Detection of Staphylococcal Enterotoxin in Food

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Abstract

Casman, Ezra P. (Food and Drug Administration, Washington, D.C.), and Reginald W. Bennett. Detection of staphylococcal enterotoxin in food. Appl. Microbiol. 13:181-189. 1965.—Methods are described for the extraction and serological detection of trace amounts of enterotoxins A and B in foods incriminated in outbreaks of staphylococcal food poisoning. Evidence is presented for the probable applicability of the methods for the detection of unidentified enterotoxins.

In 1947, a long-range program to develop methods for the detection of staphylococcal enterotoxin in foods was begun in the Division of Microbiology of the Food and Drug Administration. The biological tests which were available for the detection of enterotoxin were difficult to perform and of variable reliability. Of these, the monkey-feeding test was not sufficiently sensitive and the procedure of injecting cats or kittens was not specific. The report by Dolman and Wilson (1938) of what appeared to be a specific antibody for the staphylococcal enterotoxin suggested that the problem might be solved by means of serological procedures. It was necessary to demonstrate the antigenicity of the enterotoxin, to determine the number of antigenic types of enterotoxin that might be involved, to make available for use the specific antitoxins, and to develop methods for the serological detection of the enterotoxins in foods.

We have reported studies (Casman, 1958, 1960) in which we demonstrated (i) the antigenicity of enterotoxin, by conferring to cats a passive immunity to enterotoxin, with the use of antiserum produced in rabbits; (ii) the occurrence of two serological types of heat-resistant enterotoxin, of which only one appeared to be associated with food poisoning; (iii) the production by certain staphylococci of other apparently heat-labile emetic substances, and (iv) the use of the gel double-diffusion test for the detection of enterotoxin.

Since these studies were reported, one or more additional enterotoxins have been encountered in food-poisoning incidents in England and, in a few instances, in this country. Only one of the two serologically identified enterotoxins, however, continues to be associated with most outbreaks of food poisoning in the United States. This enterotoxin is now designated enterotoxin A (Casman, Bergdoll, and Robinson, 1963). Strains of food-poisoning origin which produce the "B" type of enterotoxin are rarely encountered. Fujiwara (1961) studied the production of enterotoxin B by strains incriminated in outbreaks of food poisoning in Japan. Enterotoxin B was produced by 5 of 25 such strains. The 5 strains originated from a common source and were later found by us to have identical phage patterns and to produce both "A" and "B" enterotoxins. Three strains isolated in 1961 in the state of Georgia are the only strains so far encountered by us which are associated with food poisoning and produce the "B" and not the "A" type of enterotoxin.

Our development of a serological test for enterotoxin A has made possible the detection of this common type of enterotoxin in foods. We demonstrated (Casman, McCoy, and Brandly, 1963) the elaboration of enterotoxin A in meat after relatively luxuriant growth of an enterotoxicogenic staphylococcus. We now report the detection of the small amounts of enterotoxin present in foods responsible for food-poisoning incidents, the nature of the problem, and our present procedures and capabilities.

Very small amounts of enterotoxin can be expected to be present in such foods. The average amount of enterotoxin A produced by staphylococci in aerated Brain Heart Infusion broth is 2 to 4 µg/ml of culture containing 15 to 20 billion organisms. [This figure is based on the sensitivity of the slide gel diffusion test (Wadsworth, 1957; Crowle, 1958) which will detect enterotoxin when present in a concentration of approximately 1 µg/ml.] In ham and in pastries responsible for food-poisoning incidents, we have recently found from 50 million to 200 million staphylococci per

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gram. If we disregard the differences in abilities of various media to support the production of enterotoxin and assume that there is a direct correlation between growth and enterotoxin production, we could expect from 0.01 to 0.04 μg of enterotoxin per gram to be present in these foods. Such foods would have to be extracted and the extracts concentrated to an enterotoxin content of 1 μg/ml to detect the enterotoxin by means of the slide gel diffusion test. Thus, to detect the presence of 1 μg of enterotoxin in 100 g of food, the extract would have to be concentrated to 1 ml. This degree of concentration of food extractives results in the production of thick pastes which cannot be examined. It is necessary, therefore, to separate the enterotoxin not only from the insoluble constituents of the foods but also from the soluble extractives.

The procedure for examining foods for the presence of enterotoxin may be divided into three steps: (i) separation of the enterotoxin from insoluble constituents, (ii) separation from soluble extractives, and (iii) concentration of the extracted and separated enterotoxin so that it may be detected by means of the gel diffusion test. We have examined three categories of foods for enterotoxin, using these procedures: (i) foods to which measured amounts of enterotoxin were added, (ii) foods inoculated with enterotoxigenic staphylococci, and (iii) samples of foods responsible for food poisoning and containing enterotoxigenic staphylococci.

