AN ABSTRACT OF THE THESIS OF

ENEFIOK JAMES NKANGA for the degree MASTER OF SCIENCE
(Name of student) (Degree)

in MICROBIOLOGY presented on June 19, 1975
(Major Department) (Date)

Title: SEROLOGICAL DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN A IN CULTURE SUPERNATANTS

Redacted for Privacy

Abstract approved: _____
A. W. Anderson

A method has been presented for the observation of a large number of enterotoxin A producers. Production of a relatively concentrated toxin is achieved by the sac culture method. Further concentration, if needed, is carried out by PM-10 membrane ultrafiltration after centrifugation. The work is further improved by trypsinization of the supernatant fluid with 0.08% trypsin to digest the interfering proteins and increase the percentage recovery during PM-10 membrane ultrafiltration. Detection is carried out by Laurell electroimmunodiffusion of the supernatant fluids. The mobility of the toxin on the antibody containing gel is increased as a result of trypsin treatment.

The usefulness of the methods for the detection of small quantities of enterotoxin A in culture supernatants was investigated. Low producers of quantities of enterotoxin usually found in implicated

foods (example S. aureus FRI #888 producing about 2.5 μg/ml and L-19 producing 0.15 μg/ml) could be detected.

Serological Detection of Staphylococcal Enterotoxin A in Culture Supernatants

by

Enefiok James Nkanga

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed June 10, 1975

Commencement June, 1976

APPROVED:

Redacted for Privacy

Professor of Microbiology
in charge of major

Redacted for Privacy

Head of the Department of Microbiology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented June 10, 1975

Typed by Velda D. Mullins for Enefiok James Nkanga

ACKNOWLEDGEMENTS

Sincere thanks is extended to my adviser, Dr. A. W. Anderson for the scientific guidance in this work.

Also, I would like to thank Dr. W. Gamble of the Department of Biochemistry for his advice and corrections, and members of the staff of the Department of Microbiology who showed concern in times of need.

This thesis is dedicated to my wife Quinta Afi for her perseverance and concern during the period of this research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SEROLOGICAL DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN A IN CULTURE SUPERNATANTS

INTRODUCTION

Staphylococci are members of the family Micrococcaceae. They are spherical cells ranging from 0.5 to 1.5 nM in diameter. They show multiple division planes hence stained smears show single, pairs and typical grapelike cluster appearances. They are non-motile organisms and young cultures are always gram positive.

Physiologically they are aerobic or fermentative and heterotrophic. Optimum growth temperatures are between 35°C and 40°C and many show salt tolerance of up to 15%. They are catalase positive.

There are only two species recognized in the genus and both are cocci. They form part of the normal microbial flora of the human hands, nose, face, respiratory and gastrointestinal tract. Staphylococcus epidermidis is the white form. It is a saprophyte, non-pathogenic and common on skin and mucous membranes and sometimes acts as secondary invader . Staphylococcus aureus is a common cause of boils, carbuncles and various other purulent diseases. Colonies are usually golden yellow, but white variants appear. They are also associated with endocarditis and food poisoning. They show β-hemolysis in blood plate, ferment mannitol, and

most often are coagulase positive.

Transmission of pyogenic staphylococci from person to person is mostly by direct contact with persons who have lesions or those who are asymptomatic carriers. In addition to their ability to cause disease by infection, Staphylococcus aureus are capable of producing intoxication which results from ingestion of contaminated foods by susceptible individuals. Staphylococcal food poisoning is a consequence of the ingestion of preformed toxin. Staphylococcus aureus produces lipochrome pigments which give the colonies a golden yellow color.

The enterotoxin is usually produced by strains of Staphylococcus aureus which are virulent and mostly golden yellow, strongly hemolytic on blood agar and coagulase positive. Numerous enterotoxigenic studies on staphylococci with these characteristics have been documented. One such study showed that 10% of the hospital strains of Staphylococcus aureus with these characteristics were toxigenic (29). Statistics by the Center for Disease Control, Atlanta, Georgia, on known food-borne disease outbreaks show that 25 to 30% of the total number of the outbreaks are attributed to enterotoxigenic staphylococci (51).

