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The purpose of this study was to develop means to facilitate the detection of staphylococcal enterotoxin and to elucidate factors that influence enterotoxin formation. It was found that semi-purification flat-gel electrophoresis in a water-cooled apparatus was more efficient than CM-cellulose and Sephadex column chromatography currently used to separate toxin from soluble food constituents. The electrophoretic process was less time consuming, less expensive and adaptable to mulitple sample analyses.

A technique combining isoelectric focusing in a polyacrylamide-stabilized ampholyte solution followed by electro-osmophoresis was successful in reducing the time required for the separation of enterotoxin A and the formation of a specific immuno-precipitate. It was possible by this method to assay enterotoxin A at a concentration of 0.5 μg/ml in five hours.

Inhibitor studies were conducted on enterotoxin A and B

production by Staphylococcus aureus 265-1 and 14458, respectively. The elaboration of enterotoxin A and B was inhibited by streptomycin sulfate, NaF, chloramphenicol, KCl and bacitracin. Inhibition by KCl and NaF was reversed by Mg^{++}. Penicillin G inhibited toxin production by S. aureus 14458 but not cell growth, whereas both toxin elaboration and growth were inhibited in studies with S. aureus 265-1.

Enterotoxin B produced by S. aureus 14458 appeared during the latter part of the exponential growth phase. Nonreplicating cells at a concentration of 8×10^{10}/ml were found capable of producing toxin in the presence of glucose and water alone. Chloramphenicol did not inhibit toxin formation in nonreplicating cells. Data suggested the existence of a toxin precursor pool and that toxin excretion may be an adjustment to static growth conditions.

Improved Detection Methods and Physiological Studies of Staphylococcal Enterotoxin

by

Charles Edward Kimble

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Redacted for Privacy

Professor of Microbiology

in charge of major

Redacted for Privacy

Head of the Department of Microbiology

Redacted for Privacy

Dean of the Graduate School

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IMPROVED DETECTION METHODS AND PHYSIOLOGICAL STUDIES OF STAPHYLOCOCCAL ENTEROTOXIN

INTRODUCTION

The accepted method for the detection of staphylococcal enterotoxin involves the use of carboxymethylcellulose or Sephadex semi-purification column chromatography followed by immuno-diffusion techniques. Although reliable, this method is time consuming and does not lend itself to mass screening tests which are sometimes necessary in the food processing industry. The primary purpose of this investigation, then, was to develop new or to improve existing methods for the detection of staphylococcal enterotoxin.

The problems involved are essentially extraction, concentration, partial purification of the enterotoxin and the application of techniques which are specific and sensitive to detect the presence and type of enterotoxin. It was sought by the use of selective ion-exchange resins, isoelectric focusing and semi-purification electrophoresis to eliminate the initial steps used in toxin detection. Electrosyneresis or electro-osmophoresis was explored as a possible alternative method for shortening the time required for the formation of specific immunoprecipitates when antigen and antibody are reacted.

Several non-specific tests have been correlated with toxin production. Chief among these is the coagulase test which is used as

a rapid method for the identification of toxigenic staphylococci. However, all coagulase-positive staphylococci do not produce enterotoxin. Therefore, the recovery of coagulase-positive Staphylococcus aureus from food implicated in a food poisoning outbreak is only presumptive evidence that the correct etiological agent has been found. Even the determination by bio-assay feeding tests that the strain isolated is capable of producing enterotoxin does not prove that it did so in the food. Conclusive proof depends on demonstration of the enterotoxin in the food itself. Moreover, the enterotoxin is characteristically stable toward heat. The cooking usually given most foods will not destroy the toxin formed therein prior to the heat process. Such foods might cause poisoning, although no live staphylococci, coagulase-positive or otherwise, are demonstrable.

Although the purification, detection and structure of enterotoxin produced by S. aureus have been the subject of detailed studies, very little is known about factors that control toxin synthesis and physiology. A second purpose of this investigation was to elucidate the factors that influence toxin formation of this important pathogenic microorganism.

LITERATURE REVIEW

Enterotoxin Detection

The etiological agent responsible for the most common type of food poisoning in the United States is staphylococcal enterotoxin. It is produced in foods or in simplified laboratory media by certain strains of S. aureus. The toxin is termed an enterotoxin because it causes gastroenteritis or inflammation of the lining of the stomach and intestines. The mode of action of enterotoxin--whether on smooth muscle of the alimentary tract, or peripheral sensory structures or directly on the emetic receptor site--is controversial and remains to be determined. However, Palmer (1951) has shown that at least part of the symptoms in man may result from a direct action of the toxin on the gastric mucosa. A characteristic feature of staphylococcal food poisoning is the relatively short period of incubation. The symptoms generally appear within two to four hours after the contaminated food is eaten, in contrast with incubation periods of 12 to 24 hours or more which are typical of botulism or of salmonella food infection. A brief period of nausea is followed by sudden paroxysms of vomiting accompanied by abdominal cramps, severe diarrhea and prostration. The symptoms persist several hours; the patient usually feels quite normal 24 hours after the beginning of the attack, although

weakness or nausea may persist for a day or two. Fatalities are rare and are probably caused by an actual tissue invasion by the staphylococci rather than by the enterotoxin alone.

Staphylococci are common organisms found in the throats of individuals, on their skin as a causative agent of pimples, boils and carbuncles and in great abundance in the postnasal drip of patients recovering from colds. The ubiquity of staphylococci and the lack of any evidence pointing to a specifically limited distribution of enterotoxigenic strains; the great variety of foodstuffs in which the enterotoxin is known to have been elaborated; and the prevailing ignorance and apathy with respect to the prevention of air, milk, finger, droplet or fly-borne contamination of foodstuffs, make a high incidence of staphylococcal food poisoning almost inevitable.

The relation of staphylococci to the intoxication type of food poisoning was indicated by a number of observations. Notably, Barber (1914) observed acute gastrointestinal upsets associated with milk obtained from a cow with staphylococcal mastitis. In 1930 Dack et al. reported finding a yellow staphylococcus in samples from a Christmas cake, which had caused acute gastroenteritis in ten persons in Chicago in 1929. Bacteria-free filtrates produced by growing the organisms from two to three days at 37 C produced violent gastrointestinal symptoms when fed to human volunteers in quantities of from two to ten milliliters. The same year Jordan (1930) reported

an outbreak of acute food poisoning affecting four people in Puerto Rico. From a sample of cheese sent to him he isolated yellow staphylococci. Broth filtrates produced typical gastrointestinal symptoms when fed to three human volunteers. In 1931 Jordan and Hall reported another instance in which two persons in the Panama Canal Zone were made ill and in which case staphylococci were isolated from chicken gravy. Broth filtrates likewise produced typical symptoms in several human volunteers.

Few attempts were made in the period 1931-1950 to ascertain the nature of the toxic agent. The investigators disagreed as to whether the enterotoxin was a carbohydrate or a protein (Davison, 1940; Hammon, 1941; Minett, 1938). In 1948 a long-range investigation was undertaken at the Food Research Institute, then associated with the University of Chicago, to elucidate the properties of the enterotoxin. It was assumed that the causative agent of staphylococcus intoxication was a single substance in the preliminary investigations at the Food Research Institute. Only after the purification of a protein responsible for the illness induced in monkeys was the existence of more than one enterotoxin discovered.

The major problem confronting investigators was the lack of a practical quantitative assay. Several species of animals, including cats (Dolman, Wilson and Cockcroft, 1936), monkeys (Jordan and McBroom, 1931; Surgalla, Bergdoll and Dack, 1953), frogs (Eddy,

1951; Robinton, 1949), dogs (Dolman et al., 1936) and pigs (Hopkins and Poland, 1942) have been used to determine the biological activity of the enterotoxins. Animals other than monkeys are relatively insensitive to the enterotoxins unless they are injected intraperitoneally or intravenously. The methods employed most frequently are intraperitoneal or intravenous injections of cats and kittens (Dolman et al., 1936; Hammon, 1941) and feeding of young rhesus monkeys (Surgalla et al., 1953). However, it is necessary to inactivate substances that may provoke symptoms similar to those caused by enterotoxin when administed by parenteral routes if the cat method is used. Heating at 100 C for 20 to 30 minutes (Davison, Dack and Cary, 1938; Dolman et al., 1936) treatment with trypsin (Denny and Bohrer, 1963) and use of antisera (Dolman, 1943) to inactivate the interfering substances have been used with success. One cannot be sure, however, that these methods inactivate all interfering substances in every instance. Another disadvantage in using the cat method is that cats are relatively insensitive to enterotoxin C. Approximately 50 times more enterotoxin C than enterotoxins A or B is required to produce emesis in cats (Casman, Bennet and Kephart, 1966). The most reliable bioassay for the enterotoxins is the feeding of young rhesus monkeys. Of the toxic moieties produced by the staphylococci, only the enterotoxins cause emesis in these animals. Assays are made by administering the enterotoxins in solution (usually 50 ml) to young

monkeys (two to three kilograms) by catheter (Surgalla et al., 1953). The animals are observed for five hours after feeding; vomiting is accepted as positive reaction for enterotoxin. To determine the presence of enterotoxin in a given sample, six monkeys are used and emesis in at least two animals is considered a positive reaction. When this method is used, direct evidence is obtained; but the cost of monkeys and the expense of their upkeep, as well as the individual variations in susceptibility and the aforementioned possibility of developing immunity, lessen their value for routine use.

The report by Dolman and Wilson (1938) of what appeared to be a specific antibody for the staphylococcal enterotoxin suggested that the problem of detection might be solved by serological methods. In 1947 the Division of Microbiology of the Food and Drug Administration (Washington, D. C.) initiated a long-range program to develop serological methods for enterotoxin detection. It was necessary to demonstrate the antigenicity of the enterotoxin, to determine the number of antigenic types, to make available for use the specific antitoxins and to develop methods for the serological detection of the enterotoxins in foods.

Casman (1958, 1960) reported studies in which he demonstrated: A) the antigenicity of enterotoxin by conferring to cats a passive immunity to enterotoxin with the use of antiserum produced in rabbits; B) the occurrence of two serological types of heat-resistant

enterotoxin, of which only one (type A) appeared to be associated with food poisoning; C) the production by certain staphylococci of other apparently heat-labile emetic substances; and D) the use of the gel double-diffusion test for the detection of enterotoxin. The development of a serological test for enterotoxin A (Casman, Bergdoll and Robinson, 1963) made possible the detection of this common type of enterotoxin in foods. Casman, McCoy and Brandly (1963) demonstrated the elaboration of enterotoxin A in meat after relatively luxuriant growth of an enterotoxigenic staphylococcus. The antigen-antibody reaction may not necessarily indicate biological activity; however, in most instances, correlation between the two is adequate to justify using the immunological reaction in assaying for enterotoxin. This method of identification was used by Casman et al. (1963) to establish a nomenclature for the enterotoxins, designating them enterotoxins A, B, C, D et cetera. To date, four enterotoxins have been definitely identified (Bergdoll, Surgalla and Dack, 1959; Bergdoll, Borja and Avena, 1965; Casman, 1960; Casman et al., 1968). All of the enterotoxins discovered so far have been implicated in food poisoning outbreaks; however, enterotoxin A is the most frequently identified with food poisoning. Enterotoxin B, although occasionally involved in food poisoning, has been associated most frequently with staphylococcus strains isolated in connection with other human ailments such as enteritis. Surgalla and Dack (1955)

reported that of 32 strains isolated from patients suffering with enteritis 30 produced enterotoxin, and 26 of these produced enterotoxin B. Enterotoxin C is produced by a number of strains isolated from foods implicated in food poisoning outbreaks (Bergdoll et al., 1965). Enterotoxin D is associated with strains implicated in food poisoning; however, many of these strains also produce enterotoxins A, B or C (Casman et al., 1966; Casman et al., 1968).

