QUANTITATIVE DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN B IN FOOD BY GEL-DIFFUSION METHODS

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RECOVERY of coagulase-positive *Staphylococcus aureus* from food implicated in a food-poisoning outbreak is only circumstantial evidence that the correct etiological agent has been found. Even the determination by animal studies that the strain isolated is capable of producing enterotoxin does not prove that it did so in the food. Positive proof depends on demonstration of the enterotoxin in the food itself. Such proof can be obtained by feeding the food to monkeys or human volunteers, but the results are subject to the variations in susceptibility known to occur among both test subjects.

The purification (*1*) of Enterotoxin B, S6 type ["E" type of Casman (*2*)] and the determination that it was an antigenically active protein (*3–5*) opened the way to use of serologic methods for direct detection of enterotoxin in foods. Although Enterotoxin B occurs in food infrequently (*2,6*) compared with the frequency of Enterotoxin A, 196E type ["F" type of Casman (*2*)], it is a useful substitute for preliminary studies because it can be produced in a highly purified form.

This report deals with the results obtained and the difficulties encountered in the use of the various gel-diffusion techniques for the direct serologic detection and quantification of Enterotoxin B in foods.

Materials and Methods

The enterotoxin used in these studies was produced by the growth of *S. aureus* strain S6 (*7*) and purified (*1,8*) until it contained approximately 96 percent pure Enterotoxin B on a dry-weight basis.

Antiserums were produced by intramuscular injection of rabbits with the purified Enterotoxin B dissolved in saline. Initial injections of 50 μg. were followed by gradually increasing doses until later injections of 10 mg. could be tolerated. Because of the toxicity of the antigen, amounts and times of the injections were determined by the reaction of each animal in terms of weight loss. Approximately 30 mg. of Enterotoxin B was used to immunize each animal over a period of 3 months. Subsequently, two booster doses of 10 mg. each were given subcutaneously in Freund's complete adjuvant (1:1).

The animals were bled from the ear by wiping the entire outer surface of the ear with xylol, severing the marginal ear vein with a sterile scalpel blade, and allowing the blood to drip into sterile glass centrifuge tubes. Approximately 50 ml. of blood were obtained at each bleeding. The serum was preserved with merthiolate (*A*) 1:10,000, frozen, and stored at —10° C. in 3- to 5-ml. volumes.

The foods used were either custard, chicken à la king, chicken pie filling, or ham salad prepared in the laboratory, or fish and meat purchased raw or cooked in the original package, usually frozen. All foods were used as slurries prepared by homogenizing one part food with two parts distilled water and blending in a Waring Blendor for 2 to 10 minutes, at approximately 8,000 rpm.

Agar gel-diffusion methods were used in the study of the serum and in the detection of Enterotoxin B. The single-diffusion tube test of Oudin, as described by Crowle (*9*), was used for quantitative work. Oxoid Ionagar No. 2 (*B*), at a concentration of 0.6 percent, was dis-

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solved in 0.02 molar phosphate-buffered saline (0.85 percent NaCl) at pH 7.2 and containing merthiolate (1:10,000). Tubes (6- by 50-mm. OD) were thoroughly cleaned, dried, and coated with 0.5 percent Oxoid Ionagar No. 2 in distilled water.

The coated tubes were dried in an evacuated jar containing silica gel as a desiccant. Equal volumes of the buffered agar and the desired dilution of serum in the same buffered saline were mixed at 50° C. and used to charge the test tubes with 0.4-ml. volumes. When the serum agar had solidified, approximately 0.4 ml. of the desired antigen was added to the tube with a disposable, Pasteur-type, glass pipette. The tubes were then sealed with a small square of Parafilm (*C*), set in a vertical position in a tray containing modeling clay, and placed in an incubator (*D*) at 29° to 30° C. for 24 hours for quantitative studies, or at room temperature for qualitative studies. Migration distances in millimeters were measured with a vernier caliper.

The double-diffusion tube test used in these studies to determine the minimum number of antigen-antibody systems demonstrable by a particular serum and to detect very low levels of Enterotoxin B most nearly resembled that described by Oakley and Fulthrope (*10*). The same reagents and equipment described for the Oudin tests were used, but the tubes were charged with 0.3 ml. of serum agar which was overlaid with 0.3 ml. of 0.3 percent buffered agar before the antigen was added.

