradient tubes were punctured and fractionated in a modified sample fractionator (Martin and Ames, 1961). A series of 38 fractions, each containing 7 drops, were obtained and the enterotoxin C content was determined by single diffusion. Lyophilized yeast alcohol dehydrogenase (Worthington Biochemical Corp.), which was used as the standard in this particular run, was assayed in a Beckman model DU spectrophotometer by

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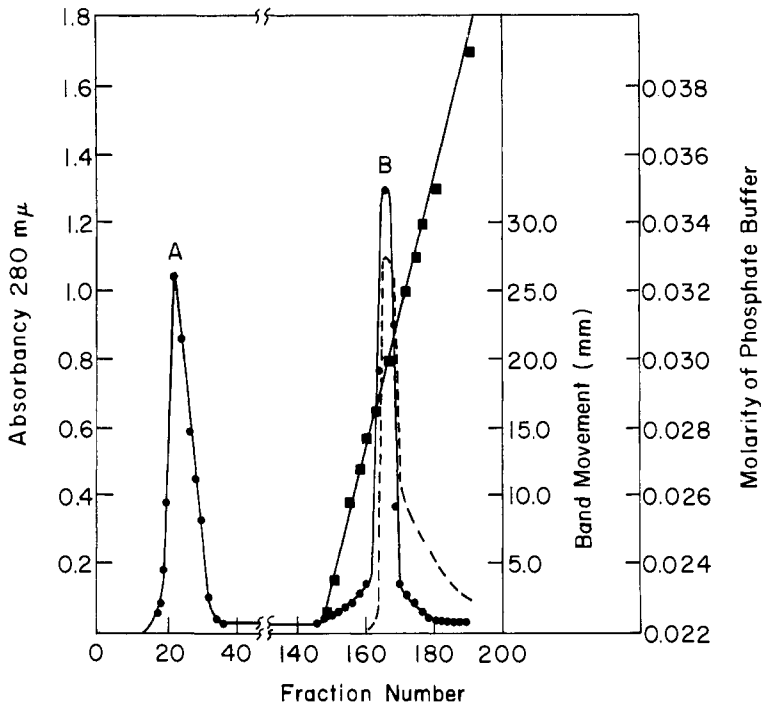

FIGURE 1: Gradient elution profile from CM-cellulose column. The partially purified sample (toxic fraction from stepwise elution on CM-cellulose column, containing 96 mg of protein) was applied to a CM-cellulose column (1.9 × 34 cm), equilibrated with 0.005 M phosphate buffer (pH 5.8), and eluted with the same buffer. The gradient was introduced in fraction number 65. Flow rate was 2 ml/min. Fraction volume was 5 ml. (—●—) absorbance. (– – –) band movement (single diffusion). (—■—) molarity of phosphate buffer.

following the increase in absorption of DPNH[1] at 340 mμ for 20 sec. The 3-ml reaction mixture contained 510 μmoles of ethanol, 159 μmoles of Tris (pH 8.5), 45 μmoles of DPN^+, and 100 μl of the enzyme fraction. Units of activity were expressed in terms of change in absorbancy/20 sec per 100 μl of the enzyme fraction.

Results

Purification Procedure. STEP I. STEPWISE ELUTION ON CM-CELLULOSE COLUMN. The freeze-dried crude enterotoxin preparation (10–12 g) (concentration of bacterial supernatant is the same as outlined in the accompanying paper, Borja and Bergdoll, 1967) was dissolved in 0.01 M NaH_2PO_4 and centrifuged in a refrigerated Servall centrifuge for 30–40 min at 15,000 rpm. This process was repeated and the pooled supernatants were dialyzed against 0.02 M sodium phosphate (pH 5.4). After 24 hr the pH of the dialyzed solution was 5.3–5.4. At this stage, 90% of the toxin contained in the original culture medium was recovered. Since the dialyzed solution was quite viscous, dilution with 0.02 M sodium phosphate (pH 5.3) was necessary. This eliminated the difficulty of maintaining a constant flow rate throughout the entire chromatographic run because of shrinkage of the column caused by the undiluted viscous mixture. The diluted dialyzed solution (300–350 ml) was introduced into a CM-cellulose column (3.5 × 67 cm) buffered with 0.02 M sodium phosphate (pH 5.4) and washed through with the same buffer. Intragastric feeding and double-diffusion plate experiments indicated that no enterotoxin was present in the "break-through" peak. The bulk of the enterotoxin was eluted with 0.06 M sodium phosphate (pH 6.6–6.7). Small amounts of four or five antigens in addition to the enterotoxin were present as revealed by double-diffusion tube tests. Single-diffusion tests indicated a recovery of about 60% of the enterotoxin present in the bacterial culture supernatant.