In the course of the investigations in the Food Research Institute laboratories, two enterotoxin C's (from separate S. aureus strains) were purified. Although they appear to be identical in most respects, their movements in an electrical field are quite different. Enterotoxin C (strain 137) has an isoelectric point of pH 8.6 and enterotoxin C (strain 361) has an isoelectric point at pH 7.0. Enterotoxin C (strain 137) will be referred to as C_1 and enterotoxin C (strain 361) will be referred to as C_2. A summary of the chemical and physical properties of the known enterotoxins is shown in Table 1.

Significant progress has been made in the methodology for detecting staphylococcal enterotoxins. Development of gel-diffusion techniques for the quantitative detection of enterotoxin marked a significant improvement over the biological test (feeding monkeys or injecting cats with toxic filtrates) previously employed. The single diffusion tube test (Oudin test) has been quite useful for detection and quantitation of enterotoxins (Hall, Angelotti and Lewis, 1963;

Table 1. Properties of staphylococcal enterotoxins

	Enterotoxin				
	A[1]	B[2]	C_1[3]	C_2[3]	D[4]
Molecular Weight	34,700	35,300	34,100	34,000	---
Partial Specific Volume	0.726	0.743	0.732	0.742	---
Nitrogen Content (%)	16.5	16.1	16.2	16.0	---
Sedimentation Coefficient (S^{o}_{20}, W), S	3.04	2.89	3.00	2.90	---
Diffusion Coefficient (D^{o}_{20}, W) X 10^{-7} cm^2 sec^{-1}	7.94	7.72	8.10	8.10	---
Reduced Viscosity (ml/g)	4.07	3.92	3.4	3.7	---
Isoelectric Point	6.8	8.6	8.6	7.0	---
Maximum Absorption (mμ)	277	277	277	277	277
Extinction ($E^{1\%}_{1\ cm}$)	14.3	14.0	12.1	12.1	---
N-terminal Amino Acid	Alanine	Glutamic Acid	Glutamic Acid	Glycine	---
C-terminal amino acid	Serine	Lysine	Glutamic Acid	Glycine	
Emetic Dose (ED_{50}) (monkey; μg/animal)	5	5	5	5-10	1.5 μg (cats)

[1] Chu et al. (1966)

[2] Schantz et al. (1965)

[3] Bergdoll, Barja and Avena (1965)

[4] Casmen et al. (1967)

Weirether et al., 1966). The limit of sensitivity of the Oudin test is approximately one μg of enterotoxin per ml. As little as 0.1 μg of enterotoxin per ml (Hall, Angelotti and Lewis, 1965) can be detected by the double-diffusion tube test (Oakley test). Unfortunately, as much as a one week incubation period is required to detect enterotoxin at this concentration. The micro-Ouchterlony slide test (Crowle, 1958) has also been applied successfully to the detection of enterotoxin (Casman and Bennett, 1965). It is more difficult to perform than the Oakley and Oudin tests, but is advantageous for determining relationships between various enterotoxin cultures. A 24 to 48 hour incubation period is required.

Of the staphylococci, type B strains usually produce large amounts of toxin in broth cultures (over 100 μg/ml) whereas type A strains produce small amounts. Casman and Bennett (1965) reported that with 15-20 billion bacterial cells per ml the production of type A toxin was approximately two to four μg per ml of aerated brain-heart infusion (BHI) broth. Since foods implicated in food poisoning rarely have such a great number of staphylococci, much less toxin would be present. Therefore, the demonstration of enterotoxin in foods involves three basic problems: A) extraction, B) concentration to detectable quantities and C) application of techniques which are specific and sensitive to detect the presence and type of enterotoxin. Experiments have been conducted by adding known amounts of toxin

to foods and then recovering them by extraction. Reported recoveries are variable. Hall et al. (1965) reported recovering 75% of enterotoxin B and 48% enterotoxin A; Casman and Bennett (1965) reported recovering 48 to 72% of type B and 68% type A. Ion-exchange chromatography and gel filtration are the accepted procedures for selective separation of enterotoxins from broth cultures or food extracts, which are concentrated to a level high enough to be within the sensitivity limit of the gel diffusion tests. The extraction, concentration and partial purification procedures are tedious, time consuming and do not lend themselves to mass screening tests which are sometimes necessary in the food processing industry. In addition, at least 20 hr are necessary for the detection of toxins when their concentration is in the area of one μg per ml of broth or food extract.

Recently, immunofluorescence has been applied to the detection of cell-associated enterotoxin B (Friedman and White, 1965) and to the detection of enterotoxin B in culture media and in foods. Presumably, the procedures for the detection of enterotoxin B in cultures and foods are based on the formation of highly fluorescent immune precipitates. According to Genigeorgis and Sadler (1966a) the detection of immune precipitates, otherwise undetectable, is made possible through fluorescence. The fluorescent antibody technique (FAT) has two major shortcomings: A) the fact that non-enterotoxigenic cells fluoresce in the presence of conjugated

enterotoxin B antiserum and B) non-specific staining caused by food protein when the method is applied to actual food samples (Genigiorgis and Sadler, 1966b).

An indirect hemagglutination-inhibition procedure has been developed (Robinson and Thatcher, 1965; Brown and Brown, 1965) and a flotation system is reported which can detect enterotoxin B at concentrations of 1 μg/ml in 2-3 hr (Hopper, 1963). The first method is subject to problems associated with the presence of potent hemagglutinins for sheep erythrocytes in the staphylococcal culture fluids examined. Both methods still need more refined techniques of extractions from foods. Recently, Silverman, Knott and Howard (1968) reported development of a rapid reversed passive hemagglutination assay. As described by the authors the sensitivity of the method would be of significant value in estimating small concentrations of toxin, such as might be found in food samples obtained from a food-poisoning event. There is considerable difficulty in end point determination and excess salts must be dialyzed out (Silverman, et al., 1968).

It is clear from the above that there has been significant progress in the methodology of enterotoxin detection. One of the primary objectives of this research project was to apply new techniques and improve existing methods for identification of staphylococcal enterotoxin.

Physiological Aspects of Enterotoxin Production

The literature on staphylococcal enterotoxin detection and chemistry is voluminous. Surprisingly enough, however, little has been done to elucidate the physiological role of enterotoxin with respect to the bacterium. Fluorescent antibody studies by Genigeorgis and Sadler (1966) and Friedman and White (1965) suggested that enterotoxin B was cell surface-associated. This was consistent with the view of Hartman and Goodgal (1959) that enterotoxin had the characteristics of a cell surface constituent of bacteria. Friedman (1968) suggested that the cell surface may contain sites involved in the synthesis of enterotoxin B. MacLean, Lilly and Alford (1968) reported that enterotoxin B production, but not cell growth, could be inhibited by growing the bacteria either at a low temperature or in a high salt medium. They showed that maximum toxin production by S. aureus ATCC (American Type Culture Collection) 14458 occurred at the beginning of the stationary phase of growth. Markus and Silverman (1969) in studies on the growth kinetics of S. aureus S-6 have shown that 95% of the toxin was released during the latter part of the exponential phase of growth and at no time was intracellular toxin detectable.

MATERIALS AND METHODS

Staphylococcal Strains

The following staphylococcal strains were used in this study:

Strain	Type of Enterotoxin	Source
13656	A	ATCC
14458	B	ATCC
19095	C_1	ATCC
23235	D	ATCC
265-1	A	FDA
D263	A and B	FDA

Reference Enterotoxins and Antisera

Source

Enterotoxins A and B and antisera containing the specific antibodies, prepared and purified by Casman, were obtained from the Food and Drug Administration (Washington, D. C.). These reagents had been serologically balanced by block titration and when diluted as directed produced a reference line of precipitation midway between the two wells. The reference toxins and antitoxins were kept in a refrigerator and diluted prior to use. Antiserum to enterotoxin C_1 was obtained from M. S. Bergdoll, Food Research Institute, University of Wisconsin, Madison. It was monospecific at a dilution of 1:50 in slide Ouchterlony gel diffusion (Crowle, 1958).

Enterotoxin Assay

A slide modification (Casman, 1967) of the Ouchterlony (1953) double-diffusion method was used to detect enterotoxin. The modification consisted of incorporation of 0.8% sodium barbital (pH 7.4), 0.85% NaCl and 0.01% merthiolate in a gel prepared with 1.2% Noble Special Agar (Difco). The agar solution was boiled, filtered with suction while hot through a double layer of Whatman no. 1 filter paper, and stored in 50 ml portions until used. Plastic squares with a distance between the centers of the central and peripheral wells of 4.5 instead of 4.0 mm were used. In this test the antibody and enterotoxin combine to form a line of precipitation in a thin layer of agar gel between a microscope slide and a square of Plexiglas containing funnel-shaped holes into which the reactants are introduced. The line of precipitation formed by the reaction of a stock enterotoxin solution and its corresponding antibody is called the reference line. The presence of enterotoxin in the material under test is verified by the coalescence of its line of precipitation with the reference line.

Production of Enterotoxin A

A significant amount of pure enterotoxin was required for definitive chemical and physiological investigations, as well as for the production of specific antienterotoxin sera. Staphylococcus

aureus strain 265-1 was selected for the production of enterotoxin A.

Cells were grown in 30-liter quantities in a Fermacell Fermentor (New Brunswick Scientific). The medium, 3.7% brain heart infusion broth (BHI, Baltimore Biological Laboratories), was adjusted to pH 6.0 before sterilization. Air was bubbled through the culture at a rate of one cubic foot per minute for 24 hr at 37 C. Two separately autoclaved 20 ml quantities of Antifoam (Dow Corning Corporation, Midland, Michigan) were added: the first before adding the inoculum and the second after 12-hr aeration of the culture. The inoculum was prepared in six 250-ml Erlenmeyer flasks each containing 50 ml of medium as described above. They were inoculated with 0.4 ml each of an aqueous suspension of a 24-hr nutrient agar slant culture containing 2×10^9 organisms per milliliter and were incubated for 12 hr at 37 C on a gyratory shaker operated at approximately 170 rev/min. The cells were separated by the use of a cooled, closed Sharples Super Centrifuge (model T-1P) with a standard clarifier bowl. Cells were washed three times with 0.85% NaCl and stored in a paste at -20 C.