**Materials and Methods**

*Staphylococcal strains.* Strain 265-1 was isolated in 1958 from cheese incruminated in an outbreak of food poisoning. It produces from 8 to 12 μg of enterotoxin A per ml of culture supernatant liquid when grown on semisolid Brain Heart Infusion Agar, pH 5.3 (Casman and Bennett, 1963). Strain D263 was received in 1957 from G. M. Dack, University of Chicago. It was isolated from the stool of a patient who developed enteritis during intensive antibiotic therapy. It produces 2 μg of enterotoxin A and 20 to 30 μg of enterotoxin B per ml of culture supernatant when grown on semisolid Brain Heart Infusion Agar, pH 5.3.

Strains 291, 293, and 294 were received in 1962 from Betty Hobbs of London, England. They produce enterotoxins which are neither enterotoxin A nor B. Strain 291 was isolated from cheese epidemiologically responsible for three outbreaks of food poisoning in which 77 individuals were made ill. Strain 293 was isolated from chicken prepared in a small airport kitchen and was responsible for the food poisoning of passengers aboard an aircraft. Strain 294 was isolated from the finger of a food handler associated with an outbreak of food poisoning caused by ham. Its phage pattern was the same as that of staphylococci isolated from the ham.

Strain 315 is an enterotoxigenic staphylococcus producing neither enterotoxin A nor B. It was isolated in 1963 from cooked turkey epidemiologically responsible for a food-poisoning incident in Houston, Tex. Six members of a family became ill with typical symptoms of staphylococcal food poisoning from 2 to 2.5 hr after eating the turkey.

*Enterotoxins used in recovery experiments.* Measured amounts of enterotoxin were added to various foods in experiments designed to determine the efficiency of the extraction and concentration procedures. The enterotoxins were produced by growing enterotoxigenic staphylococci in sausages of cellophane dialysis tubing lying on the surface of Brain Heart Infusion broth (Casman and Bennett, 1963). Strain D263 was employed for the production of enterotoxins A and B. Two batches of this toxin were used—one containing 40 μg of enterotoxin A and 800 μg of enterotoxin B, and the other, 20 μg of enterotoxin A and 2,000 μg of enterotoxin B per ml.

In an experiment designed to determine the applicability of our procedures to the detection of enterotoxins which are not yet serologically identified, similarly prepared enterotoxins were employed, by use of strains 291, 293, 294, and 315. These enterotoxins were detected and very crudely measured by the cat test (Hammon, 1941) after the removal of interfering hemolysins by pancreatic digestion (Casman and Bennett, 1963).

*Foods used in recovery experiments.* Measured amounts of enterotoxins were added to feta cheese (prepared from goat's milk), to the coconut custard portion of frozen coconut custard pie, to cooked and raw ground beef, and to cooked ground shrimp and turkey. The pie was baked as directed by the manufacturer. The beef, shrimp, and turkey were cooked in flowing steam for 25 min.

*Foods inoculated with strain 265-1.* White turkey meat, coconut custard, and shrimp were inoculated with strain 265-1. Cooked white turkey meat (150 g) was ground in a meat grinder and mixed with 100 ml of distilled water in a Waring Blender at high speed for 3 min. The resulting paste was distributed in petri dishes (diameter, 10 cm) at a depth of approximately 0.3 cm and autoclaved at 15 lb of steam pressure for 10 min. Ground cooked shrimp (100 g) was mixed with 100 ml of distilled water in the Waring Blender at high speed for 3 min, and the paste distributed in petri dishes and autoclaved in the same manner. The coconut custard of a freshly baked frozen coconut custard pie was similarly distributed in petri dishes in approximately 0.3 cm deep layers and autoclaved.

An aqueous suspension containing approximately 3 × 10⁸ staphylococci per milliliter was prepared from a 24-hr nutrient agar slant culture of strain 265-1, and 0.2 ml was spread over the surface of the food in each petri dish. The plates were incubated in air at 35 to 37 C for 48 hr. The cultures of each of the foods were mixed and stored.
at -18 C. Dilutions of the thoroughly mixed foods were streaked on Staphylococcus 110 Agar (Difeo) and on Plate Count Agar (Difeo). The counts per gram of turkey paste, shrimp paste, and coconut custard were 13.5, 15, and 2.5 billions, respectively.