Foods involved in staphylococcal food poisoning are mainly starchy, such as dressings, custards, and salads. Meats may also be involved.

The fact that staphylococci are relatively resistant to heat

(80°C for 1 hour) and can tolerate high salt concentrations reveal why staphylococcal food poisoning is very common. The prevalence of staphylococcal food poisoning has been correlated with an enormous number of commercially prepared meals catered to and consumed by American establishments (1).

Clinical symptoms of an outbreak of bacterial food poisoning include vomiting, diarrhea, enteritis and sometimes prostration in 1 to 6 hours. Gastrointestinal disturbances following the ingestion of naturally poisonous food or foods contaminated with arsenic or cyanide, are often indistinguishable from bacterial food poisoning. In bacterial food poisoning, recovery is normally complete in 24 to 72 hours and treatment, if hospitalized, is aimed at preventing the process of dehydration.

For most laboratories, it has been a process of much difficulty in trying to determine the type of enterotoxigenicity of implicated staphylococci because of high technical skill required for the detection of the enterotoxin either in foods or culture supernatants.

It is the purpose of this investigation to improve and simplify the current methods leading to enterotoxin detection in culture supernatants. If the culture isolated from food produces no identifiable enterotoxin, and if it is the culture most likely to have produced the enterotoxin in the food under examination, there is no point examining the food for enterotoxin. Also, examination of culture isolated

from foods for enterotoxigenicity is the only information that can be obtained since frequently not enough of the food is available for enterotoxin examination. Direct examination of food becomes necessary, if no cultures are present in the food in question. In this case, the organisms may have produced the toxins before pasteurization. Lastly, the toxin might be involved in the pathology of a disease in which the toxigenic Staphylococcus aureus is isolated; hence, there is need to check for enterotoxigenicity.

LITERATURE REVIEW

Enterotoxin Detection

Many laboratories that lack skilled technicians depend on relating exterotoxigenicity to other easily determined characteristics. A typical example is the correlation of coagulase with enterotoxigenicity. Coagulase, which is a protein enzyme, coagulates the plasma of a variety of animals, including humans, rabbits, horses and pigs.

When isolating Staphylococcus aureus in a plate from a variety of organisms to test for coagulase, some colonies may be untested due to overcrowding. Many investigators have gone one step further in eliminating this difficulty by developing a coagulase test media as described by Chugg (18), and by Orth and Anderson (44, 45).

The use of the coagulase test is taken as being presumptive evidence for toxin in the food in question. The test ignores the important fact that enterotoxigenic coagulase-negative strains do exist (9). Correlation of enterotoxigenic characteristic with other properties have been documented. Staphylococcus aureus produces a heat resistant nuclease (17). Following this, Lachica et al. (37) suggested that the enzyme shows a better correlation with enterotoxigenicity than coagulase. Unfortunately, not all enterotoxigenic strains

produce nuclease.

It is better to assay directly for the enterotoxin which is the active agent in food poisoning.

The enterotoxins occur as five immunological types designated as A, B, C, D and E (14, 8, 5, 16, 6). The sixth one is tentatively being identified according to Bergdoll (4). It is known that two types of enterotoxin C exist. They are designated as C_1 and C_2 and they differ in some physico-chemical properties, for example, the iso-electric point. They have both been purified (3, 13).

Enterotoxins are proteins with molecular weights ranging from 28,366 to 35,000. They are antigenic and elicit the formation of specific antibodies when injected into animals such as rabbits. Because it elicits the formation of antibodies, an antigen-antibody reaction can be envisioned. This forms the only specific and sensitive test used to detect staphylococcal enterotoxins.

Numerous methods have been developed which make use of the antigen-antibody reaction. Among these methods are Ouchterlony plate test (5), hemagglutination inhibition (43) reversed passive hemagglutination (50), double gel diffusion tube test (27), rapid capillary tube test (23, 24), microslide test (15), flourescent antibody test (48), solid phase radioimmunassay (33), reversed immuno-osmophoresis (36) and more recently Laurell immunoelectrodiffusion (25).