STEP 2. GRADIENT ELUTION ON CM-CELLULOSE COLUMN. The enterotoxin-containing fraction from the above step was lyophilized, dissolved in cold, distilled water, and dialyzed against 0.005 M sodium phosphate (pH 5.8–5.9) until equilibrium was reached (usually 20–24 hr). The dialyzed material was applied to a CM-cellulose column (1.9 × 34 cm) previously equilibrated with 0.005 M sodium phosphate (pH 5.8–5.9) followed by washing with the same buffer until the first peak (A, Figure 1) had returned to base line. To elute the adsorbed protein 400 ml of 0.05 M sodium phosphate (pH 5.8–5.9) and the same volume of 0.005 M sodium phosphate at the same pH were

[1] Abbreviations used; DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotides.

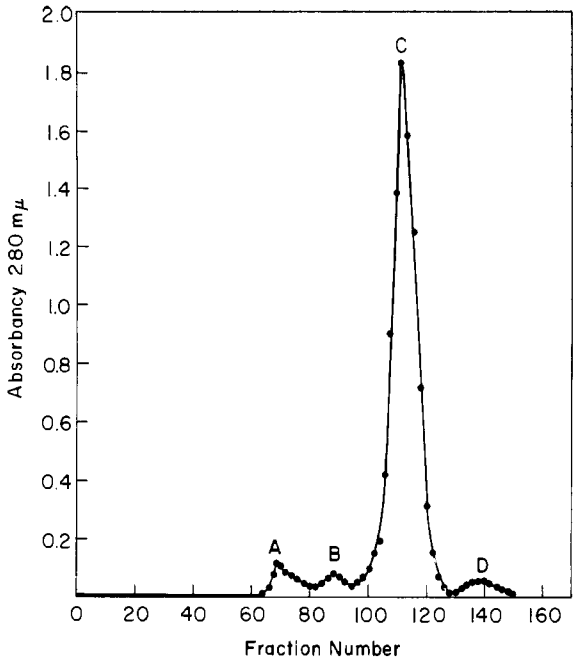

FIGURE 2: Gel filtration elution pattern from Sephadex G-75 column. About 54 mg of protein of the partially purified toxic fraction obtained from gradient elution on CM-cellulose column was introduced to a Sephadex G-75 column (2 × 145 cm) conditioned with 0.02 M phosphate buffer (pH 6.8) and eluted with the same buffer. Flow rate was 12–15 ml/hr. Fraction volume was 2.5 ml.

mixed continuously to form a linear gradient as described by Parr (1954). This procedure removed the bulk of the light yellow contaminant present in the enterotoxin-containing fraction from the preceding step. The enterotoxin appeared in the fractions between 0.02 and 0.039 M (B, Figure 1), the exact location being dependent on the amount of protein applied, length of the column, and volume of the buffers used to produce the gradient. Fraction B contained 50% of the enterotoxin present in the original culture.

An attempt to reduce the tailing of the chromatogram shown in Figure 1 was made by following the cone–sphere arrangement described by Sober and Peterson (1958). This arrangement produces a gradient which rises fairly rapidly for a brief initial period, followed by a slower rate across the center of the chromatogram and at a faster rate toward the end of the chromatogram. Gel filtration through Sephadex G-75 (following step in the purification) indicated that the enterotoxin-containing fraction was contaminated by a greater amount of impurity removed in fraction D (Figure 2) when this shape of gradient was used than when the linear gradient buffer system described by Parr (1954) was employed.

STEP 3. GEL FILTRATION THROUGH SEPHADEX G-75. The enterotoxic fractions which were obtained by linear gradient elution from CM-cellulose were lyophilized, dissolved in a minimum volume of distilled water (5°), and dialyzed against 0.02 M sodium phosphate (pH 6.8) until equilibrium was achieved. The dialyzed solution was applied to a Sephadex G-75 column (2 × 145 cm) which had been equilibrated with 0.02 M sodium phosphate (pH 6.8) and elution was accomplished with the same buffer (Figure 2).