The purification of enterotoxin A was accomplished by chromatographic and flat-gel electrophoretic methods. The supernatant was concentrated 20-fold by dialysis against 30% aqueous polyethylene glycol (PEG, Van Waters & Rogers) molecular weight 4000; it was then dialyzed 24 hr against running tap water at 4 C. The dialyzed crude

enterotoxin solution was adjusted to pH 5.6 with H_3PO_4 and centrifuged to remove insoluble material. Five hundred milliliters of the concentrated bacterial culture supernatant containing approximately 1.2 g of protein was transferred to a column of CM-cellulose (2.5 x 50 cm) which had been equilibrated with 0.01 M sodium phosphate at pH 5.6. The column was washed with the equilibration buffer until the optical density of the percolate was near zero at 280 mμ. The enterotoxin was then eluted with 0.2 M Na_2HPO_4. The fractions from the major peak were pooled, dialyzed for 5 hr at 4 C and lyophilized.

The protein concentration was estimated by measuring the optical density at 280 mμ with a Cary Model 11 recording spectrophotometer. The concentration of enterotoxin A in the various preparations was determined by a modification of the single gel diffusion technique (Bergdoll et al., 1965). In this technique an agar column containing the enterotoxin antiserum is layered with the enterotoxin solution (10-200 μg/ml). The front of the enterotoxin-antienterotoxin precipitin band formed in the antiserum-agar column moves down the column at a rate corresponding to the concentration of the enterotoxin and the concentration of the antibody. The distance that the band moves in a given time is measured and the enterotoxin concentration (micrograms per milliliter) is calculated from a standard curve obtained by plotting the log of the enterotoxin concentrations against the distance the enterotoxin-antienterotoxin band moved in millimeters into the

agar column in five days. The gel double-diffusion method of Oakley and Fulthorpe (1953) was used to follow the progress of the purification of the enterotoxin.

The lyophilized enterotoxin was dissolved in 40 ml of distilled water and dialyzed against four liters of distilled water at 4 C overnight. This solution was then adjusted to pH 5.8 and passed into a CM-cellulose column (2.5 x 40 cm) equilibrated with 0.01 M sodium phosphate, pH 5.8. The column was washed with the equilibration buffer until the optical density of the effluent was zero. The enterotoxin was removed by gradient elution with phosphate buffer from 0.01 M at pH 5.8 to 0.05 M at pH 6.6. The yield was 70% with a purity of 65%. The dialyzed lyophilized material was then further purified by polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis

Disc electrophoresis was performed by the methods of Davis (1964) and Ornstein (1964). Flat-gel electrophoresis was carried out in a device developed in our laboratory (Figure 1) as described by Levant (1969). Its design permits efficient water cooling. Multiple sample analyses may be carried out under the same conditions.

The gel is a linear polymer of acrylamide cross-linked at intervals by methylene bridges. The pore size of the gel can be controlled by the proportion of acrylamide and polymerizing agents used

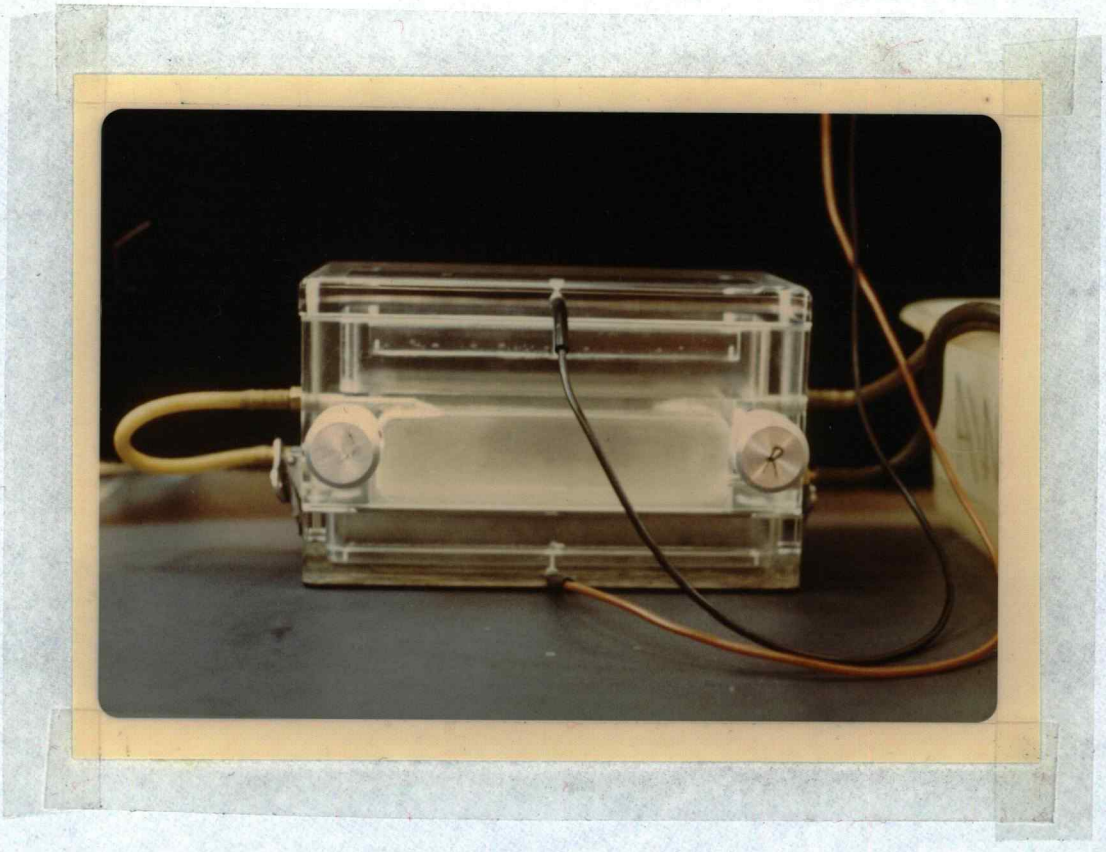


Figure 1. Flat-gel electrophoresis apparatus constructed of Lucite plastic.

to make up the gel. Acrylamide gels, then, fall into the classification of molecular sieves and like starch, separation of proteins is based not only upon electrophoretic mobility of the protein but also upon the size and shape of the protein molecule. Acrylamides produce protein patterns very similar to those obtained with starch, but possess, in addition, several inherent characteristics that make them more desirable than starch. Polyacrylamide gels: A) are less fragile and technically easier to handle, B) are more easily varied in concentration, permitting a range of pore sizes and making the sieving property more effective, C) have a clear, colorless background after destaining, making them easier to quantitate and D) have better keeping qualities after gel formation and can be stored for long periods of time submerged in buffer. The basic system is formed in these layers: lower gel (small-pore gel) which is separated from the sample by a spacer gel (large-pore gel) and the upper gel which is applied over the sample-gel mixture. In this system, the proteins are first separated according to their mobility into highly concentrated zones of protein in the spacer gel. When they enter the small-pore gel, further separation takes place based upon size of the molecules. The top layer of large-pore gel acts to prevent back diffusion of samples into the buffer reservoir. The system gives rise to extremely sharp separation of sample protein. It is quite suitable for small amounts in dilute solutions.

Purification of Enterotoxin B

Enterotoxin B was purified by a modification of the method of Schantz et al. (1965). The ion exchange resin Amberlite IRP-64 (Rohm and Haas, Philadelphia) was used instead of the CG-50 resin those investigators used. Briefly, the method was as follows: A) adsorption of the toxin on the cation exchange resin Amberlite IRP-64 from the culture supernate diluted with two volumes of water and adjusted to pH 6.4 and subsequent elution with 0.5 M sodium phosphate at pH 6.8 in 0.25 M NaCl; B) adsorption on Amberlite IRP-64 (equilibrated with 0.05 M sodium phosphate at pH 6.8) from the dialyzed eluate from step A and subsequent elution with a linear gradient phosphate buffer from 0.02 M at pH 6.2 to 0.07 M at pH 6.8; D) dialysis to reduce the buffer salts to less than 4% of the protein concentration, centrifugation, lyophilization; and E) further purification with polyacrylamide gel electrophoresis with the basic pH gel system (Davis, 1964). Gels were stained by the method of Chrambach et al. (1966).

Preparation of Anti-Enterotoxin A

After a base bleeding a goat was immunized with purified enterotoxin A from strain 265-1. Freund's Complete Adjuvant (1:1, Difco) was used with all injections. Tolerance to the enterotoxin was

established by injecting the goat first with small quantities. The animal was injected intraperitoneally with the following amounts of enterotoxin (in micrograms) at seven day intervals: 2, 10, 200, 500, 2000, 0 and 5000. Serum samples of approximately 150 ml each were obtained during the immunization and were examined for the presence of antibody for the homologous enterotoxin and for nonenterotoxic staphylococcal antigens. When a sufficiently high antibody titer (1:200) was obtained the gamma$_2$ globulin fraction was separated by a procedure involving three ethanol precipitations (Nichol and Deutsch, 1948), all carried out at 0 C or below. The procedure is outlined in Figure 2.

Detection

Two-Step Purification and Detection Method

The efficacy of a two-step process to identify and quantitate enterotoxin was evaluated. This process consists of semi-purification polyacrylamide electrophoresis followed by gel diffusion tests. Both basic and acidic acrylamide gels (Davis, 1964; Reisfeld, Lewis and Williams, 1962) were used.

Recovery experiments were conducted on cheddar cheese, ground beef, banana cream pie and chicken pot pie obtained from commercial sources. In the standard procedure the food slurry