The double-diffusion slide test used to determine relationships between antigen-antibody systems was the micro-Ouchterlony test of Crowle (*11*) and a modification of this in which holes were cut in the agar instead of using a plastic matrix. The tests were run on 3- by 1-inch slides which were cleaned and coated with hot 0.1 percent Oxoid Ionagar No. 2 in distilled water. Except for a reduction of 50 percent in agar content, because of the use of Ionagar No. 2, the directions of Crowle were followed. When wells were cut in the agar, a cork borer (ID 4.5 mm.) was used to cut and remove, by suction, the agar plugs.

The LKB6800A (*E*) immunoelectrophoresis equipment was used to separate the antibodies present in the immune serum. The directions of the manufacturer (*E*) were followed for these studies.

Cultures used included, among others, *S. aureus* strains S6 (producing both Enterotoxin A and B), 243 (Enterotoxin B), 246–3A (Enterotoxin A), 196E (Enterotoxin A), and St–1, W46, and 111 (nonenterotoxigenic). The enterotoxigenicity, or lack of it, in these strains had been confirmed by inoculation of cats or monkey-feeding tests, or by both. Antigens from these cultures were the supernatant fluids obtained from the centrifugation of sac cultures (*12*).

Enterotoxin B was produced in foods by inoculation of 125-ml. Erlenmeyer flasks containing 25 gm. of food slurry with 1 ml. of an 18-hour trypticase soy broth (TSB) culture of *S. aureus* (strain S6). The flask cultures were incubated at 35° to 37° C., as either still- or shake-cultures, for 24 to 48 hours.

Results

Production and testing of antiserum. The serums produced by the immunization procedure previously described were studied in detail to determine their specificity for Enterotoxin B. As might be expected from the work of Cohen and associates (*13*), the pre-immunization serums of the rabbits used to produce the antiserums were found to contain precipitating antibodies against substances occurring in the supernatant fluids of sac cultures of various strains of *S. aureus*. These antibodies occurred in low titer (1:2 to 1:10) and could be demonstrated by both the single-diffusion (Oudin) and double-diffusion (Oakley) tube tests.

These same pre-immunization serums did not, however, contain demonstrable levels of antibody to Enterotoxin B. Examination of the immune serums showed that these nonenterotoxin antibodies were present in higher titers than in the pre-immunization serums. They had been either specifically or anamnestically stimulated to the point where, on occasion, reactions not related to Enterotoxin B were observed to occur at dilutions as high as 1:40. The anti-enterotoxin antibodies were present, however, in titers of 1:160 to 1:320, as demonstrated by Oudin or Oakley titrations.

Several series of tests were performed in which a serum pool from two rabbits was tested for reaction to purified Enterotoxin B and supernatant fluids from sac cultures of strains of staphylococci that produced either Enterotoxin A, Enterotoxin B, neither type, or both in combination. The serum pool was tested at various dilutions by the Oudin, Oakley, micro-Ouchterlony, and immunoelectrophoretic techniques. The results of these tests are summarized in table 1.

Reactions were obtained with all the antigens at a dilution of 1:10 in the Oudin and Oakley tests. At 1:80, however, reactions were obtained only with the purified enterotoxin and the sac culture material from cultures known to produce Enterotoxin B. Furthermore, the Crowle test showed that one of the reaction bands observed with the S6 and 243 culture material produced lines of identity with the Enterotoxin B line of the purified material, whereas the other reactions, although producing lines of identify with each other, were not identified with the Enterotoxin B line.

In addition, the results of the immunoelectrophoresis studies showed that only the enterotoxin-anti-enterotoxin system produced reactions at a 1:80 dilution, although the undiluted serum contained one or two antibodies that reacted with all of the antigens. Additional studies with this and other serum pools having similar titers showed that dilutions of 1:60 to 1:80, the highest dilutions giving measurable zones of migration in the Oudin test, were specific for Enterotoxin B.

In the examination of supernatant fluids from large series of cultures, however, including strains 246–3A, 196E, St-1, W46, and 111, it was found that nonenterotoxin reactions might occur at dilutions of 1:20 and in a few instances 1:40. These serums cannot be considered truly monovalent, since their specificity depends on the type of test in which they are used and the dilutions employed. When used at dilutions of 1:60 to 1:80 in gel-diffusion techniques (Oudin and Oakley), however, their specificity for Enterotoxin B has been demonstrated.