A useful technique should be rapid, sensitive as well as practical. Laurell immunoelectrodiffusion has all these properties. Although it has not been exactly determined how much enterotoxin is required to cause illness, speculations from human volunteer studies indicate that about 1 μg is required if not less. Laurell immunoelectrodiffusion can detect as little as 0.15 μg/ml of enterotoxin A per 100 g of food within 8 to 10 hours.

Enterotoxin Production

Many methods have been employed by different investigators for enterotoxin production. These include cellophane-over-agar method (30), applied later by Jarvis and Lawrence (32); semi-solid agar method originally outlined by Casman and Bennet (15); sac culture method of Donnelly (21) and shake flask method of Kato et al. (35).

A comparative study on the efficiency of these methods of enterotoxin production has been done (14). In the study, it was found that the sac culture method was most efficient.

In this method, the culture medium is placed inside a sac of dialysis tubing and tied. The tube is then placed in an Erlenmeyer flask in a U-shaped form and the flask is stoppered and autoclaved. Measured sterile phosphate buffer in physiological saline, pH 7.4, is put in the flask and growth from slants or broth inoculated into

the phosphate buffer. The nutrients in the sac diffuse out and supply the bacteria with the necessary nutrients. The period of nutrient supply is therefore increased more than if the bacteria were in direct contact with the growth medium. Greater cell mass and toxin will result from this method. Because a little amount of phosphate buffer is used to suspend the cells, further concentration of the fluid may not be necessary.

Work has been done on the effect of different types of cellophane tubings on the production of enterotoxins. It was found that a fresh Union Carbide Company dialysis membrane was superior to others (7).

After the growth, the culture medium is centrifuged and the supernatant fluid is subjected to enterotoxin assay.

Membrane Ultrafiltration

Many times, the supernatant fluid subjected to enterotoxin assay may be negative. Concentration may be necessary to demonstrate a very low quantity (47). One of such methods that may prove useful is membrane ultrafiltration.

In ultrafiltration, solvents and solutes up to certain critical sizes are forced through a semi-permeable membrane by applying high pressure on one side of the membrane.

Two methods are regularly used: a) Pressure dialysis through

cellophane bags; b) Vacuum suction through Colloidon membranes supported on filter candles.

Pressure dialysis through cellophane bags has been described by Kabat (34). It is important to select the proper size of tubing, as different sizes of tubing have different permeabilities. Dialysis filters are now commercially available. The LKB apparatus (Stockholm, Sweden), described by Aronsson (2) has been employed by Coulter (19) and by Fish et al. (22), for concentration of staphylococcal α-toxin and anthrax toxin. The latter authors have also used diaflo ultrafiltration membranes (Amicon Corporation, Cambridge, Massachusetts) for the purification of Anthrax toxin in a shorter time period and finally by Gasper (25) for the purification of enterotoxin A in foods.

Diaflo ultrafiltration membranes are ionically cross-linked polyelectrolyte complexes manufactured into thin sheets (0.1 to 0.5 μ) and laminated to a flexible, inert backing for handling and repeated usage. Blatt et al. (11) were some of the first to apply membrane ultrafiltration to the concentration of proteins and they found the process very rapid with no evidence of denaturation. Blatt et al. (12) also reported on the fractionation of protein solutions into size graded classes.

Laurell Electroimmunodiffusion

Immunochemical analysis is based on the reaction which occurs between an antigen and its corresponding antibody resulting in the formation of an immunoprecipitate (31). The principle was applied by Ouchterlony (46) in a qualitative method of analysis called immunodiffusion. In this method, antigens and antibodies are allowed to diffuse towards each other in an agar gel. At the site where the antigen and corresponding antibody meet, they form a precipitin line. The size and form of the line reflect certain properties of the reactants, such as homogeneity.

In 1966, Laurell (38) introduced a quantitative variation called at first "Laurell rocket method," later renamed "Electro-Immuno-Assay (EIA)" (39). The EIA method is run on a thin layer of a mixture of agarose gel, a buffer substance and an antibody. The samples to be tested are placed in small circular wells punched in gel layer. An electric field is then applied over the gel resulting in an electrophoretic migration of the antigen while the antibody remains fixed in agarose. A precipitin results which resembles a rocket. The result is that for a given electric field strength and a given time of electrophoretic migration, the area under the precipitin curve or rocket is proportional to the amount of antigen applied to the wells. Commercial EIA plates are now available. An example is the Exa-Phor Ready-Made Electro-Immuno-Assay Plate (10).