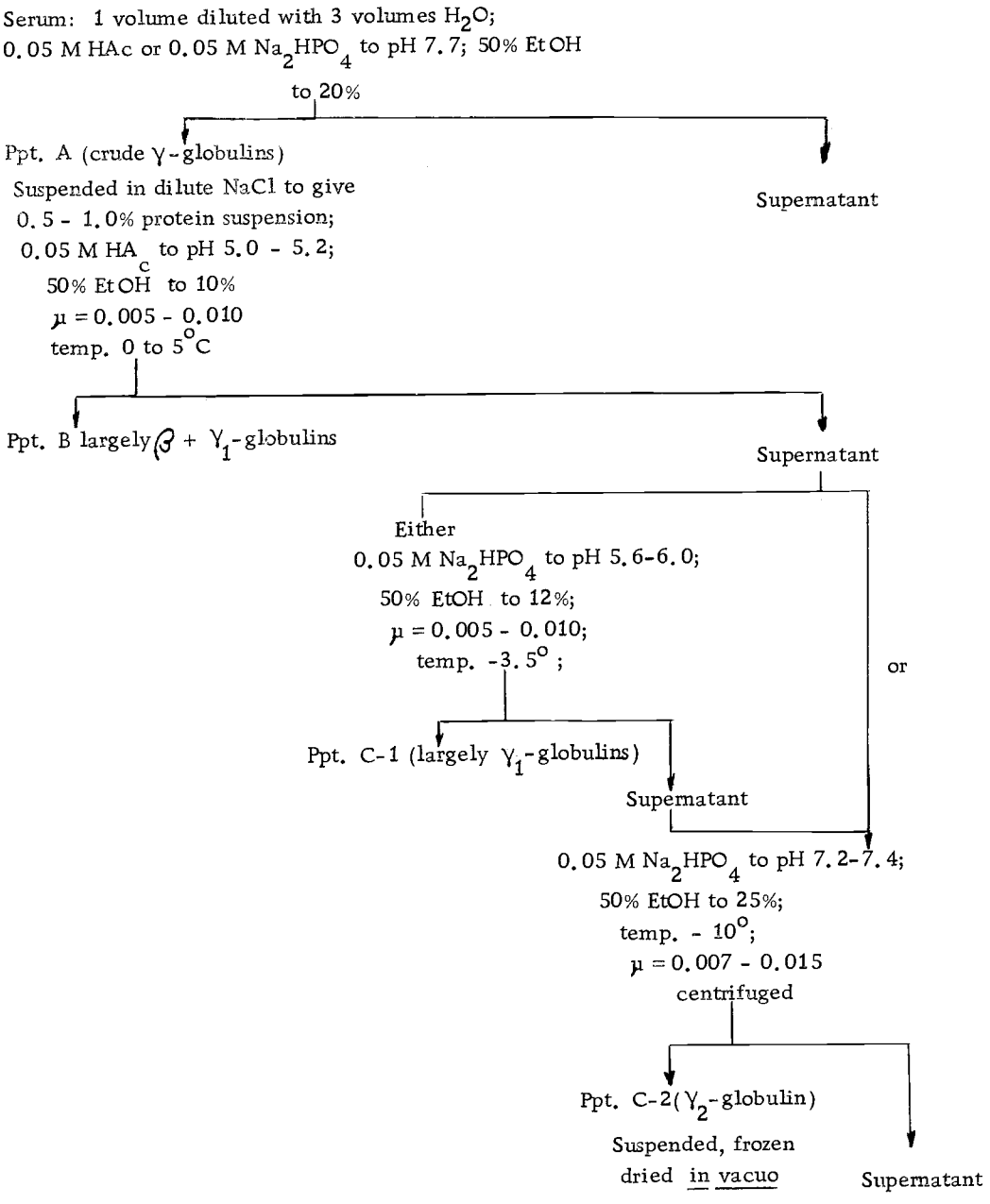


Figure 2. Fractionation scheme to isolation of $γ_2$-globulin from goat serum .

was prepared by homogenizing 20 g of food with 40 ml of 0.02 M phosphate buffer, pH 6.4, in a Waring Blendor for three minutes. The slurry was then poured into plastic centrifuge tubes, placed in a 50 C water bath for 20 minutes and upon removal allowed to remain at room temperature for an additional 20 minutes. This treatment accelerated the precipitation of certain insoluble phosphates that form in food slurries. The tubes were then centrifuged at 13,000 x g for 25 minutes in a Servall centrifuge at 4 C. The supernatant fluid was poured into a flask through a layer of cheese cloth which removed the fat particles. An aliquot was removed and tested immediately, using the Oudin, Oakley and micro-Ouchterlony procedures. The remaining supernatant fluid was diluted 1:3 and subjected to CM-cellulose chromatography (Casman and Bennett, 1965). The eluate was then concentrated by dialysis against 30% PEG and again examined by gel-diffusion techniques. The modified procedure differed from the above in that after removal of the lipid material the fluid was dialyzed against 30% PEG 4000 and submitted directly to polyacrylamide gel electrophoresis. After electrophoresis the gel was sliced and a section stained (Chrambach et al., 1966). An unstained section was taken and the area containing enterotoxin cut out. A pure enterotoxin sample was run along with the food sample as a reference. The enterotoxin-laden gel section was then macerated with a small volume of 0.85% NaCl, the liquid centrifuged and

subjected to serological tests. Alternatively, the sliced gel was placed on a slide, overlaid with agar and subjected to serological tests against highly specific anti-enterotoxin serum.

Isoelectric Focusing

The feasibility of using gel electrofocusing to fractionate and concentrate enterotoxin from both food and culture supernatants was examined. The technique of gel electrofocusing has been described by Wrigley (1968). There is a distinct difference between gel electrofocusing and gel electrophoresis. In the process of gel electrophoresis, proteins applied as a zone move continuously through the gel and are separated on the basis of their charge and molecular size. Spreading of the zones due to diffusion continues throughout the run. In contrast, with gel electrofocusing proteins are concentrated from any part of the gel to the portion of the pH gradient at which each is isoelectric. A suitable pH gradient can be produced by applying a voltage to a mixture of ampholytes, possessing a range of isoelectric points, in a solution stabilized against convective disturbances. The components remain stationary as long as the pH gradient is maintained constant. Spreading caused by diffusion is reduced. In electrofocusing the gel serves to stabilize the pH gradient and does not act as a molecular sieve. Fractionation of a protein which has a known isoelectric point is enhanced by using ampholytes over a narrower

pH range.

The method is adaptable to both the apparatus for disc electrophoresis (Davis, 1964) and flat-gel electrophoresis (Levant, 1969). Solutions necessary for preparing the electrofocusing gels are shown in Table 2. The anodic and cathodic vessels were filled with 0.2% sulfuric acid and 0.4% triethanolamine, respectively. A current of 2 ma per tube was used with the disc apparatus and 10 ma was used with the flat-gel apparatus. Four hours were generally required for satisfactory electrofocusing.

For concentrated protein samples, the protein in 10% sucrose was applied to the top of the gel and under a protecting layer of carrier ampholyte solution (see Table 2).

Table 2. Stock solutions for electrofocusing

Catalyst Solution		Acrylamide Solution	
N, N, N', N' - tetramethylethylenediamine	1.0 ml	N, N'-methylene bisacrylamide	0.8 g
		Acrylamide	30 g
Distilled Water	to 100 ml	Distilled Water	to 100 ml

Gel Mixtures

Concentrated Gel Mixture		Gel Mixture for One Tube	
Catalyst Solution	0.8 ml	Concentrated Gel Mixture	0.5 ml
Acrylamide Solution	3.0 ml	Protein Sample	30-300 µg
		Potassium persulfate	1 mg
Carrier Ampholytes (40%, pH 7-10; LKB)	0.3 ml	Distilled water	to 1.5 ml

Alternate Loading Procedure

Ampholyte Layer		Sample Layer (apply 0.05 ml)	
Carrier ampholytes (40%)	0.05 ml	Protein sample	1-5 mg
sucrose	100 mg	Sucrose	100 mg
Distilled water	to 2.0 ml	Distilled water	1.0 ml

Electro-osmophoresis

The identification of antigens by means of immunoelectrophoresis or gel diffusion requires a significant period of time. In the course of this investigation it was sought to develop means to shorten the time involved yet retain or increase the sensitivity. One method investigated was electro-osmophoresis. With this method both the antigen mixture to be investigated and the antiserum were simultaneously submitted to electrophoresis. If there is a positve reaction, visible precipitation bands are formed. The analysis lasts approximately one hour. In addition to saving a great deal of time, this method requires very small amounts of antiserum and antigen sample. The method has been described by Macheboeuf et al. (1953) and Bussard (1959). The former used paper electrophoresis and the latter agar. Nakamura (1966) has recently reviewed the literature on cross electrophoretic techniques.

Antibodies are localized in the γ-globulin fraction of the antiserum. This fraction exhibits little electrophoretic mobility in a weakly alkaline solution. During electrophoresis in agar gel, a strong electroendo-osmosis occurs which causes a flow to the cathode, while the proteins with greater mobility such as albumins and α-globulins, move toward the anode. Therefore, if two slots are made in the agar gel and the slot on the anode side is filled with antiserum

and the cathode with antigen solution, during electrophoresis the antibody fraction of the antiserum and the fast moving antigens from the antigen solution come into contact between the two slots. If the reaction is positive, precipitation takes place at that point.

The slides were cleaned with a weak detergent, rinsed three times in distilled water, then acetone and air-dried. Three ml of a solution of 1.5% agar (Noble, Difco) in Veronal buffer (17.0 gm sodium veronal, 23 ml 1N HCl per liter, pH 8.4) were added to each slide. When the agar had set, slots or punch holes were cut in it. The slots were dried by inserting a piece of filter paper. Then the preparations under investigation and the antiserum were placed in the slots or holes. Forty µl of solution was needed for a 2-cm slot and approximately 5 µl of solution for a punch hole. The slots for the antigens were 4 mm long and for the antiserum 20 mm; they were approximately 0.5 mm wide. The punch holes were 1.5 mm in diameter. The slides were placed in the electrophoresis apparatus, covered with pentane and subjected to electrophoresis as described by Wieme (1965). Electrophoresis was conducted with the slides upside down, supported at each end by blocks of agar or filter paper strips which were in contact with the electrode compartments. The samples were electrophoresed at 15 volts/cm for 30 minutes. The slides were then examined under a microscope.

Physiological Studies

The Effect of Inhibitors

The effect of nutritional inhibitors on the formation of enterotoxins A and B was studied. Experiments were also conducted to test the ability of S. aureus to produce enterotoxin in the presence of compounds known to affect bacterial cell walls.

Cultural Conditions. S. aureus strains 265-1 and 14458 were used for the inhibitor studies. Stock cultures, maintained on Trypticase Soy Agar (BBL), were stored at 2 C and transferred every three weeks. Broth cultures were grown in a medium described by Rosenwald and Lincoln (1966). It was composed of 4.0% N-Z-Amine type A (Sheffield Chemical, Norwich, N.Y.), 0.4% yeast extract (Difco) and 0.1% K_2HPO_4 and distilled water. The medium was adjusted initially to pH 7.0. Streptomycin, chloramphenicol, oleic acid, penicillin and bacitracin were prepared as sterile solutions and added aseptically to the autoclaved medium. The remaining compounds were added with the other medium constituents and autoclaved.

Cells for the preparation of inocula were grown at 37 C for 9 hr on Trypticase Soy Agar slants, removed with sterile distilled water and adjusted to an optical density of 0.20 (650 mμ). This represented approximately 1×10^8 cells/ml. This suspension (0.1 ml)

was inoculated into 50 ml of medium in a 250-ml Erlenmeyer flask. Incubation was for 18 hr at 37 C on a gyratory shaker. After 18 hr, when the cultures had reached the stationary phase, the contents of triplicate flasks were pooled and turbidity measurements made at 650 mμ on samples diluted 10-fold in fresh medium. Fresh medium was also used as a blank. A supernatant solution collected by centrifugation of the culture at 5,000 x g for 20 min then was subjected to serological assay for enterotoxins (Bergdoll et al., 1965; Oakley and Fulthorpe, 1953).

Chemicals. The following chemicals and reagents were used: penicillin G (Charles Pfizer & Co., New York), streptomycin sulfate (Consolidated Midland Corporation, Katonah, N. Y.), bacitracin (Calbiochem, Los Angeles) and chloramphenicol (Sigma Chemical Co., St. Louis). Sodium deoxycholate, oleic acid, KCl and NaF were of reagent grade.

Production of Enterotoxin B by Nonreplicating Cells of S. aureus

Enterotoxin B production by S. aureus ATCC 14458 was studied by the use of replacement cultures. The bacteria were cultured for 16-20 hr in the medium described by Rosenwald and Lincoln (1966), medium A. The cells were harvested, washed three times in 0.01 M phosphate buffer at pH 7.0, resuspended in fresh medium A at a

concentration of 8 X 10^{10} cells/ml and incubated at 37 C for 4-6 hr. The toxin was then assayed by the single gel-diffusion method (Weirether et al., 1966) using the appropriate reference curves for toxin assay in the presence of electrolytes. Toxin release by stationary phase cells was also examined in a nitrogen-free medium described by Markus and Silverman (1969). This medium contained 3.5% K_2HPO_4 and 0.2% glucose and was designated medium B. The effect of inhibitors on toxin release was examined.