Sensitivity of gel-diffusion tests. The minimum amount of enterotoxin capable of eliciting food-poisoning symptoms in man is unknown, but work with animals and human volunteers indicates that considerable variation in the susceptibility among individuals is to be expected. Any test used to detect enterotoxin must be highly sensitive. Consequently, the various gel-diffusion techniques were evaluated for their ability to detect low concentrations of enterotoxin.

Purified Enterotoxin B was dissolved in buffered saline to produce a stock solution contain-

Table 1. Reactions obtained with pooled serum from two rabbits to purified Enterotoxin B and other antigens

Antigen	Enterotoxin type produced	Oudin test serum at—		Oakley test serum at—		Micro-Ouchterlony (Crowle) serum at 1:40; lines of identity with—		Immunoelectrophoresis; number of antigen-antibody systems detected	
		1:10	1:80	1:10	1:80	Enterotoxin B	Other substance	Serum undiluted	Serum 1:80
Purified Enterotoxin B (96 percent)	B	+	+	+	+	+	−	1	1
Other culture materials:[1]									
S6	A & B	+	+	+	+	+	+	2	1
243	B	+	+	+	+	+	+	2	1
246–3A	A	+	−	+	−	−	+	1	0
196E	A	+	−	+	−	−	+	1	0
St–1	None	+	−	+	−	−	+	2	0
W46	None	+	−	+	−	−	+	2	0
111	None	+	−	+	−	−	+	2	0

[1] Supernatant fluids from sac cultures of strains of staphylococci.

ing 1,000 μg. per ml. Further dilutions of this stock were made in various salt solutions, such as 0.85 percent sodium chloride, phosphate-buffered saline, Michaelis universal buffer, and others. In addition, a number of organic diluents were used, such as food extracts, brain heart infusion broth, and other broth media. These dilutions, containing from several hundred to less than 0.001 μg. of toxin per ml., were then used as antigens, with the serum at 1:60 to 1:80 dilution to determine the sensitivities of the Oudin and Oakley tests. The Oudin test detected as little as 1 μg. per ml., or 0.4 μg. per tube (0.4 ml. × 1 μg. per ml.), and the Oakley test was even more sensitive, consistently detecting 0.05 μg. per ml., or 0.015 μg. per tube (0.3 ml. × 0.05 μg. per ml.).

During the course of these studies, greater diffusion distances were observed, and, therefore, lower levels of enterotoxin were detected when the Enterotoxin B was suspended in an organic diluent, such as brain heart infusion broth, than in a buffered salt or salt solution. Examples are shown in table 2.

With a serum dilution of 1:80 in phosphate buffered saline agar, Enterotoxin B levels of 100 to 12.5 μg. per ml., diffused at least 1 mm. further when diluted in organic diluents such as brain heart infusion broth or food extracts than when diluted with 0.85 percent sodium chloride, phosphate-buffered saline, or Michaelis universal buffer.

The cause of the migration-enhancing effect noted with the organic diluents has not been completely determined, but it appears to be related to the molecular concentration in the diluent. For example, the results with various concentrations of sodium chloride (table 3) indicate that a concentration of 5×10^5 micromoles per ml. gives migration distances equivalent to those obtained with food extracts and broth filtrates, whereas lower concentrations are not stimulatory. There may also be some relation to molecular size, since sucrose had an effect similar to that of sodium chloride, but at a concentration of 10^5 micromoles per ml. or higher (table 3).

Table 3. Diffusion in millimeters obtained with various concentrations of NaCl and sucrose, with Enterotoxin B at 50 μg. per ml. and a serum dilution of 1: 80

Diluent	Substance added	Concentration in micromoles per ml.	Migration distance in 24 hours at 29°–30° C.
Distilled H_2O	None	0	4. 4
Distilled H_2O	NaCl	5, 000	4. 3
Distilled H_2O	NaCl	50, 000	4. 5
Distilled H_2O	NaCl	100, 000	4. 5
Distilled H_2O	NaCl	500, 000	5. 9
Distilled H_2O	Sucrose	5, 000	4. 4
Distilled H_2O	Sucrose	50, 000	5. 0
Distilled H_2O	Sucrose	100, 000	5. 8
Brain heart infusion broth.	None	0	5. 7