MATERIALS AND METHODS

Staphylococcal Strains

A number of strains of Staphylococcus aureus were used in this investigation. Listed below are the sources:

State Department of Agriculture

1 SDA 402 (1)
2 " 402 (2)
3 " 402 (3)
4 " 247 A
5 " 246 A
6 " 249 A

Dr. Merlin Bergdoll, Food Research Institute (FRI)
University of Wisconsin, Madison, Wisconsin

1 FRI #722
2 " #888
3 " #100
4 " #196E
5 " #L19

Department of Home Economics, Oregon State University, Corvallis, Oregon

1 265-1

Department of Microbiology, Oregon State University, Corvallis, Oregon (Stock culture)

1 ATCC 25923
2 FDA 209

All of the cultures supplied by the State Department of Agriculture, Salem, were obtained during routine laboratory isolation of coagulase positive Staphylococcus aureus from foods.

Standardization of Cells by Optical Density

For comparative work on enterotoxin production, it was necessary to standardize the number of cells. The method often used by some investigators has been nephelometric approximation (15). Since this is not very accurate it was thought necessary to use the turbidimetric method. FRI #722 strain of Staphylococcus aureus, freeze-dried and supplied by Dr. Merlin Bergdoll, was used for this investigation. Fifty mls of brain heart infusion broth (BHI broth), supplemented with 0.5 mg/liter thiamine-HCl (Matheson Coleman and Bell, Norwood, Cincinnati, Ohio) and 50 mg/liter nicotinic acid (Nutritional Biochemicals, Cleveland, Ohio) was inoculated with one bead of the preserved bacterial culture. It was then shaken at 120 rpm New Brunswick Shaker (Scientific Co., Inc., New Brunswick, New Jersey) at 37°C for 48 hrs. The growth from the initial broth culture was inoculated on to nutrient agar incubated at 37°C, and a loopful of the agar growth was re-inoculated into another 50 ml BHI broth supplemented with 0.5 mg/l thiamine-HCl and 50 mg/l nicotinic acid. It was shaken at 120 rpm for 24 hrs in 37°C incubator and the growth was used as inocula for the standardization study.

One ml of cells from the 50 ml re-inoculated flask of BHI broth was inoculated at 150 ml of BHI broth supplemented with

nicotinic acid 50 mg/1 and thiamine-HCL 0.5 mg/1 and shaken at 120 rpm at 37°C. Four ml samples were taken every 30 minutes thereafter for measurements to detect first change in optical density. Then the cells were treated as follows:

Before optical density measurements and viable cell-counts were taken, the cells were first shaken up with glass beads for 8 minutes after harvest, and 15 ml samples were harvested every 30 minutes for this purpose. OD_{530} was taken in duplicate and viable cell counts in triplicate. The glass bead treatment was to insure the dispersion of cells. Staphylococcus 110 medium was used for the spread plating to estimate viable cell count.

Standardization of Laurell Electroimmunodiffusion

Since the amount of toxin in the concentrated supernatant fluid was to be determined by Laurell electroimmunodiffusion, it was necessary to prepare a standard curve using known concentrations of enterotoxin A and antibody.

Buffer: 0.1 μ barbital buffer pH 8.6 was used for the electro-immunodiffusion. The buffer contained 21.35g/1 5,5' - diethylbar-bituric acid (Sigma Chemical Corporation, St. Louis, Missouri) and 4.0 g/1 NaOH in deionized distilled water. A buffer of 0.05μ used for diluting both the antigen and the antiserum was prepared by diluting the 0.1μ buffer with an equal volume of deionized distilled

water and preserved with 0.1 mg/ml merthiolate.

Antigen: The antigen used was staphylococcal enterotoxin A. The enterotoxin was supplied in lyophilized form and reconstituted in 0.37% BHI broth (Difco, Detroit, Michigan) as suggested by Bergdoll. The stock solution was stored at -20°C. Working solutions were then obtained by serial dilutions in 0.05μ barbital buffer preserved with 0.1 mg/ml merthiolate. They were stored in screw-capped vials at 4°C. Typical concentrations were 10 μg/ml, 5 μg/ml, 2 μg/ml, and .5 μg/ml of toxin protein.