RESULTS AND DISCUSSION

Highly purified enterotoxin A was necessary for toxin recovery experiments and the preparation of specific antiserum. Based on the initial culture supernatant content, the final yield of enterotoxin A was 50% with a purity of 95%. A comparison of the partially purified enterotoxin A after the first CM-cellulose step and the final preparation is shown in Figure 3. Toxin purification was also followed by a slide modification (Casman, 1967) of the double-diffusion method (Ouchterlony, 1953) as seen in Figure 4.

The double-diffusion method (Bergdoll et al., 1965) was used to estimate the concentration of impurities. A range of enterotoxin concentrations (2.5 mg-1 μg/ml of 0.02 M potassium phosphate, pH 7.4, 0.85% sodium chloride) was used against a range of antiserum concentrations (one-plus-one dilution to a dilution that resulted in a visible preciptate band with 1 μg or less of the enterotoxin). The minimal enterotoxin concentration, at which a precipitin band appeared resulting from the presence of an impurity, was compared to the minimal enterotoxin concentration that was required to produce a precipitin band to the enterotoxin. The relationship of the two concentrations provided a rough estimate of the impurity concentration. Tests for α- and β-hemolysins (Bergdoll et al., 1965) were negative. No proteinase activity was found in the purified

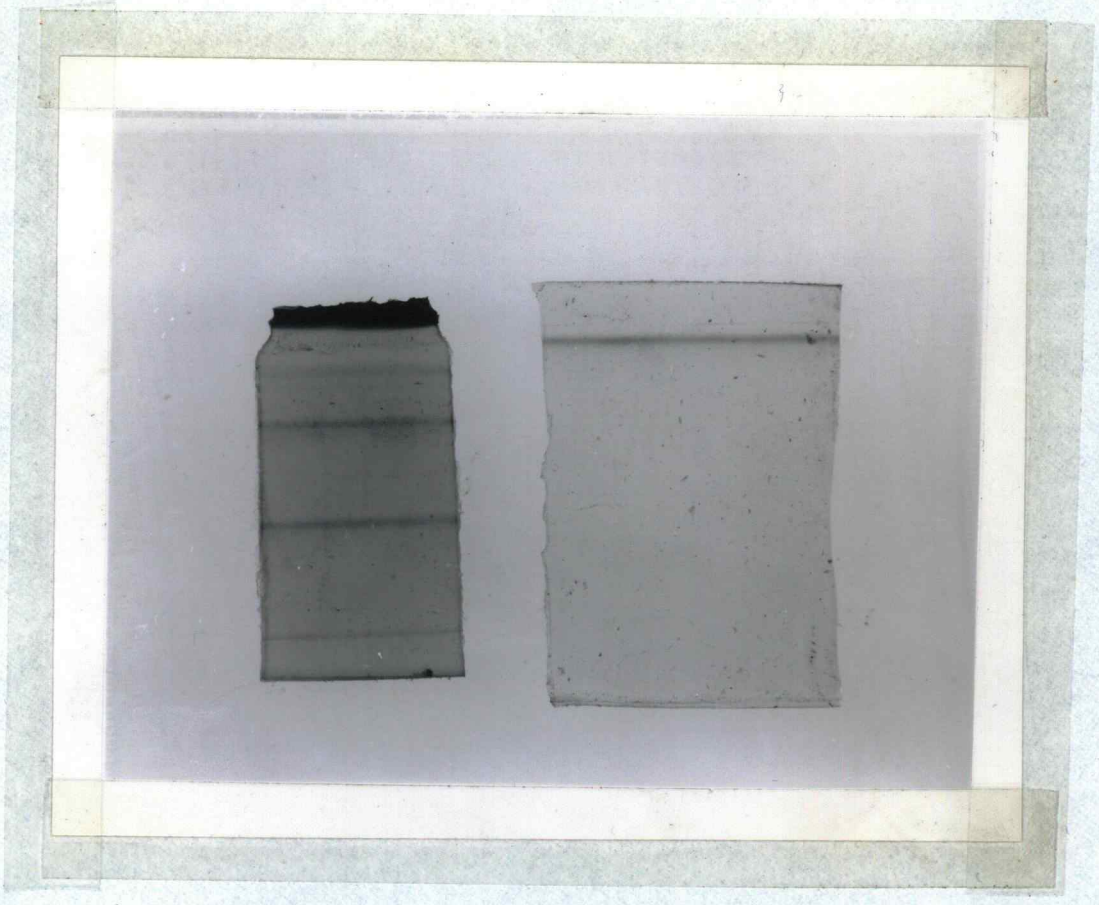


Figure 3. A comparison of a partially purified enterotoxin A preparation (left) and a purified preparation (right) stained after flat-gel electrophoresis. Samples run with standard high pH gel system.

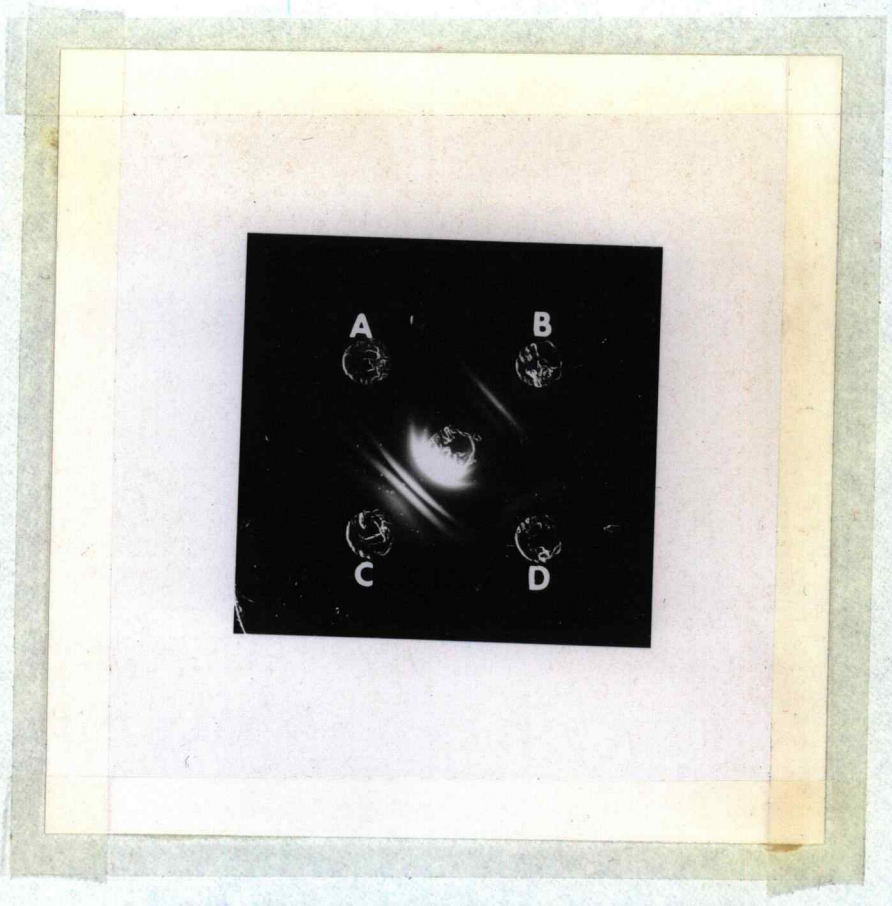


Figure 4. Slide gel diffusion of enterotoxin A purification sequence. (A) Highly purified entrotoxin B; (B) Partially purified enterotoxin A after second CM-cellulose column elution; (C) Culture supernatant from S. aureus 265-1; (D) Purified enterotoxin A after flat-gel electrophoresis with high pH system. Central well: rabbit antiserum prepared against crude enterotoxin A. Slide stained with 0.5% amidoblack 10B in methanol-glacial acetic acid (9:1); photographed using dark field illumination.

enterotoxin A.

The yield of enterotoxin B was 65% with a purity of 97%. The Amberlite IRP-64 cation exchange resin was effective in separating the toxin from the bulk of the impurities. The toxin could not be satisfactorily isolated directly from the culture with CM-cellulose because of the great variety of substances in the culture. When fractionation was carefully performed, high-purity enterotoxin usually was obtained in two passes through the Amberlite resin; and subsequent chromatography did not increase purity significantly, as indicated by electrophoretic studies and by serological tests on micro-Ouchterlony slides (Figure 5). Further examination of both purified enterotoxin preparations showed negative Molisch, orcinol, and diphenylamine tests; and insolubility in and lack of extractability by both chloroform and ethyl ether.

Antiserum produced against enterotoxin A was specific and considerably more potent that that produced against crude enterotoxins (Casman 1958, 1960). The goat serum possessed a titer of 1:320. It was unnecessary to absorb the serum with nonenterotoxic antigens. The production of antiserum to enterotoxin A has been especially difficult because only small amounts of this enterotoxin are produced by the strains which have been examined.

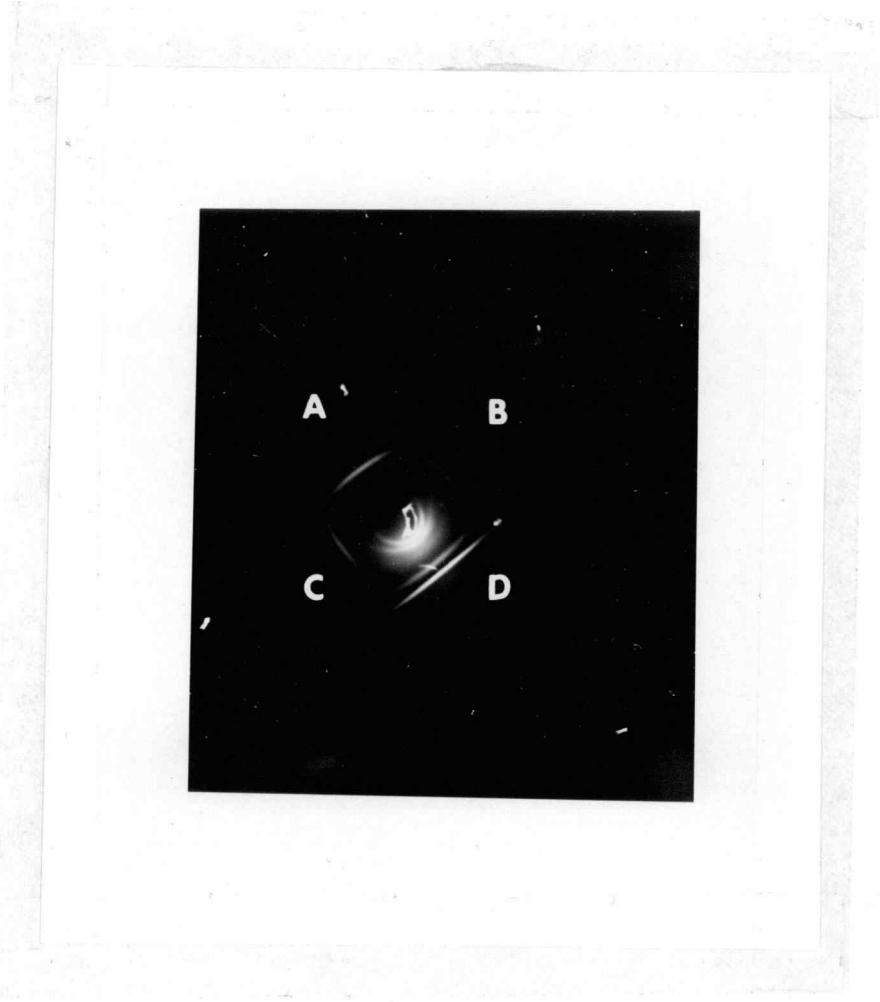


Figure 5. Slide gel diffusion of enterotoxin B purification process. (A) and (C) show the enterotoxin eluate after one passage through Amberlite IRP-64 column. Note lines of identity. (B) Enterotoxin B after second column chromatography step. Faint line present in (A) and (C) has disappeared. (D) Culture supernatant (10X) from S. aureus ATCC 14458. Central well: rabbit antiserum prepared against crude enterotoxin B. Protein concentration in A, B and C was 0.5 mg/ml.