Rapidity of reactions in gel-diffusion tests. To select a method that would yield results in as short a time as possible, repeated observations were made of the three gel-diffusion tests (Oudin, Oakley, and Crowle). When 50 μg. or more of enterotoxin per ml. were present in

Table 2. Millimeters of diffusion developed in serum agar (serum 1:80) with Enterotoxin B dissolved in various diluents

Concentration of Enterotoxin B (μg./ml.)	Diluents					
	Salt solutions			Organic diluent		
	0.85 percent NaCl	PO_4 buffer 0.02 M in 0.85 percent NaCl, pH 7.2	Michaelis universal buffer, pH 8.6	Fish extract 1:1 in PO_4 buffer	BHI broth 1:1 in PO_4 buffer	Custard extract 1:1 in PO_4 buffer
100	5. 4	5. 5	5. 4	6. 6	6. 9	6. 7
50	4. 3	4. 4	4. 3	5. 7	5. 7	5. 6
25	3. 0	3. 1	2. 9	4. 5	4. 6	4. 6
12.5	1. 6	1. 6	1. 8	3. 5	3. 6	3. 7

the buffer-salt solutions or food slurries, a precipitate formed almost immediately at the serum-agar meniscus in the Oudin tubes. Food slurries or salt solutions containing less than 50 μg. of enterotoxin per ml. yielded positive results by the Oudin tests within a 24-hour incubation period at either 29° to 30° C. or room temperature.

In most instances, positive results could be observed in the Oakley tubes after 48 hours of incubation, but very low levels of enterotoxin, 1 μg. or less, were not detectable in less than a week at room or incubator temperature. The Crowle technique could also be read after 24 hours of incubation, but in a number of instances the full development of lines of identity required a further incubation period of 24 to 48 hours.

Quantification of enterotoxin. Having demonstrated that the Oudin test could be used to detect enterotoxin specifically, rapidly, and directly in foods, attention was turned to the quantitative aspects of the method. It is not, however, the purpose of this paper to review in detail the several excellent studies of quantitative gel-diffusion techniques published by other workers such as Crowle (*9*) and Kabat and Mayer (*14*).

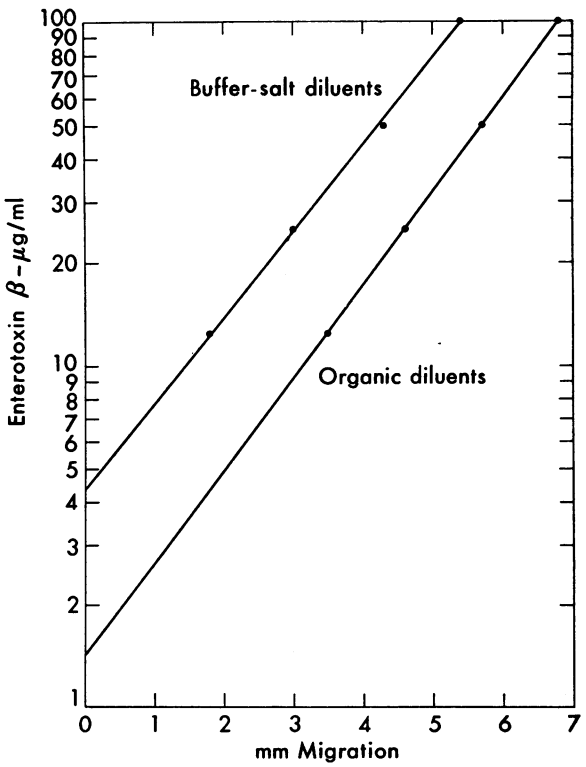
Our choice of a method for quantification was based on the need for relative simplicity and a short incubation period. Becker and associates (*15*) have postulated that the front of a precipitation band moves at a rate directly proportional to the antigen concentration in the single-diffusion tube test under the conditions of excess antigen in the presence of low antibody concentration. By selecting the highest dilution of serum that would give a measurable zone of precipitation, and using a short incubation period (24 hours) to prevent antigen depletion, it appeared that these criteria could be met by the Oudin test for enterotoxin in foods.