Antibody: The antibody was also supplied by Dr. Merlin Bergdoll, Food Research Institute, University of Wisconsin, Madison, Wisconsin. It was lyophilized type-A specific antiserum made in rabbit. The antibody was restored to its original volume by adding 1.0 ml of 0.05 barbital buffer preserved in 0.1 mg/ml merthiolate. A working solution was prepared by diluting 1:10 and stored in several small vials at 4°C. Further dilutions were made in agarose when needed for a run. The optimum sensitivity of the antiserum was not achieved till after 8 days of standing at 4°C.

Preparation of antibody containing agarose gels was as follows: The solid support medium was 1% agarose (Marine Colloids, Inc., lot #501600) prepared in 0.05μ barbital buffer, pH 8.6. Several milliliters were prepared at once, dispensed into pyrex bottles and stored at 4°C until needed. This was done to insure

reproducibility. When required, the contents of the pyrex bottles were re-melted in boiling water and equilibrated to 50°C in a constant temperature water bath. Measured volumes were dispensed into pre-warmed vials.

For a final dilution, antiserum working solution was now added (v/v) to the pre-warmed agarose containing vial with a pre-warmed pipette. The contents of the vial was rotated gently and immersed carefully in a 50°C water bath.

Using a pre-warmed diSPo pipette 5-3/4" long (Scientific Products, McGraw Park, Illinois), the antibody containing agarose was pipetted into a pre-formed mold by the method of Gasper (25) and left to solidify for 5 minutes in 4°C.

The preparation of the agarose gel after hardening was achieved by removing the clamps and the remainder of the mold placed on a levelled surface and the closed ege of the template rested against a stop plate. The top glass slide was carefully pushed off the gel over the stop plate. The plastic template was then peeled away. Four small wells 4 mm diameter were cut into the gel with a sharp edged glass tube by placing a lucite cover template with four 6-mm diameter holes on the gel surface and reaching through the holes to cut the wells. The wells were 10 mm apart.

Electroimmunodiffusion: The prepared gel, resting on the glass slide was placed on a surface cooled by a mixture of ice and

water in a conventional immunoelectrophoresis unit (Universal Electrophoresis Apparatus, Colab Laboratories, Chicago Heights, Illinois). The cooling bath was replenished with ice crystals when the temperature got above 15°C. The idea was to dissipate the heat generated by the electrophoresis to maintain a constant mobility of the antigen and avoid denaturation. Both electrode vessels contained 0.1μ barbital buffer pH 8.6 with electrical bridge contact between the buffer and gel cut from Whatman #3 mm filter paper to sizes of 55 mm by 70 mm. The two wicks were lightly placed on both ends of the gel after the entire lengths were pre-moistened with the buffer. The short edges were just placed flushed on the agarose.

A constant current of 25mA or 5 mA/cm was applied for 20 mins or until a constant voltage was reached. The wells were filled with 10 μl of standard toxin solutions containing varying concentrations of enterotoxin A. Typical concentrations were 0.5 μg/ml, 2 μg/ml, 5μg/ml and 10 μg/ml. Electrophoresis lasted 9 hrs.

Detection of Cones: The precipitin cones were made visible by immersion of the agarose gel in 0.2 M NaCl for 1 hr at room temperature. At the end of 1 hr, it was briefly rinsed with distilled water. Absorbent wipers were gently touched to the sides of the gel and glass slide to remove excess water. The surface of the gel slab was overlaid with sheep anti-rabbit globulin (purified globulin fraction, Nutritional Biochemicals Corporation, Cleveland, Ohio)

diluted 1:10 in 0.05μ barbital buffer pH 8.6 and preserved with 0.1 mg/ml merthiolate. It was allowed to react at room temperature for 1 hr. After the reaction, it was rinsed with distilled water and wiped off with absorbent wipers and allowed to dry. The gel was then immersed in 1% cadmium acetate for 30 mins to impregnate the complex, hence, visibly enhancing the precipitate. The precipitin cones were visible enough at this point to be measured. The concentrations of enterotoxin A in unknown bacterial culture supernatant fluids were determined by relating the length of the precipitin cones to the standard curve. The immunoelectrophoretic conditions remained the same as for the standard curve except that toxin concentrations were unknown.