*Foods incriminated in outbreaks of food poisoning.* Foods which were epidemiologically responsible for four outbreaks of food poisoning were also examined for the presence of enterotoxin. In three of these outbreaks involving cream pies, ham, and macaroni salad, staphylococci which produced enterotoxin A were found in the incriminated foods. In another outbreak involving ham, only nonenterotoxigenic staphylococci were detected.

Banana cream pie and coconut cream pie, consisting of flour, sugar, eggs, flavoring, and coconut or banana, were responsible for an outbreak of food poisoning in which seven individuals became ill 4 hr after consuming the pies. The staphylococci isolated produced enterotoxin A. The coconut cream pie contained 200 million and the banana cream pie 72 to 73 million staphylococci per gram.

In another food-poisoning incident, three individuals developed the symptoms of staphylococcal food poisoning 1 hr after consuming ham. The ham contained 50 million staphylococci per gram, and the isolated organisms produced enterotoxin A.

In a third outbreak of food poisoning, macaroni salad, consisting of cooked macaroni, salad dressing, canned milk, and sugar, was the common food consumed by 83 individuals who became ill 3 to 9 hr after eating lunch in the cafeteria of an elementary school. The symptoms were nausea, vomiting, and headache. The salad contained 1 million staphylococci per gram, and the isolated organism produced enterotoxin A.

The fourth outbreak was apparently caused by ham which was cooked, sampled without incident, wrapped in aluminum foil, and stored without refrigeration for approximately 12 hr. Over a period of 5 days, seven individuals became ill with symptoms typical of staphylococcal food poisoning within 2 to 6 hr after eating the ham. The ham contained approximately 3 billion coagulase-positive staphylococci per gram. Cultures were prepared from eight isolated colonies and were found to produce neither enterotoxin A nor B. Four of the eight cultures were tested for enterotoxigenicity, by use of the cat test, with negative results.

*Separation of enterotoxin from insoluble food constituents.* Foods were homogenized with diluent at high speed in a Waring Blender. After centrifugation, the supernatant liquids were examined for their enterotoxin contents by the gel diffusion test, either directly or after further processing. Generally, 20 g of food were blended for 3 min in 100 ml of 0.2 M NaCl and, after adjusting the mixture to pH 7.4 to 7.5, centrifuged for 10 min at 32,800 X g. The supernatant liquid was decanted into a beaker. The sedimented food was resuspended by blending in 50 ml of 0.2 M NaCl at pH 7.4 to 7.5 and centrifuged again at 32,800 X g for 10 min. Floating particles of lipoidal material were removed. The pooled extracts were generally placed in cellophane dialysis tubing and immersed in 30% (w/w) polyethylene glycol 20,000 (PEG) (Fisher Scientific Co., Pittsburg, Pa.) to reduce their volume.

The presence of 0.2 m NaCl in the diluent diminished markedly the removal of enterotoxin during centrifugation. There appeared to be more retention of the enterotoxin by the supernatant at pH 7.3 to 7.5 than at lower or higher pH values. This was especially evident when water, rather than saline, was the suspending fluid. Amounts considerably larger than 20 g were examined when the presence of very small amounts of enterotoxin was indicated by the numbers of staphylococci detectable by plate count and microscopic examination.

*Separation of enterotoxin from soluble extractives.* Two procedures used by others in purification of staphylococcal enterotoxin B were examined for the separation of enterotoxin from food extractives. Of these, adsorption onto carboxymethyl-cellulose (CM-cellulose; Schantz, 1964) was more applicable than filtration through Sephadex G-100 (Frea, McCoy, and Strong, 1963).

*CM-cellulose.* Prior to the separation of enterotoxin with CM-cellulose, the extract was generally concentrated by dialysis against PEG to approximately one-fourth its original volume. The dialysis tubing was emptied and rinsed with two or three 2-ml quantities of 0.2 M NaCl, and the pooled concentrate and washings were adjusted to pH 7.4 to 7.5 with 1.0 N NaOH. After centrifugation at 32,800 X g for 10 min to remove any precipitate that appeared on concentration, the extract was diluted with 20 volumes each of distilled water and 0.01 M sodium phosphate (pH 6.0). The extract was adjusted to pH 6.0 with 0.01 M H2PO4 and passed through a column of CM-cellulose. A 1-g amount of CM-cellulose (Whatman Powercer, CM-70, manufactured by W. R. Balston, Ltd., 0.7 meq/g capacity) was suspended in approximately 100 ml of 0.01 M sodium phosphate (pH 6.0). The suspension was adjusted to pH 6.0 with 1.0 N NaOH. The column was packed by pouring the suspended CM-cellulose over a plug of glass wool in a chromatographic tube 2 cm in diameter and 40 cm long. The particles were allowed to settle under flow conditions until the adsorbent reached a height of approximately 2 cm. A loosely packed plug of glass wool was placed just above the CM-cellulose to prevent disturbing the column, and fines were removed by washing the column with 100 to 200 ml 0.01 M sodium phosphate (pH 6.0).