Accordingly, a number of experiments were performed to determine whether Enterotoxin B could be recovered quantitatively from food slurries and buffer-salt solutions. The results revealed that a straight line relationship existed between the log of the concentration of Enterotoxin B and the diffusion distance in millimeters over a range of enterotoxin concentrations from several hundred to approximately 1 μg. per ml. However, as has been noted, the diffusion distance for any given concentration depended upon the enterotoxin diluent.

The relation of the line obtained by plotting the average diffusion distances obtained with 14 organic diluents, including food extracts, culture filtrates, and uninoculated culture media, and the line obtained by averaging the diffusion distances observed in repeated experiments with phosphate-buffered saline, 0.85 percent sodium chloride, Michaelis universal buffer, and other buffer-salt solutions are shown in the figure.

The original purpose of these titrations was to establish a reference curve from which micrograms of Enterotoxin B per milliliter of diffusion could be calculated. Since two markedly different reference curves were obtained, it became necessary to determine which gave quantitative recovery of enterotoxin that had been added to food in known amounts. To do this, purified Enterotoxin B was added at vari-

Relationships between Enterotoxin B concentration and millimeters of migration in the Oudin test using buffer salt and organic diluents [1]



[1] Serum dilution of 1:80; 24-hour incubation at 29° to 30° C.

ous levels to a number of food slurries, incubated at 37° C. overnight, and the diffusion distances determined by the Oudin test. Representative results are shown in table 4. These data indicated that a reference curve representing diffusion distances in organic diluents gave the most accurate results. In every case, the indicated recovery, based on the organic reference curve, was close to the addition level; whereas the indicated recovery based on the salt-solution reference curve was between two and three times greater than that of the addition level.

Further studies showed that an organic reference curve could be prepared, using brain heart infusion broth as the diluent, that gave excellent agreement with levels of addition of Enterotoxin B to food slurries, thus eliminating the need to prepare a reference curve for each food. A comparison of diffusion distances obtained with nine organic diluents and with five titrations in brain heart infusion broth is shown in table 5. The mean of the five brain heart infusion titrations is slightly higher than the mean of the nine titrations in organic diluents. The difference is, however, within the range of variation of the method employed, since repeated measurements on duplicate or triplicate sets of Oudin tubes vary by a factor of ± 0.1 mm.

Recovery of enterotoxin from foods. The detection of Enterotoxin B in food slurries has been mentioned repeatedly in the preceding sections as part of the developmental work done to establish the validity of the gel-diffusion technique. All of this work was based on the serologic assay of purified Enterotoxin B (96 percent) that had been added to foods, broth cultures, or buffer solutions, usually in carefully measured amounts.

Table 4. Recovery of Enterotoxin B from food slurries to which it had been added in known amounts

Food slurry	Enterotoxin B added (μg. per ml.)	Migration distance in mm. in 24 hours at 29°–30° C.	Calculated recovery of Enterotoxin B	
			Organic standard curve (μg. per ml.)	Salt solution standard curve (μg. per ml.)
Custard	50	4. 5	50	108
Chicken pie filling	50	4. 6	53	114
Shrimp	25	3. 3	23	59
Custard	25	3. 4	25	61
Chicken liver	10	1. 9	9. 3	28
Chicken pie filling	10	1. 8	8. 7	27

The Enterotoxin B could be assayed in broth cultures and buffer solutions by adding them directly to the Oudin and Oakley tubes. In food slurries, however, substances were present that combined with the phosphate buffer to precipitate insoluble phosphates in the agar, which interfered with reading the Oudin tubes, especially when the levels of enterotoxin were low. Consequently, studies were carried out to deter-

Table 5. Diffusion distances in millimeters obtained in the Oudin titration of known levels of Enterotoxin B added to various organic diluents (serum at 1:80)