Physiological Studies

The Effect of 2, 4-Dinitrophenol (2, 4-DNP)

The effect of 2, 4-DNP on the synthesis of enterotoxin A was studied.

S. aureus FRI #722 was used for the inhibitor studies. Stock cultures, maintained a Trypticase Soy Agar (BBL), were stored at 2°C and transferred every three weeks.

In the experiment, cells for the preparation of inocula were grown in 37°C for 12 hrs on Trypticase Soy Agar slants. The growth from the slant was inoculated into 50 mls of single strength

BHI broth and grown at 37°C for 12 hrs shaken at 120 rpm. The growth medium was adjusted to 10^8 cells/ml by dilution to required O. D. to fit the standard curve. This suspension (0. 2 ml) was inoculated into 50 ml BHI broth supplemented with appropriate concentration of 2, 4-DNP in a 250 ml Erlenmeyer flask. Incubation was for 24 hours at 37°C on a gyratory shaker. The supernatant solution was collected by centrifugation of the culture at 12, 000 g for 30 min, and subjected to serological assay for enterotoxin A.

Development of Culture Medium for Production of Enterotoxin A

The method used for the study was the sac culture technique (21). Different media were designed in an attempt to increase cell growth and toxin production, and are represented below:

Quantities indicated are those added to 1 liter of distilled water

MEDIUM 1		MEDIUM 2-A	
BHI broth	37 gms	Nicotinic Acid	50 mgs
Nicotinic Acid	50 mgs	Thiamine-HCl	0.5 mg
Thiamine-HCl	0.5 mg	Aspartate	1 mg
pH	7.5	Calcium Pantothenate	50 mgs
		BHI broth	37 gms
		pH	7.5

MEDIUM 2-B

Aspartate	1 gm
Sodium Acetate	2.0 gms
Nicotinic Acid	50 mgs
BHI broth	37 gms
Calcium Pantothenate	50 mgs
pH 7.5	

MEDIUM 3-A

Nicotinic Acid	50 mgs
Thiamine-HCl	0.5 mg
Aspartate	1 gm
Calcium Pantothenate	50 mgs
BHI broth	37 gms
Succinate	3.70 gms
pH 7.5	

MEDIUM 3-B

Nicotinic Acid	50 mgs
Thiamine-HCl	0.5 mg
Aspartate	1 gm
Calcium Pantothenate	50 mgs
BHI broth	37 gms
Succinate	1.85 gms
pH 7.5	

MEDIUM 4-A

BHI broth	37 gms
Succinate	1.35 gms
Calcium Pantothenate	50 mgs
Thiamine-HCl	0.5 mg
Nicotinic Acid	50 mgs
Guanine Monophosphate	0.5 mg
Adenosine	0.5 mg
Magnesium Phosphate	20 mgs
Sodium Acetate	3.0 gms
pH 7.5	

MEDIUM 4-B

BHI broth	37 gms
Succinate	2.70 gms
Calcium Pantothenate	50 mgs
Thiamine-HCl	0.5 mg
Nicotinic Acid	50 mgs
Guanine Monophosphate	0.5 mg
Adenosine	0.5 mg
Magnesium Phosphate	20 mgs
Sodium Acetate	6.0 gms
pH 7.5	

Cells for the preparation of inocula were grown at 37°C for 9 hrs on Trypticase Soy Agar slants. A loopful from this was inoculated into 50 mls of BHI broth supplemented with 0.5 mg/l thiamine-HCl and 50 mg/l nicotinic acid. It was then grown at 37°C for 16 hrs shaken at 120 rpm. The test flasks were then inoculated with 0.2 ml of 10^8 cells/ml. They were grown for 16 hours at 37°C shaken at 120 rpm. The cells were removed by centrifugation at 12, 000 g and the supernatant assayed for enterotoxin. The above media were investigated as possible growth media.