Double-diffusion plate tests revealed that fractions A and D were virtually devoid of the enterotoxin. Minute amounts of the enterotoxin were present in fraction B with the bulk of it being present in fraction C. At a level of 1.6 mg of protein/ml, the latter fraction exhibited a major precipitin band and two very faint lines in double-diffusion tube tests. However, a single antigen–antibody line was observed when the concentration was 150 μg of protein/ml. It is impossible to determine visually whether the precipitin line common to fractions B and C is due to the same molecular species of the enterotoxin. Fraction B was not toxic when fed intragastrically at the same dosage level as fraction C. The intensity of the line given by 15 μg of protein/ml of fraction C was comparable to the intensity of the line produced by 60 μg of protein/ml of fraction B. These observations, together with the findings that fraction B was excluded earlier than fraction C from the pores of Sephadex G-75, would suggest that fraction B might possess a molecular weight corresponding to that of a dimer. The molecular weights of the two fractions were, therefore, determined by sucrose density gradient centrifugation. Both fractions sedimented 1.21 cm from the center of the applied protein layer as did the purified enterotoxin C. Yeast alcohol dehydrogenase as the standard (mol wt 150,000) sedimented 2.68 cm from the meniscus. Assuming that the partial specific volumes of the standard and enterotoxin C from both fractions are identical and using the formula of Martin and Ames (1961), the molecular weight of each fraction was calculated to be 32,000. This would demonstrate that the precipitin line common to both fractions is given by the monomeric form of the enterotoxin. No dimerization of the enterotoxin occurred during all subsequent treatments in the purification procedure.

Refiltration of fraction C through a Sephadex G-75 column under the same experimental conditions as the previous step was necessary in order to remove additional traces of impurities found in fractions B and D. Essentially all of the enterotoxin was recovered in one peak. About 45% recovery of the toxin was estimated.

STEP 4. GEL FILTRATION THROUGH SEPHADEX G-50. The enterotoxin-containing fraction from the refiltration process was lyophilized, dialyzed against 0.02 M sodium phosphate (pH 6.8), and applied to a Sephadex G-50 column. The same experimental conditions as those described for Sephadex G-75 filtration were used. A single protein peak was obtained (Figure 3). Double-diffusion tube tests revealed the presence of minute amounts of one impurity in the enterotoxin fraction when tested at 1 mg of protein/ml, but no impurities were detectable at a concentration of 0.5 mg.

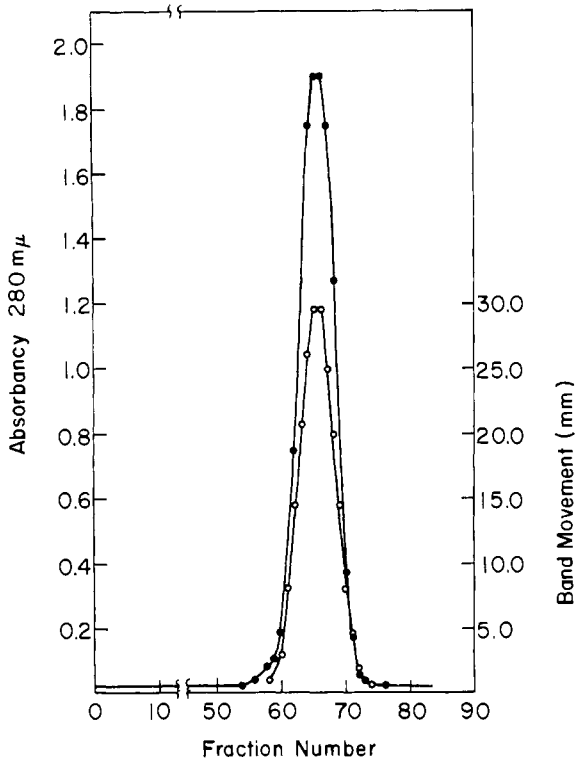

FIGURE 3: Gel filtration elution diagram from Sephadex G-50. Protein (40 mg) of fraction C from filtration through Sephadex G-75 was applied to a Sephadex G-50 column (2 × 145 cm) equilibrated with 0.02 M phosphate buffer (pH 6.8) and eluted with the same buffer. Flow rate was 15 ml/hr. Fraction volume was 2.5 ml. Fractions were diluted twofold for a single-diffusion assay. (—●—) absorbance. (—○—) band movement.