Diluents: type and concentration	Micrograms of Enterotoxin B added per ml. of diluent				
	100	50	25	12.5	6.25
Trypticase (1 percent)	6. 6	5. 6	4. 6	3. 6	2. 5
Casamino acids (1 percent)	6. 7	5. 7	4. 6	3. 4	2. 4
Trypticase soy broth	6. 9	5. 8	4. 7	3. 7	2. 7
Sucrose (1 percent)	6. 9	5. 7	4. 3	3. 2	2. 3
Dextrose (1 percent)	6. 6	5. 6	4. 2	3. 6	2. 2
Gelatin (1 percent)	6. 7	5. 4	4. 3	3. 3	2. 4
Soluble starch (1 percent)	6. 6	5. 4	4. 4	3. 4	2. 2
Haddock (1:1 with PO_4 buffer)	6. 8	5. 8	4. 5	3. 6	2. 5
Custard (1:1 with PO_4 buffer)	6. 8	5. 7	4. 6	3. 6	2. 6
Average of 9 titrations	6. 8	5. 6	4. 5	3. 5	2. 4
Average of 5 brain heart infusion titrations	6. 9	5. 7	4. 6	3. 6	2. 6

mine how this interfering precipitation could be eliminated.

The use of phosphate-buffered saline to extract enterotoxin from the food slurries was adopted because the work of Bergdoll (*1*) had established it as a highly efficient elution agent. Therefore, rather than abandon this method, a procedure to pretreat the food extract was adopted to eliminate the precipitation. Equal parts of the food slurry and the 0.02 molar phosphate-buffered saline (0.85 percent NaCl, pH 7.2) were thoroughly mixed in heavy-walled glass centrifuge tubes. The tubes were placed in a 50° C. water bath for 15 minutes, removed, and left at room temperature for an additional 45 minutes. They were then centrifuged at 10,000 X g. for 20 minutes in a Servall centrifuge (type SS4 with head type SS34), and the resultant, clear supernatant fluid was used as the antigen.

Comparative studies showed that although the pretreatment at 50° C. did not reduce the amount of detectable enterotoxin, it hastened precipitation of insoluble phosphates and eliminated the interference previously observed with the Oudin tests. By this method, Enterotoxin B, at levels ranging from 100 μg. to approximately 10 μg. per ml., was added to a variety of foods, including custards, meat pie fillings, chicken à la king, meat salads, meat, and fish slurries; and in each instance detection and quantification were achieved.

Throughout this study, Enterotoxin B was produced by inoculating various substrates with 1-ml. volumes of 18-hour trypticase soy broth cultures of *S. aureus* strain S6. Detectable levels of enterotoxin were consistently produced in brain heart infusion and trypticase soy broths, as well as in numerous simpler substrates, such as NZ-amine A, trypticase, proteose peptone, tryptone, neopeptone, casitone, and tryptose in 2 percent concentrations in distilled water.

The production of detectable levels of enterotoxin in food slurries, however, was not as consistently successful. Although good growth was obtained (10^7 to 10^9 organisms per ml. in 24 to 48 hours at 35° C.) with all of the food slurries, detectable levels of enterotoxin were obtained consistently only in shrimp, scallop, lobster, and crabmeat slurries, once in lake perch, and once in laboratory-prepared custard filling. In the last food slurries, detection was possible only in the Oakley tubes and only after incubation for 1 week at room temperature. In the four other food slurries, however, rapid detection was made in the Oudin tubes, and levels ranging from approximately 4 μg. to 68 μg. per ml. were obtained, depending on the culture conditions used. Repeated tests on various food slurries showed that detectable levels of enterotoxin were not added with the inoculum.

Discussion

Although Crowle (*9*) stated that production of a monovalent antiserum to enterotoxin has become simple since the purification of the enterotoxin by Bergdoll, most rabbit serums contain low levels of antibodies against various staphylococcal antigens. Furthermore, the purified enterotoxin now available is Enterotoxin B, which rarely occurs in food (*2, 6*). At present, the anti-enterotoxin A serum from rabbits must be made monovalent by absorption (*2*), or, if sufficiently high titered serum can be obtained, by dilution. When such serum is produced, it can be used in the same gel-diffusion techniques as have been described for Enterotoxin B.

The results of our studies convinced us that the serum to be used in detection of enterotoxin must be carefully studied before full confidence can be placed in the results of precipitin tests performed according to the gel-diffusion techniques of Oudin or Oakley. Study of the serum should include determination of the number and strengths of the various antibodies present and their relationships to the enterotoxin being detected, as well as to other substances that might be present in food extracts or broth filtrates. The reactions produced by antigens known to contain the enterotoxin in question should be carefully compared with the reactions produced by other antigens, and the specificity of the serum must be determined for each condition for which it is to be used.