Growth and Toxin Production Kinetics in Medium 1

S. aureus strain FRI #722 was used. Cells were grown by the sac culture method. Seventy-five mls of culture medium were delivered into each of the cellophane sacs, tied and put in U-shaped forms in 250 ml Erlenmeyer flasks. The flasks were stoppered and autoclaved. Twenty mls of sterile 0.02 M sodium phosphate buffer in physiological saline pH 7.4 was put in each of the flasks aseptically. The flasks were inoculated with 3×10^6 cells each. Twenty-four Erlenmeyer flasks (250-ml capacity) were treated as such. The 24 flasks were then incubated in 37°C shaken at 120 rpm. The harvest was centrifuged at 12, 000 g and the supernatant fluid assayed by Laurell electroimmunodiffusion. Viable cell count was assayed by spread plate technique in Staphylococcus 110 medium. Toxin and

growth were assayed in triplicate and results are averages.

In order to make accurate comparisons of the enterotoxin produced by S. aureus FRI #722, measurement had to be made during the first 17 hours.

Membrane Ultrafiltration

Since enterotoxin A is produced in very small quantities, it was necessary to concentrate the supernatant fluid for testing its toxigenicity. Diaflo ultrafiltration membrane with 10, 000 molecular weight cut-off was chosen since it prevents partial entry into the membrane of the enterotoxin A which has a molecular weight of 34, 700.

Concentration was carried out as described by Gasper (25). Low molecular weight compounds were forced out through a PM-10 membrane (25 mm) in a 10 ml capacity Diaflo ultrafiltration cell (Amicon Corporation, Cambridge, Massachusetts). Nitrogen pressure of 55 psi was applied on one side of the membrane to concentrate till a final volume of 1 ml was reached.

Effect of Trypsin on Recovery of Enterotoxin A From PM-10 Membrane

The effect of trypsin on the recovery of enterotoxin A from PM-10 membrane was investigated. The strain of S. aureus used

for this investigation was FRI #722. Cells for preparation of inocula were grown at 37°C for 12 hrs on trypticase Soy Agar slant. The growth from the slant was then inoculated into 50 mls of BHI broth supplemented with 50 mg/l nicotinic acid and 0.5 mg/l thiamine-HCl. The growth was then diluted to $3x10^6$ cells/ml and used as final inocula. Trypsin used was purchased from Difco Laboratory, Detroit, Michigan. A concentration of $3 x10^6$ cells were suspended in 40 mls of 0.02 M phosphate buffer and grown by method of Donnelly. The nutrient medium consisted of 75 mls of single strength BHI broth supplemented with 50 mg/l nicotinic acid and 0.5 mg/l thiamine-HCl. The growth was centrifuged at 12,000 g for 30 mins, divided equally, and treated with the following concentrations of trypsin: 0%, 0.01%, 0.08%, and 0.16%. The trypsinized samples were subjected to concentration and immunoelectrodiffusion to determine percentage recovery.

RESULTS AND DISCUSSION

Standardization of Cells by Optical Density Method

To standardize the cells, it was necessary to do both turbidimetric measurements as well as viable cell count. Even though bacterial suspensions do not actually follow Lambert-Beer's law, it was a close enough approximation in dilute suspensions at a wavelength of 530 nM to obtain a usable growth curve. Bausch and Lomb spectronic 20 spectrophotometer was used for the measurements.

Standardization of S. aureus strain FRI #722 was done as described in the Materials and Methods and the results are represented as follows:

Table 1. Standardization of S. aureus strain FRI #722 by turbidimetric measurements.

Time in hrs[a]	5	5.5	6	6.5	7
Average O. D.[b]	.04	.11	.24	.45	.72
Average # cells[c]	2.1×10^7	6.0×10^7	1.8×10^8	3.8×10^8	6.5×10^8

[a] Time is given in hours after inoculation and shaking had commenced.

[b] O. D. readings are at a wavelength of 530 nM, and results are averages of two readings.

[c] Average number of cells are given per ml of sample. They are averages of three separate platings.