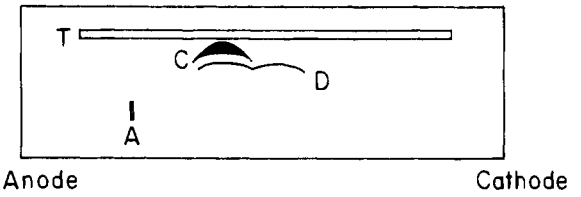

FIGURE 4: Schematic representation of the immunoelectrophoretic patterns on agar gel. Electrophoresis was carried out in agar gel plates (10 × 30 cm) using Veronal–acetate buffer (pH 8.6) with an ionic strength of 0.1 for 8 hr at 12 v/cm. Antiserum–agar (1 : 1) mixture was layered into the trough (T) which was 1 cm from the point of antigen application (A) and diffusion proceeded for 2 days. (C) Saturated solution of enterotoxin C from strain 361. (D) Mixture containing 50 μg of enterotoxin C from strain 361 and 50 μg of toxin from strain 137.

The enterotoxin fraction was dialyzed against distilled water for 48 hr, centrifuged, and the supernatant was freeze dried. The percentage recovery of the enterotoxin present in the bacterial culture supernatant was estimated by single-diffusion tests to be 40%.

After enterotoxin C was thoroughly identified as the biologically active substance present in cultures of strains 137 and 361 of *S. aureus* (Bergdoll *et al.*, 1965), the specific immunoprecipitin line given by this antigen to its specific antibody was taken advantage of to show that the purification was progressing. Specific antiserum was used also to estimate the degree of purity of the enterotoxin. Double-diffusion tube tests with serial dilutions of both the antiserum and the purified enterotoxin demonstrated that the intensity and position of the precipitin band obtained with 0.5 μg of enterotoxin/ml was comparable to the intensity and position of the precipitin band resulting from the contaminating protein in 125 μg of the purified enterotoxic fraction. A percentage purity greater than 99% was indicated from such dilution experiments.

Homogeneity. Detailed sedimentation velocity studies showed that solutions of the purified enterotoxin exhibited a single symmetrical boundary. Experiments in which the boundary was initially formed near the center of the synthetic boundary cell at a low speed (21,740 rpm) gave a similar Gaussian-like ultracentrifugal pattern. In all probability, the sedimenting as well as the diffusing molecules are noninteracting, a sound but insufficient criterion for monodispersity. Unless a different molecule of the same molecular size and shape is present in the purified sample, such a preparation can be considered homogeneous.

As a further test for homogeneity, high-voltage paper electrophoresis was performed using buffers of varying pH values from 4.6 to 9.6. In all cases, the protein migrated as a single unit with different electrophoretic mobilities at different pH values. The pH at zero mobility was calculated to be 7.0 ± 0.02.

Electrophoresis on agar gel using Veronal buffer at pH 8.6 and 0.1 ionic strength gave one spot which migrated 1.2 cm from the point of antigen application toward the cathode, indicating the presence of a single component. Similar electrophoretic runs were carried out in which immunodiffusion was allowed to proceed after gel electrophoresis. A very distinct single precipitin line was observed (Figure 4). This observation offers strong support for other experimental evidence presented that the enterotoxin preparation is indeed of high purity.

Properties of Purified Enterotoxin C. The lyophilized purified enterotoxin preparation is a white, fluffy material that is highly hygroscopic. It is very soluble in water and salt solutions. Tests for carbohydrate, nucleic acid, α- and β-hemolysins, and coagulase were negative. The nitrogen content of the dry salt-free protein was determined (F & M Scientific Model 185 CHN analyzer) to be 16.0%.