The column was then placed in the cold room, and the diluted extract was allowed to pass through it at a flow rate of approximately 1 to 2 ml/min, mainly because it was convenient to do this step overnight. When the last of the extract just entered the adsorbent bed, the column was washed with 100 ml of 0.01 M sodium phosphate.
(pH 6.0). When passage of the column was completed prematurely, resulting in a drying of the column, the latter was hydrated by passing 25 to 50 ml of distilled water through it and then washed with 100 ml of 0.01 M sodium phosphate (pH 6.0). The enterotoxin was eluted from the column with 150 ml of 0.2 M sodium chloride in 0.2 M sodium phosphate (pH 7.4).

With some foods, excessive amounts of soluble food constituents were adsorbed by the CM-cellulose at pH 6.0 so that the eluates could not be concentrated to the degree possible with other foods.

**Gel filtration.** To separate enterotoxin from soluble extracts by gel filtration, the extract was concentrated by osmotically forced dialysis to 20 ml or less, clarified by centrifugation at 32,800 X g for 10 min and filtration through a membrane filter (average porosity, 0.5 μm), and placed on a column (4 by 21 cm) prepared with 12 g of 140 to 400 mesh Sephadex G-100 (Pharmacia Laboratories, Inc., New York, N.Y.) equilibrated with 0.2 M NaCl (pH 7.3 to 7.4). After the sample entered the Sephadex, the column was eluted by gravity flow with 0.2 M NaCl, maintaining a head of 15 to 20 cm. An elution pattern determined with a mixture of enterotoxin A (20 μg) and B (2,000 μg) in 20 ml of 0.2 M NaCl revealed that enterotoxin appeared in the eluate at 95 ml and was no longer detectable at 210 ml. The eluates from 90 through 210 ml (total volume 120 ml) were combined and concentrated.

**Comparison of CM-cellulose and Sephadex G-100.** In comparing adsorption to CM-cellulose to gel filtration, 40 g of food, to which measured amounts of enterotoxins A and B had been added, were extracted at pH 7.4 to 7.5 with 200 ml of 0.2 M NaCl. The extract was concentrated to at least 40 ml by osmotically forced dialysis, centrifuged at 32,800 X g for 10 min, and filtered through a membrane filter (average porosity, 0.5 μm). The filtrate was divided into two equal quantities. One portion was passed through CM-cellulose at pH 6.0, as described above, after dilution with 20 volumes each of distilled water and 0.01 M sodium phosphate, and the other was passed through Sephadex G-100.

**Concentration of the eluates.** Eluted toxin was concentrated by dialysis against PEG at 5°C. The 150 ml of eluate from the CM-cellulose column or the 120 ml of eluate from the Sephadex column were placed in 75 to 80 cm of cellophane dialysis tubing (2.9 cm flat width) and immersed overnight in 100 ml of PEG. The concentrated contents were collected in one end portion (about 10 cm) of the cellophane sac. The remaining 65- to 70-em portion of tubing was rinsed with several 2- to 3-ml quantities of saline which were added to the smaller sac. Concentration was continued by immersing the end portion of the sac in fresh PEG until almost complete dehydration was effected. The sac was immersed briefly in saline to hydrate the contents. A Pasteur pipette was used to add the concentrate to the well in the plastic matrix of the slide gel diffusion assembly.

**Gel diffusion test.** A slide modification (Wadsworth, 1957; Crowle, 1958) of the double-diffusion test of Ouchterlony was used to detect the enterotoxin. In this test, lines of precipitation develop in a thin layer of agar gel formed between a glass slide and a 0.3 cm thick square of plastic. The plastic contains funnel-shaped holes in which the reactants are placed. Identification of the material under test is permitted by coalescence of its line of precipitation with a reference line of precipitation formed by the interaction of antibody with known enterotoxin. Results obtained with antigens, approximately 95% pure, indicate that the sensitivity of the test is such that an enterotoxin concentration of approximately 1 μg/ml is required for the formation of a line of precipitation. The test may be employed to determine, semiquantitatively, the micrograms per milliliter of the material under examination, by multiplying its volume by the reciprocal of the highest dilution giving a line of precipitation. Only 0.02 to 0.03 ml of antigen or serum is required to fill a well in the plastic matrix. The test was performed as described by Crowle (1958) except for the incorporation of 0.04 M sodium barbital (pH 7.4), and 0.01% thimerosal (Merthiolate) in the agar gel. In the examination of certain foods such as uncooked beef, it was necessary to add 1 ml glycine (Halbert, Swick, and Sonn, 1955) to the agar and to lower the sodium barbital concentration to 0.02 M to reduce clouding which obscured the lines of precipitation.