Detection Procedures

Significant progress has been made in the past several years in the procedures for detecting staphylococcal enterotoxins. It has been possible to replace the difficult feeding of monkeys or injection of cats with serological procedures. One purpose of this investigation was to improve on the methodology of enterotoxin assay. At present carboxymethylcellulose and Sephadex columns are used in the initial steps of enterotoxin assay of foods (Casman and Bennet, 1965). It was found in the course of this investigation that these column procedures could be effectively replaced by semi-purification polyacrylamide electrophoresis in a water-cooled flat-gel electrophoresis unit. It was possible to shorten the time required for the semi-purification steps from one to two days to less than three hours. The method consisted of extraction of food samples with distilled water, concentration of the extract with PEG 4000, polyacrylamide gel electrophoresis, elution of the sample and the application of immunodiffusion techniques as illustrated in Figure 6 (bottom). It was also possible to overlay a sliced section of gel with agar and thus apply serological techniques without eluting the toxin as can be seen in Figure 7.

Tables 3 and 4 show the relative recovery efficiency of the electrophoretic and column chromatographic procedures. The

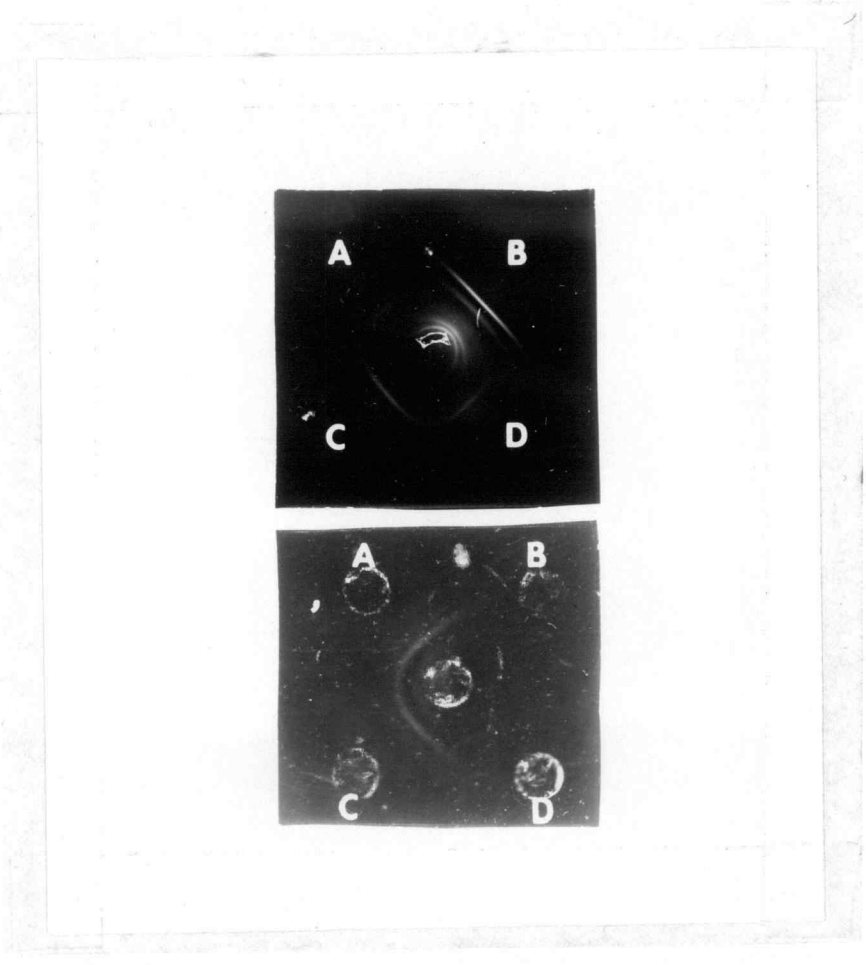


Figure 6. Micro-Ouchterlony slide diffusion tests. Top: Same as Figure 5 but all constituents at 1/10th the concentration. (C) and (D) show enterotoxin preparation after one column passage and (A) after two passages. (B) Culture supernatant from S. aureus 14458. Bottom: (A) Enterotoxin A sample eluted from polyacrylamide gel after being recovered from ground beef; (B) Culture supernatant from S. aureus 23235; (C) Reference enterotoxin A; and (D) Culture supernatant from S. aureus 19095.

Figure 7. Agar overlay technique. Polyacrylamide gel containing enterotoxin A (on left) overlaid with immunodiffusion agar and reacted with specific anti-enterotoxin A (γ_2 fraction).

Table 3. Efficiency of toxin concentration and recovery using CM-cellulose column chromatography

Material or Condition	Enterotoxin A	Enterotoxin B
Amount of ground beef (cooked) used	20 g	20 g
Amount of enterotoxin added	500 µg	500 µg
Volume of extracting buffer	40 ml	40 ml
Total volume of food slurry	60 ml	60 ml
Calculated enterotoxin per ml of slurry	8.3 µg	8.3 µg
Enterotoxin per ml of slurry detected by Oudin assay	7.7 µg	7.1 µg
Volume of supernatant fluid used	33 ml	33 ml
Water added to supernate	33 ml	33 ml
Calculated enterotoxin content	274 µg	274 µg
Amount of enterotoxin in 30 ml of eluate	118 µg	146 µg
Amount of enterotoxin per ml in 20X concentration	78.6 µg	97.3 µg
% recovery of enterotoxin in supernate passed through column	43	64
% recovery of enterotoxin in original food	24	29

Table 4. Efficiency of toxin concentration and recovery using semi-purification electrophoresis

Material or Condition	Enterotoxin A	Enterotoxin B
Amount of ground beef (cooked)	20 g	20 g
Amount of enterotoxin added	500 µg	500 µg
Volume of water for extraction	40 ml	40 ml
Total volume food slurry	60 ml	60 ml
Calculated enterotoxin per ml of slurry	8.3 µg	8.3 µg
Enterotoxin per ml of slurry detected by Oudin method	7.7 µg	7.1 µg
Volume of supernatant fluid concentrated 10X	10 ml	10 ml
Calculated enterotoxin content	83 µg	83 µg
Amount enterotoxin recovered after electrophoresis and elution	71 µg	76 µg
% recovery of enterotoxin submitted to electrophoresis	86	92

amount of enterotoxin contained in the column eluates were 118 μg of A and 146 μg of B. This represented 24% of enterotoxin A and 29% of enterotoxin B present in the original meat sample. These percentages of recovery are low, but with meat samples such values are to be expected since much of the enterotoxin is held in the solid material after centrifugation. With the electrophoretic procedure 86% of enterotoxin A and 92% of enterotoxin B was recovered of that subjected to analysis. Semi-purification has several advantages over column techniques. It is less time consuming and much less expensive. In addition, the method is easily adaptable to multiple sample analyses. The latter advantage is significant, since, if one had to analyze several food samples, it would be necessary to set up a CM-cellulose column for each sample. The electrophoretic procedure necessitates elution of the toxin from the gel for quantitative studies. This is a disadvantage that can be alleviated by the adaption of a continuous elution device (presently under construction in our laboratory).

The efficiency of the semi-purification electrophoresis procedure was tested with a number of different foods (Table 5). The values presented in Table 5 represent an average of two trials. In each case the recovery percentage was significantly higher than that obtained with carboxymethylcellulose. The recovery of enterotoxin from raw ground beef presented considerable difficulty. The soluble

meat components were adsorbed by the carboxymethycellulose to an extent that interfered with the concentration of the eluate. In addition, the extracts possessed material which diffused into and clouded the agar gel in the Oudin and slide diffusion tests. This problem was solved by the electrophoretic technique. The sample was first electrophoresed using the standard 7.5% gel. The enterotoxin containing section was sliced out and submitted to electrophoresis again using an 8% gel. Elution was unnecessary; the sliced gel was simply placed on top of the modified small-pore gel. The altered gel pore size prevented most of the interfering protein from entering the gel.

Table 5. Recovery of entrotoxin from food slurries to which it had been added in known quantities by use of carboxymethylcellulose and polyacrylamide gel electrophoresis

Food Slurry	Carboxymethylcellulose				Polyacrylamide gel electrophoresis			
	Enterotoxin A		Enterotoxin B		Enterotoxin A		Enterotoxin B	
	Added (µg)	Recovered %	Added (µg)	Recovered %	Added (µg)	Recovered %	Added (µg)	Recovered %
Ground beef (raw)	50	25	50	31	50	43	50	55
Banana Cream Pie	30	40	30	52	30	55	30	60
Chicken Pot Pie	20	45	20	50	20	53	20	52
Cheddar Cheese	30	42	30	47	30	47	30	52

Since the trace amounts of enterotoxin present in foods must first be separated and concentrated to be detected by the gel diffusion precipitation test, it is clear that the successful application of this test depends on the efficiency of the procedure for extracting and separating the enterotoxin from the food prior to its specific precipitation with antibody. From the data it can be seen that semi-purification electrophoresis offers a significant advantage over accepted column techniques.

The development of gel-diffusion techniques for the quantitative and qualitative detection of staphylococcal enterotoxin marked a significant improvement over animal tests. There are essentially three different applications of the gel-diffusion principle: single-diffusion (Oudin) and double-diffusion (Oakley) tube methods and the double gel-diffusion (micro-Ouchterlony) slide test. All of these methods require a time period of 16 hours to as much as seven days. During the course of this investigation it was sought to shorten the time period necessary for the formation of specific immunoprecipitates. A combination of electrofocusing and electro-osmophoresis was successful in reducing the time for qualitative analysis to less than a working day. Enterotoxin A samples were first focused in polyacrylamide gel using an ampholyte solution with a range of pH 6-9. Protein bands were located by treating a slice of the gel with 10% TCA for 15 minutes. That section of sample gel corresponding to that of

the purified reference toxin was sliced out, eluted with distilled water and subjected to electro-osmophoresis as previously described. To facilitate identification of the faint precipitin lines only the γ_2 fraction of the antiserum was used. This method was compared with the Oakley double-diffusion method for efficiency (Table 6). Toxin samples could be assayed at a concentration of 0.5 μg/ml in five hours. This is a significant improvement over current gel-diffusion techniques although it lacks the sensitivity of the micro-titer system described by Silverman et al. (1968).