Administration *per os* of 5–10 μg of the purified enterotoxin/animal and intravenous injection of 0.5 μg/animal caused emesis in approximately 50% of the monkeys. Treatment of the enterotoxin with urea or

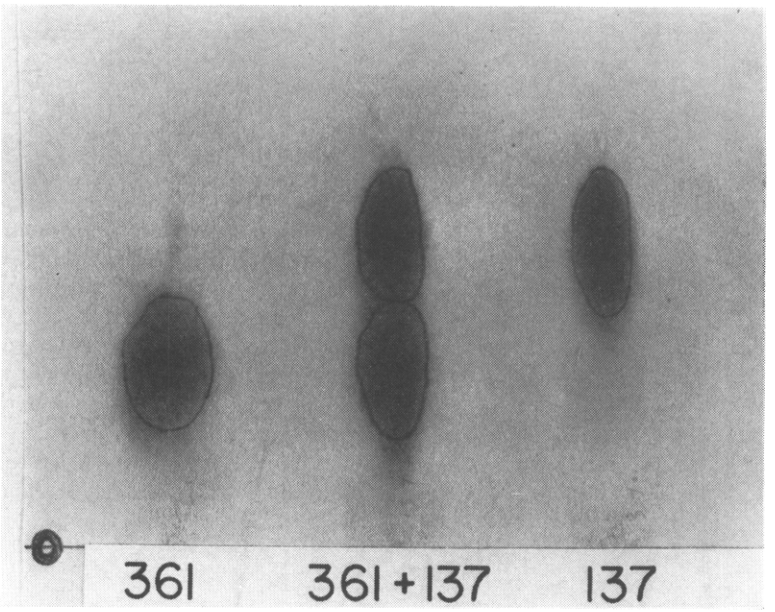

FIGURE 5: Paper chromatograms showing the difference in electrophoretic mobilities of enterotoxin C from strains 361 and 137. Electrophoresis was carried out in Veronal–acetate buffer (pH 8.6) and ionic strength 0.1 for 6 hr at 600 v. The heavy circle at the left indicates the line of sample application. Movement is toward the cathode. (left) Enterotoxin C from strain 361. (middle) Mixture containing equal amounts of enterotoxin C from strain 361 and 137. (right) Enterotoxin C from strain 137.

guanidine hydrochloride before its intravenous administration did not affect the emetic action of the toxin.

The calculated pH at zero mobility, 7.0 ± 0.02, obtained from high-voltage paper electrophoretic studies (sodium phosphate buffer at 0.1 ionic strength), was taken as the isoelectric point of the protein. Its ultraviolet absorption spectrum is that of a simple protein with an absorbance ratio, $A_{280}:A_{260}$, of 2.23. The two maxima at 220 and 277 mμ are characteristic absorption bands for polypeptides. The $E^{1\%}_{1\text{ cm}}$ at 277 mμ was determined to be 12.1.

Measurements of specific viscosity at pH 6.8 in 0.05 M sodium phosphate solution over the concentration range of 0.5–0.9 g indicated no affinity between the solvent and the protein. The intrinsic viscosity, $[\eta]$, of native enterotoxin C was determined to be 3.7 ml/g.

A plot of sedimentation coefficient against protein concentration when extrapolated linearly to zero concentration yielded an $s^0_{20,w}$ of 2.9×10^{-13} sec. Moreover, extrapolation to infinite dilution of the linear plot of diffusion coefficient versus protein concentration gave a $D^0_{20,w}$ of 8.1×10^{-7} cm²/sec. By the use of the Svedberg equation the molecular weight of the enterotoxin was calculated to be 34,000 based on the above values of $s^0_{20,w}$, $D^0_{20,w}$, and the partial specific volume, $\bar{V}$, of 0.742 ml/g determined by pycnometry. A molecular weight of 32,000 was obtained from sucrose density gradient centrifugation studies which within experimental error is in good agreement with the value obtained from the $s^0_{20,w}$ and $D^0_{20,w}$ data. Using the determined values for $s^0_{20,w}$ as 2.9 S, $D^0_{20,w}$ as 8.1×10^{-7} cm²/sec, $[\eta]$ as 3.7 ml/g, $\bar{V}$ as 0.742 ml/g, and molecular weight as 34,000, the frictional ratio, $f:f_0$, is calculated to be 1.19. The hydrodynamic parameter β of Scheraga and Mandelkern (1953) for purified enterotoxin C produced by strain 361 is 2.21×10^6.