**Results**

**Evaluation of CM-cellulose and Sephadex for the separation of enterotoxin from food extracts.** To determine the relative merits of adsorption onto CM-cellulose and filtration through Sephadex for the separation of enterotoxin from soluble food constituents, approximately 20 μg of enterotoxin A and 2,000 μg of enterotoxin B contained in 1 ml of culture toxin of strain D263 were added to 40-g quantities of feta cheese, coconut custard, cooked beef, cooked ground shrimp, and cooked ground turkey. After blending and extracting with 200 ml of 0.2 M NaCl (pH 7.4 to 7.5), as described above, the extracts were concentrated by dialysis against PEG to at least 40 ml. They were clarified by centrifugation and filtration through a membrane filter (average porosity, 0.5 μm) and divided into two equal quantities. One was subjected to gel filtration, and the other was diluted with 20 volumes each of distilled water and 0.01 M sodium phosphate (pH 6.0) and passed through 1 g of CM-cellulose. The eluates were concentrated, when possible, to a final volume of approximately 0.1 ml. Because of their viscosity, however, it was not
possible to concentrate the eluates from the Sephadex column to this degree.

The average final volumes of the concentrates and the titers and percentages of recovery of the enterotoxins of at least two experiments are presented in Table 1.

The use of Sephadex G-100 generally resulted in the recovery of slightly more enterotoxin but did not permit the degree of concentration possible after adsorption to and elution from CM-cellulose. The latter procedure permitted the detection of smaller amounts of enterotoxin and was, therefore, selected for the separation of enterotoxin from food extracts.

Recovery of enterotoxin added to foods. Separate 0.4-ml quantities of the sac culture toxin of strain D263, each containing approximately 16 \( \mu \)g of enterotoxin A and 320 \( \mu \)g of enterotoxin B, were added to 20-g quantities of coconut custard and cooked and raw ground beef. By use of the procedures described above (except for dilution of the extracted foods with 10 volumes rather than 20 volumes each of distilled water and 0.01 M sodium phosphate), 0.72 ml of cloudy, colorless end product was obtained from the mixture of enterotoxin and coconut custard. It contained 11 \( \mu \)g of enterotoxin A and 230 \( \mu \)g of enterotoxin B, representing recoveries of 69 and 72\%, respectively. With cooked beef, 38\% enterotoxin A and 48\% enterotoxin B were recovered. When the same procedure was applied to raw beef, soluble meat constituents were adsorbed by the CM-cellulose to such an extent that the eluates could not be concentrated to the degree possible with extracts of other foods. Furthermore, the extracts contained substances which diffused into and clouded the agar gel in the slide diffusion test, obscuring the specific lines of precipitation. The clouding was not prevented by the addition of 1 M glycine to the agar or by lowering the sodium barbital content of the agar to 0.02 M. The substances in uncooked beef which caused the clouding were not removed when the extract was passed through a column of Sephadex G-100 prior to passage through CM-cellulose.

Because less of the extractives from raw beef were adsorbed by the CM-cellulose at higher pH values, attempts were made to separate the enterotoxin by adsorption at pH 6.5 and also at pH 6.5 and pH 6.8 at reduced ionic strength. The reduction in ionic strength was accomplished by dialyzing the 0.2 M NaCl extract of the meat against PEG until approximately one-fourth its original volume was reached, and then diluting the concentrated extract with 20 volumes each of water and 0.01 M sodium phosphate (pH 6.5 or 6.8).

The results of these studies (Table 2) reveal that at pH 6.5, when the ionic strength was not reduced, there was a marked reduction in recovery of enterotoxin. At pH 6.5 and reduced ionic strength, there was only a slight reduction in recovery. At pH 6.8 and reduced ionic strength, the recovery was one-half that obtained at pH 6.0. Since considerably less adsorption of meat extractives took place at higher pH values and especially at pH 6.8, the loss of enterotoxin could be compensated for by greater reduction of the final volume. The loss of enterotoxin at higher pH values and the excessive amounts of meat extractives at pH 6.0 limit the effectiveness of the procedure to the detection of more than trace amounts of enterotoxin in raw meat. Clouding of the gel by extracts of raw meat obtained