Table 6. Sensitivity of the double-diffusion and electrofocusing - electro-osmophoresis assay systems for entrotoxin A

Double-diffusion			Electrofocusing - Electro-osmophoresis		
Assay period	Enterotoxin concentration (μg/ml)	Results	Assay period	Enterotoxin concentration (μg/ml)	Results
4 days	1.0	3+	5 hr	1.0	3+
3 "	0.75	4+	"	0.75	3+
4 "	0.50	3+	"	0.50	1+
10 "	0.25	2+	"	0.25	-

Some justification must be offered for using the gel diffusion technique since it is not as sensitive as other seriological procedures, such as complement-fixation and hemagglutination-inhibition. Gel diffusion is preferred to the latter because of its simplicity and the presence in many foods of substances which affect the specificity

of the two more sensitive tests (Silverman et al., 1968). Flotation and fluorescent antibody methods offer no increase in sensitivity since they must be preceded by precipitation with specific antibody. Fluorescent antibody techniques are applicable only when there is concentration of the enterotoxin to a degree required for precipitation. FAT offers the advantage of speed but this is countered by nonspecific fluorescence when the technique is applied to a food such as meat. Flotation methods (Hopper, 1963) require the presence of considerably greater amounts of enterotoxin than are involved in food poisoning.

Physiological Studies

A second purpose of this study was to elucidate the factors affecting enterotoxin synthesis. A number of inhibitors were tested for their effect on enterotoxin synthesis and release (Tables 7, 8, 9, 10 and 11). Streptomycin sulfate and KCl (Tables 8 and 9) inhibited the elaboration of both enterotoxins A and B produced by S. aureus 265-1 and 14458, respectively. Chloramphenicol and NaF also inhibited the elaboration of enterotoxin A and B from the two toxigenic strains. However, the inhibitors shown in Tables 8 and 9 did not adequately distinguish between factors directly involved in toxin formation and those required for normal cellular growth. Magnesium at a concentration of 0.001 μmoles/ml did reverse the inhibition by

Table 7. The effect of inhibitors on the production of enterotoxin A by S. aureus 265-1

Inhibitor	Amount (μ moles/ml)	OD* (650 mμ)	Enterotoxin A (μg/ml)	Inhibition (%)
Streptomycin sulfate	0	0.34	13	
	0.0005	0.35	0	100
NaF	0	0.31	10	
	50	0.15	0	100
Chloramphenicol	0	0.36	10	
	0.005	0.32	2	80
KCl	0	0.32	12	
	40	0.33	3	75

* Optical density of 10-fold diluted culture

Table 8. The effect of inhibitors on the production on enterotoxin B by S. aureus 14458

Inhibitor	Amount (μ moles/ml)	OD (650 mμ)	Enterotoxin B (μg/ml)	Inhibition (%)
Streptomycin sulfate	0	0.38	63	
	0.0005	0.36	0	100
NaF	0	0.36	58	
	50	0.19	5	91
Chloramphenicol	0	0.34	65	
	0.005	0.28	30	58
KCl	0	0.34	63	
	40	0.32	0	100

Table 9. Inhibition of staphylococcal enterotoxin A and B by bacitracin

Bacitracin (μmoles/ml)	S. aureus 265-1			S. aureus 14458		
	OD (650 mμ)	Enterotoxin A (μg/ml)	Inhibition (%)	OD (650 mμ)	Enterotoxin B (μg/ml)	Inhibition (%)
0	0.32	12		0.30	55	
0.002	0.32	10	17	0.31	52	5
0.004	0.30	6	50	0.30	47	15
0.008	0.29	0	100	0.27	32	42
0.016	0.30	0	100	0.19	10	82
0.032	0.12	0	100	0.00	0	100

Table 10. Inhibition of staphylococcal enterotoxins A and B by penicillin G

Penicillin G (mμmoles/ml)	S. aureus 265-1			S. aureus 14458		
	OD* (650 mμ)	Enterotoxin A (μg/ml)	Inhibition (%)	OD (650 mμ)	Enterotoxin B (μg/ml)	Inhibition (%)
0	0.34	10		0.33	57	
0.0025	0.33	8	20	0.32	57	-
0.0083	0.29	2	80	0.33	-	-
0.0133	0.20	0	100	0.30	55	5
0.0182	0.18	0	100	0.34	-	-
0.0225	0.18	0	100	0.33	-	-
0.0264	0.20	0	100	0.34	-	-
0.0311	0.11	0	100	0.30	52	9

* Optical density of 10-fold diluted culture

Table 11. Inhibition of staphylococcal enterotoxins A and B by detergent-like compounds

Inhibitor (μ moles/ml)	S. aureus 265-1			S. aureus 14458		
	OD[1] (650 mμ)	Enterotoxin A (μg/ml)	Inhibition (%)	OD (650 mμ)	Enterotoxin B (μg/ml)	Inhibition (%)
None	0.36	13		0.32	52	
Sodium deoxycholate						
0.024	0.34	4	69	0.30	20	64
0.126	0.24	0	100	0.25	8	85
0.223	0.20	0	100	0.23	0	100
Tween 80[2]						
4.0	0.27	6	54	0.26	83	0
8.0	0.24	4	69	0.23	91	0
12.0	0.20	0	100	0.25	101	0
Oleic acid						
0.216	.35	3	78	0.36	11	79
0.547	.37	0	100	0.39	0	100
1.217	.33	0	100	0.35	0	100

[1] Optical density of a 10-fold diluted 18-hr culture.

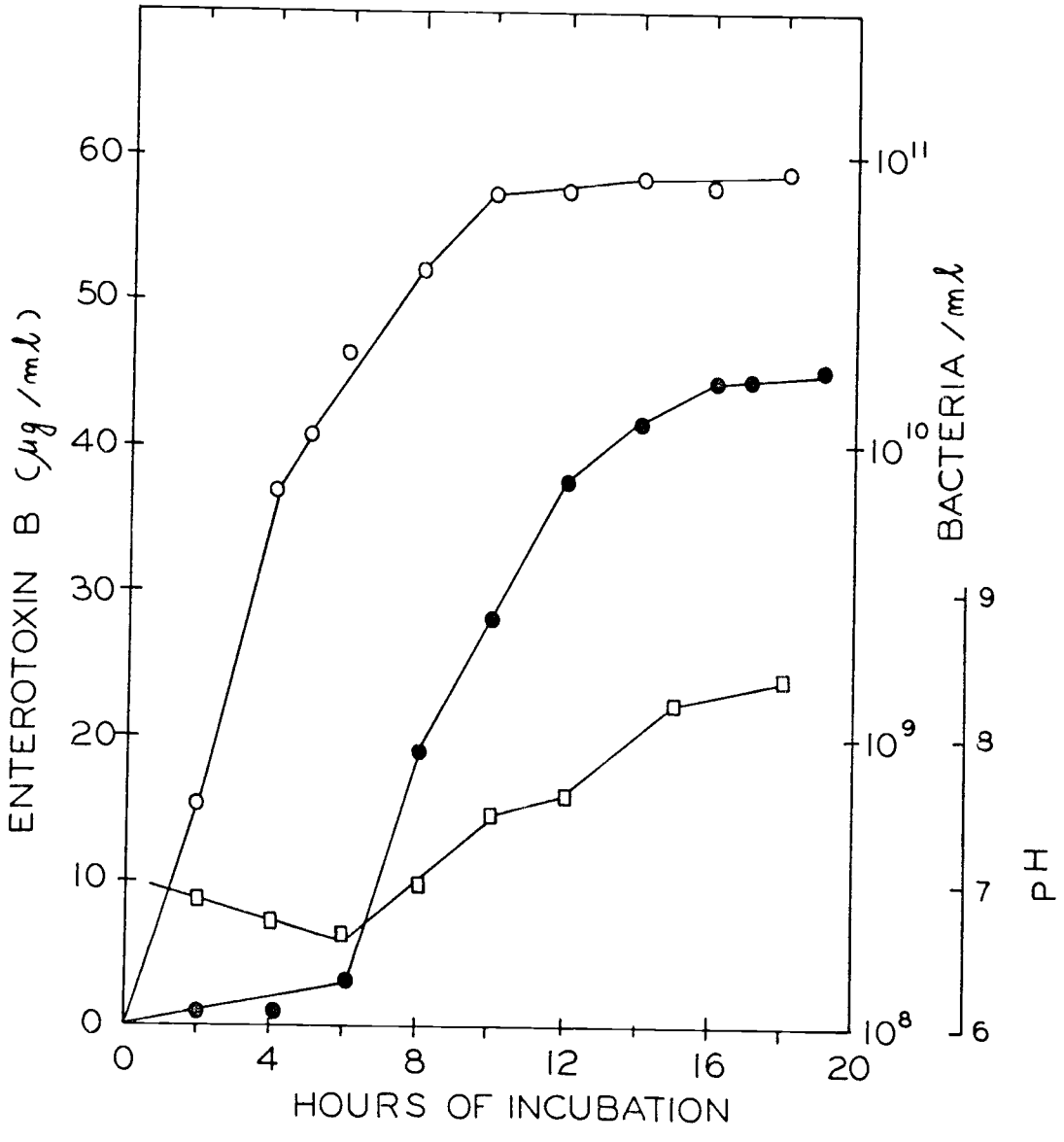
[2] Milligrams per milliliter.

both NaF and KCl. This is similar to inhibitor experiments reported by Friedman (1966) on an enterotoxin B producing strain of S. aureus. Since NaF is known to inhibit enzymatic reactions by complexing with Mg^{++}, this suggests that Mg^{++} may be required for the enzymatic processes leading to toxin formation. Bacitracin inhibited both the growth and toxin production of S. aureus strains 265-1 and 14458. However, penicillin G did not inhibit the growth of S. aureus 14458 but did inhibit the synthesis of enterotoxin B. In contrast, both enterotoxin A production and growth by strain 265-1 were inhibited by this agent.

The effects of surface active agents (Table 11) were variable. Sodium deoxycholate inhibited toxin synthesis and growth of the two strains used for this investigation. Tween 80 did not have a great effect on cell growth but accounted for 69% inhibition at 8 μmoles/ml and complete inhibition of enterotoxin A elaboration at 12 μmoles/ml. Conversely, this agent stimulated the synthesis of enterotoxin B by S. aureus 14458 54% at a concentration of 4 μmoles/ml without appreciably affecting growth. This suggests a difference in the mode of synthesis of the two enterotoxins. Oleic acid had no effect on growth but completely inhibited the elaboration of both toxins at a concentration of 0.547 μmoles/ml.

The relationship of bacterial growth and toxin release was studied (Figure 8) using S. aureus 14458. Enterotoxin was released

Figure 8. Enterotoxin B production, growth and pH changes in S. aureus 14458 at 37 C. Symbols: ○ , bacteria; ● , enterotoxin B; □ , pH.