The suggested use of both the Oudin and Oakley methods in the detection of enterotoxin, although it requires preparation of two sets of tests, is justified in our minds on the basis of

a greater sensitivity in the Oakley and a more rapid result with the Oudin. Kabat and Mayer (*14*), in discussing sensitivities of the various gel-diffusion techniques, stated that, in terms of the amount of antigen detectable, the Preer test (double-diffusion tube) is the most sensitive (*16*), the Ouchterlony (plate) method the least sensitive, and the Oudin method occupies a midpoint position. Rubinstein (*17*) detected 0.8 μg. of antigen-nitrogen with a semimicro form of the Oudin test, and Preer (*16*) detected as little as 0.01 μg.

The Ouchterlony plate method has been shown to be about one-fifth as sensitive as other gel-diffusion techniques, but the micro (slide) Ouchterlony method appears to approach the sensitivity of the single-diffusion tube methods as indicated by Brown and Crick (*18*), who reported the detection of as little as 1 μg. of antigen. The tests using the pooled serums of rabbits indicated that similar degrees of sensitivity can be expected with an enterotoxin-anti-enterotoxin system.

A relatively simple and rapid method has been suggested for the quantification of enterotoxin, since our interest is in developing a rapid method for the study of foods suspected of containing this substance. The work reported here with known concentrations of Enterotoxin B indicates that this method possesses an acceptable degree of accuracy. We acknowledge, however, that other methods (*17, 19, 20*) may offer a somewhat more refined approach and a greater degree of accuracy.

Any method that appears acceptable to the interested investigator is subject to the same critical control as that employed in this study. Time and temperature of incubation, tube size, agar concentration, antigen diluent, and serum concentration must all be kept constant if valid comparisons between different specimens are to be made.

In our work, the two factors of serum dilution and antigen diluent seemed to be the most significant variables. It was convenient in this study to use the same solution for preparing the food extracts as for making serum dilutions and buffered agar; but 0.04 molar phosphate-buffered saline, 0.85 percent sodium chloride solution, and distilled water have also been tried successfully.

Other buffer systems have been used successfully with the enterotoxin-anti-enterotoxin system. Casman (*2*) employed 0.04 molar veronal, and members of the Milk Sanitation Research Laboratories (Robert A. Taft Sanitary Engineering Center, unpublished data) have found that veronal or barbital-acetate buffers can be used to advantage in studies of the enterotoxin content of milk. Furthermore, the pH of these buffers in the range of 7.0 to 8.6 appears to have no adverse effect on the migration of enterotoxin in agar gel. The important aspect of these possible variations is that any system chosen must be used consistently, since valid comparisons cannot be made between results obtained with different systems.

The enterotoxin-anti-enterotoxin system employed in this and most similar studies uses rabbit antiserum. The reactions are referred to as the "R" type (*11*) because zones of precipitation with a relatively flat front and a fairly dense trail of specific precipitate are formed in the Oudin test. The double-diffusion tube test is similiarly characterized by the formation of a relatively wide and somewhat hazy-edged band. Since the antigen-antibody complexes formed with the "R" type of antibody are relatively insoluble in the presence of excess antigen, a clear zone below the antigen-serum agar interface is rarely seen. Furthermore, because of the relatively low molecular weight (21,000 to 27,000) of the enterotoxin (*5*), it tends to behave like a polysaccharide antigen in that the migration rate is high and there appears to be a relatively broad range in the equivalence ratio (*21*) between antigen and antibody.

These facts suggest that the single-diffusion tube test will probably give more accurate quantitative results than the double-diffusion tube test, because the leading edge of the zone of precipitation can be measured more accurately than a somewhat fuzzy line in the buffer-agar layer of the Oakley-type test. In addition, the zone formed in Oudin tubes, when the serum is used in high dilutions, does not appear to be of sufficient density to significantly impede the further diffusion of antigen. Preliminary work with Enterotoxin A indicates that it behaves in a comparable manner in all the test systems so far studied.

Both Enterotoxin A and Enterotoxin B pro-

duce similar responses in test animals and in human beings, but we do not yet know whether the same relationships to pH, substrate, incubation temperatures, and culture conditions in food hold true for both types. Possibly the failure of the S6 strain to produce demonstrable levels of enterotoxin in so many of the food slurries may account for the rarity of its occurrence in food-poisoning outbreaks. However, more detailed studies will be required to prove this, and to show that under similar conditions Enterotoxin A would be produced in detectable amounts.