| Table 1. Recovery of enterotoxins A and B from food extracts by use of carboxymethylcellulose and Sephadex G-100 |
|---|---|---|---|---|---|---|---|---|
| Food extracted | Carboxymethylcellulose | | | | Sephadex G-100 | | |
| | Concentrate | Titer | Recovered | Titer | Recovered | Concentrate | Titer | Recovered |
| | ml | | % | | | | | % |
| Feta cheese . . . | 0.13 | 1:37 | 43 | 1:2,500 | 30 | 0.33 | 1:13 | 35 | 1:1,090 | 30 |
| Coconut custard . . . | 0.1 | 1:32 | 32 | 1:1,800 | 19 | 0.3 | 1:19 | 46 | 1:2,200 | 50 |
| Cooked beef . . . | 0.15 | 1:33 | 41 | 1:2,850 | 29 | 0.3 | 1:20 | 60 | 1:1,600 | 48 |
| Cooked shrimp . . . | 0.1 | 1:23 | 23 | 1:3,500 | 35 | 0.2 | 1:21 | 41 | 1:1,890 | 37.5 |
| Cooked turkey . . . | 0.1 | 1:48 | 48 | 1:3,750 | 37.5 | 0.4 | 1:12 | 48 | 1:1,640 | 55 |
at pH 6.0 would also interfere with the detection of lines of precipitation formed by traces of enterotoxin.

Detection of enterotoxin A in foods inoculated with an enterotoxigenic staphylococcus. Foods which were inoculated with strain 265-1 and incubated at 35 to 37 C for 48 hr supported excellent growth. Enterotoxin could be detected in the 20% extracts of the turkey paste and shrimp paste without concentration.

Supernatant liquids from the 20% slurries, prepared in 0.2 M NaCl in a Waring Blender and centrifuged at pH 7.5 to 7.6 for 10 min at 32,800 X g, were concentrated by dialysis against PEG and titrated by the gel diffusion test. The numbers of staphylococci and amounts of enterotoxin A produced are presented in Table 3. It is evident that the turkey and shrimp are superior to coconut custard in support of growth of the staphylococcus and the production of enterotoxin A. The numbers of staphylococci per gram of the inoculated foods are considerably greater than those generally found in foods responsible for food poisoning.

The inoculated foods were diluted with uninoculated food to approximate the numbers of staphylococci encountered in food-poisoning incidents. These diluted foods were examined for their enterotoxin contents. A 2-g amount of inoculated coconut custard, containing approximately 0.4 μg of enterotoxin A, was diluted with 18 g of coconut custard obtained from a freshly baked frozen coconut custard pie. A 19-g amount of ground steamed turkey meat was mixed with 1 g of turkey paste containing approximately 8 to 9 μg of enterotoxin A; also, 19 g of ground steamed shrimp were mixed with 1 g of shrimp paste containing approximately 14 μg of enterotoxin A. Slurries of the mixtures were prepared in 0.2 M NaCl and examined for their enterotoxin contents. The coconut custard slurry was processed as described above (see Materials and Methods). The supernatant liquids obtained on centrifuging the turkey and shrimp slurries were similarly processed except for dilution of the supernatant liquids with 10 volumes rather than 20 volumes each of distilled water and 0.01 M sodium phosphate before passage through the CM-cellulose. The final volumes after elution and concentration were 0.1, 0.9, and 0.75 ml for the custard, turkey, and shrimp, respectively. The approximate percentages of recovery of enterotoxin A are presented in Table 4.

Detection of enterotoxin A in foods responsible for poisonings. The procedures which were employed successfully for the detection of enterotoxin added to food and for the detection of enterotoxin in foods which were inoculated with an enterotoxigenic staphylococcus were tested with foods which were responsible for outbreaks of food poisoning. The types of foods (cream pies, cooked ham, and macaroni salad) which were available for these tests were such that CM-cellulose equilibrated at pH 6.0 was used to adsorb the enterotoxin from diluted extracts adjusted to pH 6.0. The numbers of staphylococci in the foods, as determined by the plating of dilutions of their suspensions and by microscopic examination, indicated that 20-g quantities of

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**Table 2. Recovery of enterotoxins of strain D265 after addition to foods**

<table>
<thead>
<tr>
<th>Food</th>
<th>Adsorption pH</th>
<th>Ionic strength reduced</th>
<th>Clouding of gel</th>
<th>Enterotoxin A</th>
<th>Enterotoxin B</th>
<th>Per cent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coconut custard</td>
<td>6.0</td>
<td>-</td>
<td>-</td>
<td>69</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Cooked beef....</td>
<td>6.0</td>
<td>-</td>
<td>-</td>
<td>38</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Raw beef.......</td>
<td>6.0</td>
<td>-</td>
<td>+</td>
<td>30</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Raw beef.......</td>
<td>6.5</td>
<td>-</td>
<td>-</td>
<td>9</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td>Raw beef.......</td>
<td>6.5</td>
<td>+</td>
<td>-</td>
<td>25</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Raw beef.......</td>
<td>6.8</td>
<td>+</td>
<td>-</td>
<td>15</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

* Containing 1 M glycine and 0.02 M sodium barbital.
the cream pies and ham samples could be examined successfully.