ENTEROTOXIN B (μg/ml)
BACTERIA/ml
pH
HOURS OF INCUBATION
0
10
20
30
40
50
60
10^8
10^9
10^{10}
10^{11}
6
7
8
9
0
4
8
12
16
20

approximately 4-6 hrs after incubation. The greatest toxin release occurred during the late exponential and early stationary phases. This is consistent with the work of MacLean et al. (1968) on the same organism. These data indicate that enterotoxin B production is not inseparably bound to cell multiplication. In agreement with the results of Markus and Silverman (1969) on S. aureus S-6, it was found that the lag period in toxin production could not be eliminated by propagating cells of S. aureus 14458 in the supernatant fluid obtained from a 6 hr culture which was just prior to optimal toxin production.

After bacteria had reached the stationary phase of growth they were washed and resuspended in medium A minus yeast extract. After five hours 20 μg/ml of enterotoxin B were excreted into the medium (Figure 9). The bacterial concentration, DNA and protein content remained nearly constant during this period. In a medium devoid of nitrogen (medium B) enterotoxin B was still produced by cells in the stationary phase, 20 hr cells, but not by those in the exponential phase (Figure 10). Chloamphenicol at a concentration of 0.0005 μmoles/ml did not inhibit toxin formation in medium B. These experiments indicate the existence of a toxin precursor pool as suggested by Markus and Silverman (1969) in studies with S. aureus S-6.

Figure 9. Enterotoxin B, protein and DNA synthesis by nonreplicating cells of S. aureus 14458. Symbols: ○ , enterotoxin B; ● , DNA; □ , protein; △ , bacteria.

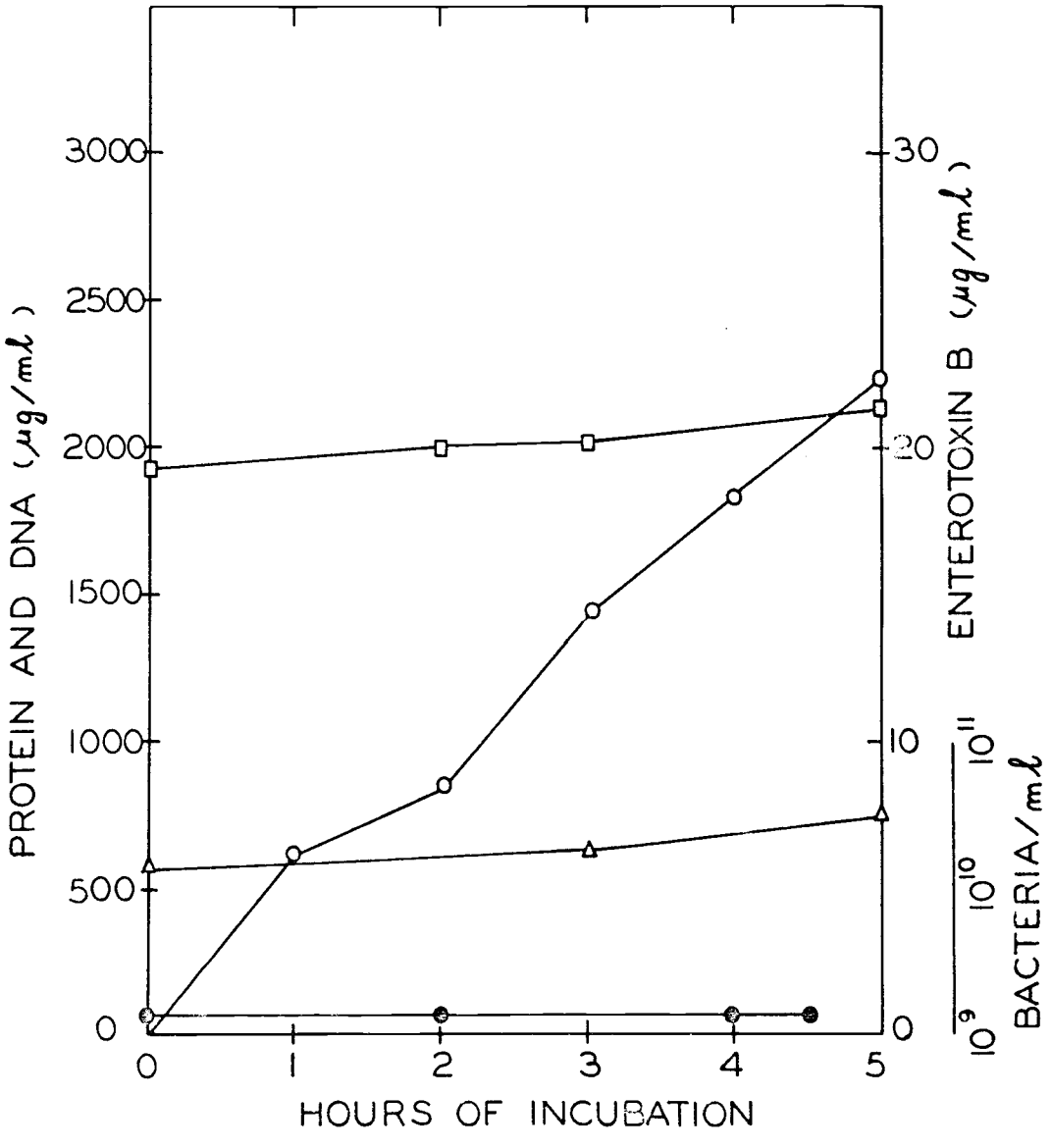
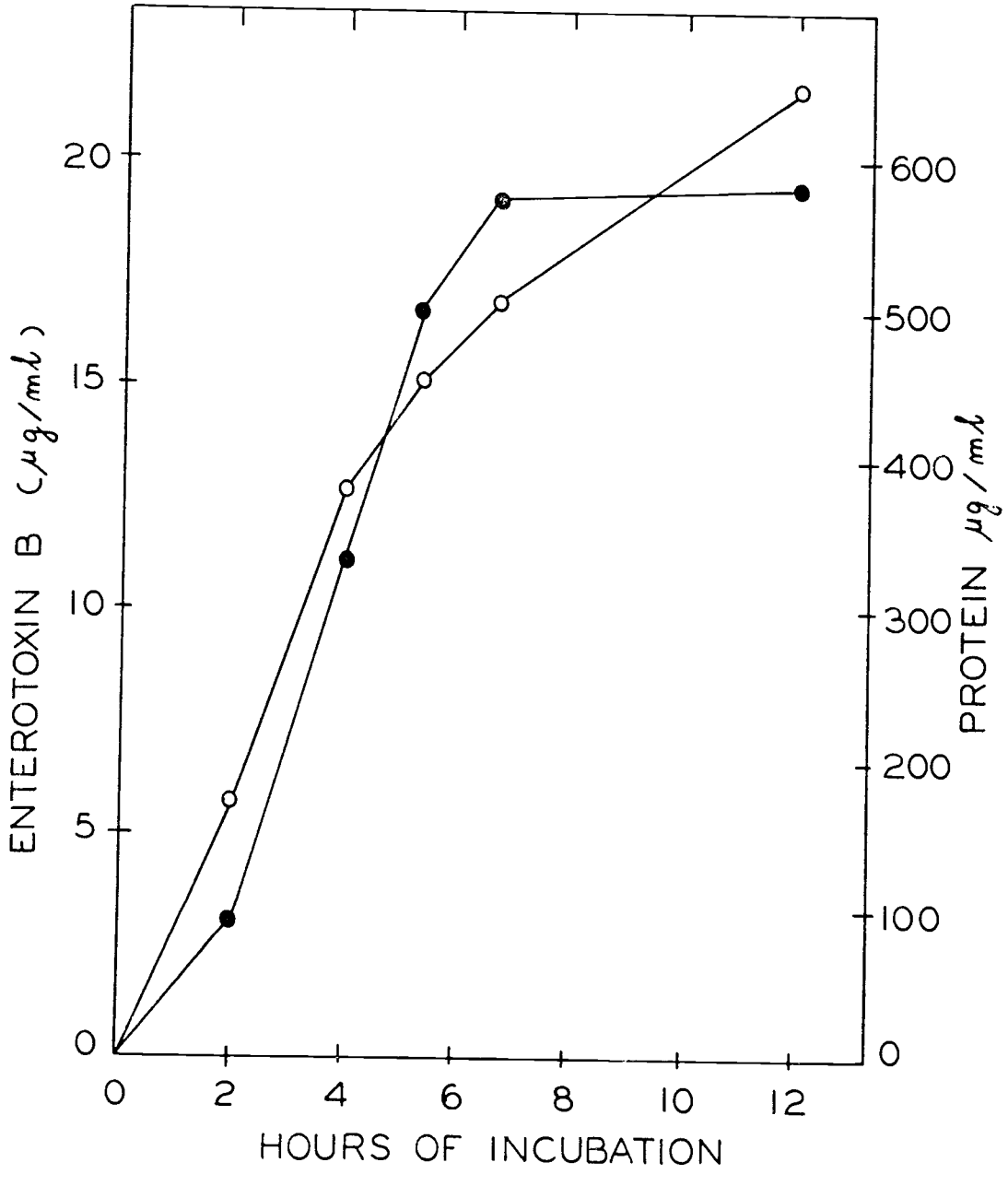

PROTEIN AND DNA (μg/ml)
3000
2500
2000
1500
1000
500
0
ENTEROTOXIN B (μg/ml)
30
20
10
0
BACTERIA/ml
10^9
10^10
10^11
0
1
2
3
4
5
HOURS OF INCUBATION

Figure 10. Enterotoxin B formation in a nitrogen free medium by nonreplicating cells of S. aureus 14458. Bacteria inoculated in medium B. Symbols: ● , toxin; ○ , protein.


ENTEROTOXIN B (μg/ml)
PROTEIN μg/ml
HOURS OF INCUBATION
0
5
10
15
20
100
200
300
400
500
600
2
4
6
8
10
12

The function of enterotoxin with respect to the bacterial cell is still an unanswered question. MacLean et al., 1968, have suggested that it is a secondary metabolite, like many antibiotics. Further investigation must be conducted before the function of enterotoxin, if it has one, can be fully understood.

SUMMARY

Means of facilitating the detection of staphylococcal enterotoxin were investigated. It was found that semi-purification polyacrylamide flat-gel electrophoresis was more efficient than currently accepted CM-cellulose and Sephadex column chromatography methods in separating and enterotoxin from soluble food constituents. The electrophoretic process was less time consuming, less expensive and adaptable to multiple sample analyses.

A technique combining isoelectric focusing followed by electro-osmophoresis was successful in reducing the time required for the formation of specific immuno-precipitates. It was possible by this method to assay enterotoxin A at a concentration of 0.5 μg/ml in five hours.

Inhibitor studies were conducted on enterotoxin A and B production by S. aureus 265-1 and 14458, respectively. The elaboration of enterotoxin A and B was inhibited by streptomycin sulfate, NaF, chloramphenicol, KCl and bacitracin. Inhibition by KCl and NaF was reversed by Mg^{++}. Penicillin G inhibited toxin production by S. aureus 14458 but not cell growth, whereas both toxin elaboration and growth were inhibited with S. aureus 265-1.

Replacement culture experiments with S. aureus 14458 demonstrated the existence of a toxin precursor pool.

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