Summary

Single-diffusion (Oudin) and double-diffusion (Oakley and Fulthrope) techniques were studied for serologic detection and quantification of Enterotoxin B in foods in which known enterotoxigenic strains of *Staphylococcus aureus* were grown or to which Enterotoxin B was added.

Antiserum was produced by intramuscular injection of rabbits with purified Enterotoxin B. At dilutions of 1:60 and 1:80 in the Oudin and Oakley tests, this antiserum was specific for Enterotoxin B.

Oudin and Oakley tubes were prepared for each food sample, with the serum at a dilution of 1:60 or 1:80. Extracts of food slurries were added to the tubes with a disposable, Pasteur-type pipette. The tubes were sealed with Parafilm, incubated in air at 29° to 30° C. for 24 hours, and removed from the incubator. Diffusion distance in the Oudin tube was measured with a vernier caliper.

Concentration of enterotoxin was calculated by referring the diffusion distance obtained in the sample tubes to a reference curve prepared from results obtained with known concentrations of enterotoxin dissolved in brain heart infusion broth.

The tubes were left at room temperature and observed after an additional 24 hours and at the end of 1 week. Reactions noted in the Oakley tubes were used to confirm the specificity of equivalence reaction (precipitate at the antigen-agar interface), and to detect levels of enterotoxin too low to give observable reactions in the Oudin tubes.

The Oudin method detected as little as 1 μg. of Enterotoxin B per ml. of food extract or culture filtrate in 24 hours at 29° to 30° C. The Oakley method detected as little as 0.05 μg. per ml., but a 1-week incubation period at room temperature was necessary.

Although detectable levels of Enterotoxin B were consistently produced by the growth of *S. aureus* (S6) in brain heart infusion, trypticase soy, 2 percent tryptose, and casitone broths, many food slurries failed to support similar levels of toxin production. Slurries of shrimp, scallops, lobster, and crabmeat, however, consistently yielded levels of enterotoxin of 4 μg. to 68 μg. per ml. Occasionally, Enterotoxin B was produced in lake perch and custard slurries, but at levels detectable only by the Oakley technique.

Because the rate of antigen migration in the gel-diffusion tubes was enhanced when the antigen was placed in organic diluents, such as food extracts and liquid media components, it was necessary to prepare standard reference curves for use in quantitative work in brain heart infusion broth.

Preliminary work with Enterotoxin A indicated that it behaves similarly to Enterotoxin B in all of the gel-diffusion techniques.

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(*A*) Merthiolate: Eli Lilly and Company, Indianapolis, Ind.
(*B*) Oxoid Ionagar: Consolidated Laboratories, Inc., Chicago Heights, Ill.
(*C*) Parafilm: Marathon Division of American Can Company, Menasha, Wis.
(*D*) Thelco Model 2 Incubator: Precision Scientific Company, Chicago, Ill.
(*E*) LKB6800A immunoelectrophoresis equipment: LKB Instruments, Inc., 4840 Rugby Ave., Bethesda, Md. (manufactured by LKB-Produkter AP, Stockholm, Sweden).

New PHS Hospital for Apaches

A new Public Health Service Indian hospital on the San Carlos (Ariz.) Apache Reservation was dedicated September 7, 1963. The 36-bed, air-conditioned building, replacing one built in 1929, was completed in August 1963 at a cost of $1,665,500. Equipped with the most modern surgical, laboratory, pharmacy, and X-ray apparatus and with a nursery, a pediatric section, a general medical section, and a large outpatient department, the hospital will provide a full range of community health services for the 5,000 Apache tribal members. The staff of the hospital, 1 of 50 the Public Health Service administers as part of its medical services for some 380,000 American Indians and Alaska Natives, consists of 59 persons, including 4 physicians, 1 pharmacist, 1 dentist and 1 assistant, 11 nurses and 14 assistants, 1 community health worker, 2 public health nurses and 1 assistant, and 1 sanitarian aide.

Dr. Carruth J. Wagner, Assistant Surgeon of the Public Health Service and chief of the Division of Indian Health, made the address dedicating the new hospital.