The number of staphylococci in the macaroni salad was so small that the entire 360 g of sample were examined as a 40% suspension in 0.2 M NaCl (pH 7.5 to 7.6). After centrifugation at 32,800 \( \times g \) for 10 min, the large volume of supernatant fluid was placed in dialysis tubing and concentrated to 64 ml by dialysis against PEG. The concentrated supernatant was diluted with 15 volumes of distilled water and 15 volumes of 0.01 M sodium phosphate (pH 6.0), and passed through the CM-cellulose equilibrated to pH 6.0 and eluted and concentrated as described above.

The 0.15 ml of concentrated extract from 20 g of the ham involved in the fourth food poisoning incident was only faintly positive for enterotoxin A. Concentration to a smaller volume was not attempted because the concentrate absorbed moisture and overflowed its well in the plastic matrix of the slide gel diffusion assembly.

A definitely positive test was obtained, however, when the extract of an 80-g sample of the ham was passed through Sephadex G-100 and then through CM-cellulose. The 80-g portion was extracted as a 40% slurry in 0.2 M NaCl, and was concentrated by osmotically forced dialysis. The concentrate was clarified by centrifugation and filtration through a membrane filter and divided into two 14-ml quantities. These were separately passed through Sephadex G-100 (see Materials and Methods), and the two eluates were combined and concentrated to 26.8 ml. The 26.8 ml of concentrate were diluted with 20 volumes each of distilled water and 0.01 M sodium phosphate (pH 6.0), and passed through CM-cellulose, and eluted and concentrated as described above to 0.2 ml of a brick-red suspension which did not absorb moisture in the gel diffusion test.

No attempt was made to measure the enterotoxin in these foods. The undiluted concentrated extracts were placed in the wells of the plastic matrix of the gel diffusion assembly. As indicated in Table 5, positive results were obtained with all of the samples.

When 20-g portions of the coconut cream pie and banana cream pie were examined by gel filtration on Sephadex G-100 (see above), the column eluates became viscous on concentration and could not be reduced in volume sufficiently to permit detection of the enterotoxin.

**Detection of enterotoxins other than "A" and "B."** The applicability of the above procedures to the detection of enterotoxins which have not been serologically identified was determined. Such enterotoxins were produced with strains 291, 293, 294, and 315 by use of the sac culture technique. They were studied with respect to their adsorption to and elution from CM-cellulose and their detection when mixed with meat by means of the procedures developed for the detection of enterotoxins A and B. The intravenous injection of cats was used for their detection and assay.

To determine the approximate cat-vomiting doses (Casman, 1958) of the toxins, portions were digested with pancreatin to remove hemolysins and injected intravenously. From 2 to 4 cat-vomiting doses of each toxin were diluted with 20 volumes each of distilled water and 0.01 M sodium phosphate (pH 6.0), and passed through 1 g of CM-cellulose equilibrated at pH 6.0. The CM-cellulose columns were washed, and each was eluted with 150 ml of 0.2 M NaCl in 0.2 M sodium phosphate (pH 7.4 to 7.5). The eluates were reduced in volume by dialysis against PEG, digested with pancreatin, and injected intravenously into cats. The results of the toxin assays and the emetic responses to the digested eluates from the CM-cellulose columns are presented in Table 6.

Having determined that the enterotoxins were adsorbed to and eluted from CM-cellulose, we blended 5 ml of each toxin with 20 g of cooked meat.
ground beef and 100 ml of 0.2 M NaCl and attempted to demonstrate the presence of the toxins by use of the separation and concentration techniques described above for the detection of enterotoxins A and B. The eluates from the CM-cellulose columns were concentrated and digested with pancreatin, and one-half of each was injected intravenously into cats.

The eluates obtained on processing beef to which enterotoxins produced by strains 291, 293, 294, 315, and 304 had been added produced emetic responses at 136, more than 300, 36, 47, and 39 min, respectively. Two 20-g quantities of the cooked beef were processed in the same manner without the addition of enterotoxin. Neither of two cats gave emetic responses on injection of the entire amount of each eluate.