Effect of Heat. Solutions of enterotoxin C that were heated to 52° developed turbidity which increased with an increase in temperature. The reaction of the enterotoxin with antienterotoxin was reduced to about 20% of normal when the protein was heated at 100° for 1 min.

Effect of pH. Enterotoxin C treated with 0.02 M HCl (pH 1.85) and 0.02 M NaOH (pH 12.0) did not react with the specific antibody made to the native enterotoxin (after adjustment of the pH to 7). The enterotoxin–antienterotoxin precipitin reaction was not affected by adjustment of enterotoxin solutions at any pH between 4.5 and 9.0.

Effect of Urea and Guanidine Hydrochloride. Enterotoxin solutions were treated with aqueous solutions of 8 M urea and 5 M guanidine hydrochloride for 24 hr or longer. The final pH values of the two solutions were 8.0 and 5.0, respectively. Both urea and guanidine hydrochloride treated samples did not react with the specific antibody to the native protein (single gel diffusion tubes). However, when the urea and guanidine hydrochloride were removed from the enterotoxin solutions by dialysis against distilled water and 30 mM

KOH solution, respectively, the reaction of the enterotoxin with its native specific antibody was completely restored. The failure of the enterotoxin to react with its specific antibody in 8 M urea may be attributed to the effect of the urea on the antibody, since spectrophotometric titration of the tyrosine groups of the enterotoxin in 8 M urea from pH 8.0 to 12.6 at 295 mμ (unpublished observation) indicated no change in the conformation of the toxin molecule. Other investigators have shown that urea affects the binding capacity of antibodies. Wright and Schomaker (1948) showed that urea irreversibly inactivated staphylococcal α-hemolysin antitoxin and Karush (1958) found that in 8 M urea an antibody induced by a haptenic dye lost its binding capacity. The binding capacity was recovered, however, when the urea was removed by dialysis. Failure of the antigen–antibody reaction to take place in guanidine hydrochloride may be attributed to the effect of this reagent on both the antigen and antibody since the titration curve obtained for enterotoxin C (strain 361) in 5 M guanidine hydrochloride was different from that obtained with the native enterotoxin (unpublished observation). The curve returned to normal, however, after removal of the guanidine hydrochloride by dialysis.

Discussion

The method described for the purification of enterotoxin C produced by *S. aureus* strain 361 yields a preparation with a high degree of homogeneity as indicated by ultracentrifugation, electrophoresis, immunodiffusion, and immunoelectrophoresis. Data concerning end-group determinations on the purified preparation (Huang *et al.*, 1967) demonstrated that no detectable amount of contaminating protein with different terminal groups is present. Only one C-terminal and one N-terminal acid was found per molecule. The purified preparation is a simple, antigenic, and highly toxic protein. Five to ten micrograms per animal when administered intragastrically cause emesis in approximately 50% of young rhesus monkeys, a finding which is similar to that observed for enterotoxin C from strain 137. Its emetic activity (intravenous route) and its reaction with its specific antibody are not markedly modified by a typical denaturing reagent such as urea. These results seem to indicate that some relation exists between that portion of the molecule responsible for provoking emesis and that group(s) which confers its antigenic specificity. This apparent direct relationship is in conformity with the earlier observation that the toxic action is inhibited when the enterotoxin is treated with its specific antibody (Bergdoll *et al.*, 1965).

The hydrodynamic parameter β (Scheraga and Mandelkern, 1953), for enterotoxin C produced by strain 361, suggests that the molecule under consideration is a prolate ellipsoid. Data on intrinsic viscosity and frictional ratio indicate molecular compactness.