**Table 6. Adsorption of unidentified enterotoxins to CM-cellulose**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Emetic responses* to toxins</th>
<th>Toxin passed through CM-cellulose</th>
<th>Per cent eluate per cat</th>
<th>Emetic responses* to eluates</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 ml</td>
<td>1.0 ml</td>
<td>ml</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>291</td>
<td>1/2</td>
<td>1/2</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>293</td>
<td>1/1</td>
<td>2/3</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>294</td>
<td>1/2</td>
<td>1/2</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>315</td>
<td>0/2</td>
<td>2/3</td>
<td>4</td>
<td>50</td>
</tr>
</tbody>
</table>

* Number of cats vomited/number of cats injected.

**DISCUSSION**

In previously reported studies (Casman et al., 1963) on staphylococcal growth and enterotoxin production in meats, we used less efficient procedures for extracting, separating, and concentrating enterotoxin. The use of water instead of 0.2 M NaCl resulted in extraction of less enterotoxin but was responsible also for extraction of less of the meat constituents. The use of CM-cellulose at pH 6.8, rather than pH 6.0, similarly resulted in the adsorption of smaller amounts of both enterotoxin and meat extractives. Finally, because concentration of the eluates by means of the LKB ultrafilter was used, there was an appreciable loss of enterotoxin. We have found that approximately 25% of crude enterotoxin passes through the ultrafilter. The amounts recovered were much smaller than amounts one would expect to be present as a result of the excellent growth of the staphylococcus with which the meats were inoculated.

In the present studies, extraction with 0.2 M NaCl followed by adsorption to CM-cellulose and concentration of the eluate by dialysis against PEG permitted the serological detection of 38 to 69% of the enterotoxin A and 48 to 72% of the enterotoxin B which was added to selected foods. The method was used successfully to detect very small amounts of enterotoxin added to custard, cooked turkey, and shrimp, as well as the enterotoxin in foods responsible for food poisoning. The presence of extractives which clouded the gel of the gel diffusion test for enterotoxin and limited the degree of concentration of the eluates reduced the applicability of the procedure for the detection of small amounts of enterotoxin in raw beef. This deficiency may not be important from a practical point of view, however, since raw meats are generally not involved in staphylococcal food poisoning.

As indicated by our study of the macaroni salad which was responsible for an outbreak of food poisoning, amounts of food considerably greater than 20 g may be examined. This procedure is especially applicable when the food does not contain extractives which are adsorbed by and eluted from the CM-cellulose.

When excessive amounts of these interfering extractives are present in a food, the applicability of the CM-cellulose method is limited to extracts of samples considerably larger than 20 g which are first passed through Sephadex G-100 or to samples containing relatively large numbers of enterotoxigenic staphylococci. For example, the ham which was incriminated in an outbreak of food poisoning, and which contained 50 million staphylococci per gram, contained just enough enterotoxin A to permit its detection when the extract from 20 g was concentrated to 0.1 ml. Further concentration was not possible because of the presence of other extractives, so that the presence of fewer, or less enterotoxigenic, staphylococci could have resulted in a negative test for enterotoxin. Under such circumstances, the availability of a serological test more sensitive than the slide double-diffusion test would be very desirable. Preliminary trials with the hemagglutination-inhibition test in which erythrocytes were sensitized with partially purified enterotoxin, indicate that an approximately 10-fold increase in sensitivity may be expected, and that its application for the detection of enterotoxin in foods will depend on the availability of antigenically pure enterotoxin.

The methods developed for the detection of enterotoxins A and B may be applicable to the detection of staphylococcal enterotoxins not yet identified serologically. Generalization, however, is not possible at this time, because we know...
neither the number of types represented by the toxins included in this study, nor the number involved in food poisoning.

The slide double-diffusion test of Crowle (1958) and Wadsworth (1957) is especially valuable for the detection of enterotoxin in foods. It is technically simple and requires very small amounts (0.02 to 0.03 ml) of reagents. Enterotoxin is detected at concentrations of 1 µg/ml, a sensitivity which is the same as that of the tube double-diffusion test of Oakley and Fulthorpe (1953). The lines of precipitation are easily identified through their coalescence with lines formed by reference toxins—a property which is valuable in the examination of food extracts which may produce lines of precipitation not caused by specific reaction with antibody. Such precipitation would present a considerable problem in the single-diffusion test of Oudin (1952) or the double-diffusion test of Oakley and Fulthorpe (1953).

**Literature Cited**