Concurrent studies on enterotoxin C produced by strains 137 and 361 have made possible partial comparison between immunologically similar enterotoxins from different strains. The calculated values for molecular weight, sedimentation coefficient, and frictional ratio of enterotoxin C produced by strain 137 (Borja and Bergdoll, 1967) and strain 361 do not seem to reveal any differences between the two. This has to be stated with a certain degree of reservation because the limits of sensitivity in the techniques employed have to be considered. Differences between the two enterotoxins have been observed, however, the most significant of which is the dissimilarity in their electrophoretic mobilities (Figures 4 and 5). Simultaneous electrophoretic runs on paper using the two enterotoxins either singly or in mixtures within the pH range of 4.6–9.6 revealed this dissimilarity. An isoelectric point of approximately 7.0 is indicated for enterotoxin C from strain 137. Similar observations were made on human immunoglobulins, 7-S_γ and γ_1. These proteins have identical values for molecular size, sedimentation coefficient, and frictional ratio but possess different electrophoretic mobilities and isoelectric points (Putnam, 1965). The observed difference in basicity of the two purified enterotoxins is borne out from chromatographic experiments on CM-cellulose columns. Enterotoxin C from strain 361 is eluted with 0.02 M sodium phosphate (pH 5.7) conditions under which enterotoxin C from strain 137 is still adsorbed by the ion exchanger. Different chromatographic and electrophoretic behavior was observed for adrenocorticotrophins (A_1 and A_2) having identical amino acid composition (Dixon and Stack-Dunne, 1955). The higher anodic migration rate of A_2 has been ascribed to the discovery of a glutamic acid residue in position 30 of A_2 *vs.* a glutamine residue in this position for A_1. Data on the amino acid composition (Huang *et al.*, 1967) indicate a possible difference in the ratio of the number of acidic to basic residues in the two enterotoxins. The dissimilarity in electrophoretic mobilities and isoelectric pH values might be explained on this basis.

Single-diffusion tube tests show that both enterotoxins give a more dense precipitin band with a sharper leading edge when assayed against their respective specific antibodies than when assayed against the antiserum prepared from the other enterotoxin. A probable difference in the antigenicity of the two enterotoxins exists, but this slight difference, if significant at all, does not invalidate the use of either antiserum for the identification of enterotoxin C. It is very likely that the determinant group(s) is common to both enterotoxins, but that segments of the molecule other than patches of amino acid residues which confer its antigenic specificity are not identical. The differences presented above do warrant consideration of assigning appropriate designations to distinguish the two immunologically similar enterotoxins as separate and nonidentical entities.

Acknowledgments

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Amino Acid Composition and Terminal Amino Acids of Staphylococcal Enterotoxin C*

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ABSTRACT: The amino acid compositions of enterotoxin C strain 137 and enterotoxin C strain 361 were determined with a Spinco amino acid analyzer. Glutamic acid was determined as the N-terminal amino acid and glycine as the C-terminal amino acid of both enterotoxins.

This indicates that their structures are single polypeptide chains.

The staphylococcal enterotoxins have been classified according to their reactions with specific antibodies as enterotoxins A, B, etc. (Casman *et al.*, 1963). Identification of a new enterotoxin as enterotoxin C produced by staphylococcal strains 137 and 361 was accomplished (Bergdoll *et al.*, 1965a). Purification procedures and molecular weights calculated from sedimentation, diffusion and viscosity data for enterotoxin C (strain 137) and enterotoxin C (strain 361), respectively, are reported by Borja and Bergdoll (1967) and Avena and Bergdoll (1967). This paper presents a complete amino acid analysis of the enterotoxins C (strains 137 and 361) as well as information about the N- and C-terminal amino acids.

Experimental Section

Materials. The enterotoxins C (strains 137 and 361) used in this investigation were prepared according to the methods of Borja and Bergdoll (1967) and Avena and Bergdoll (1967). The nitrogen content of the enterotoxins C (strains 137 and 361) was determined with an F & M Scientific automatic CHN analyzer to be 16.2 and 16.0%, respectively. All the reagents used in paper chromatography were reagent grade. FDNB[1] and anhydrous hydrazine (95%+) were obtained from Eastman Organic Chemicals. No further purification was made on these two reagents. Benzaldehyde was distilled before use.

Amino Acid Analysis. The amino acid composition of the two enterotoxins C was determined with a Spinco Model 120B amino acid analyzer. In the analyses of calibration mixtures, an average deviation of ±3% was obtained. Trace amounts of ammonia present in the reagents were taken into consideration for calculation of amide nitrogen in enterotoxin C. Hydrolysates were prepared by heating in 6 N HCl at 110° for 24 and 72 hr in evacuated, sealed tubes. The threonine and serine values were calculated by

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[1] Abbreviations used: FDNB, 1-fluoro-2,4-dinitrobenzene; PTH, phenylthiohydantoin; PTC, phenylthiocarbamyl.