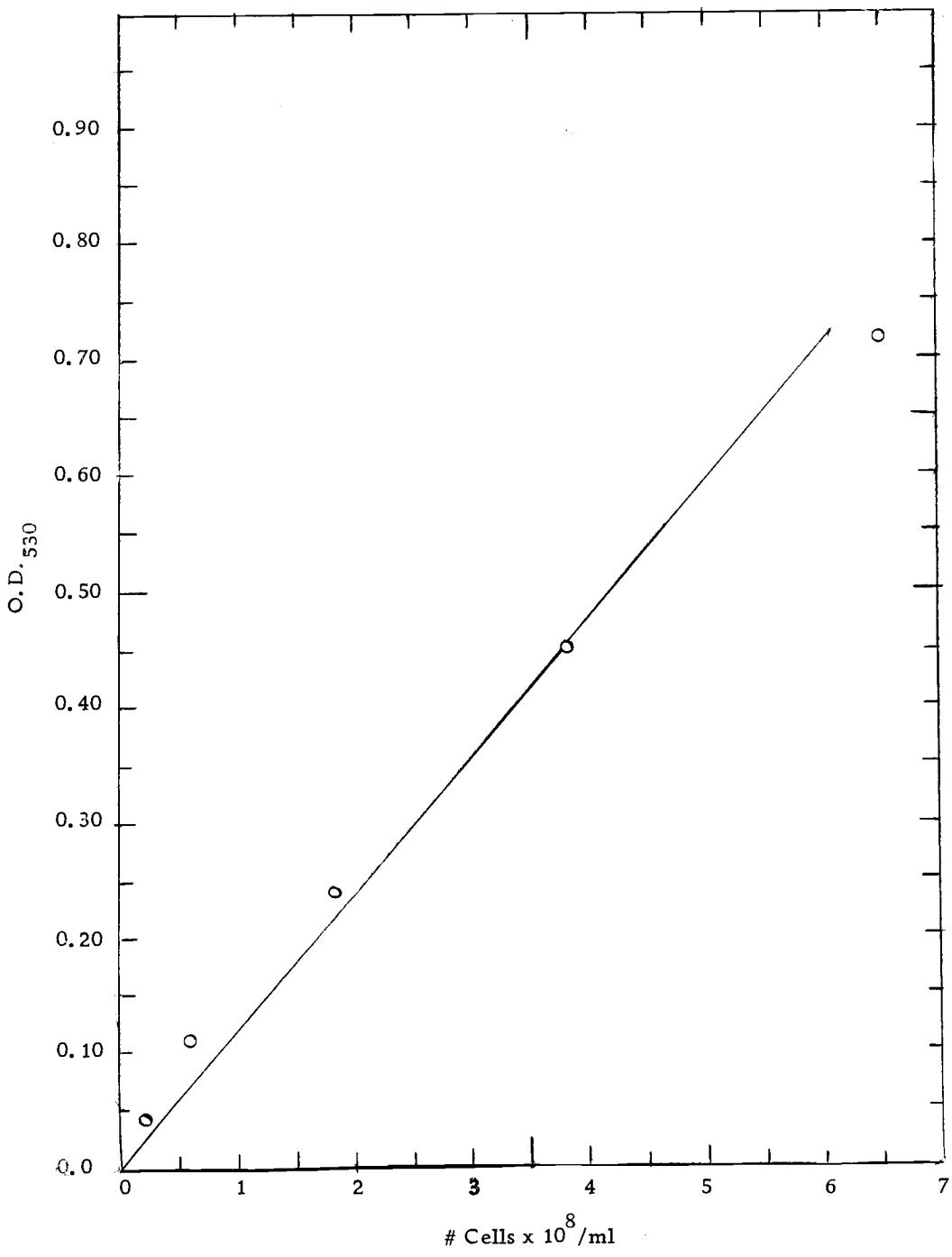


Figure 1. Standard curve of viable cell count as a function of its optical density at 530 nM. Cells were grown in BHI broth supplemented with 0.5 mg/1 thiamine-HC1 and 50 mg/1 nicotinic acid; shaken at 120 rpm at 37°C.

Standardization of Laurell Electroimmunodiffusion

Laurell electroimmunodiffusion was used for the determination of the concentrations of enterotoxin A in the culture supernatant fluids. Known concentrations of enterotoxin A were subjected to electroimmunodiffusion in an antibody containing agarose to obtain the standard curve. The antibody concentration in the agarose was 1:200. The protocol for the preparation of the standard curve is described in the Materials and Methods and the results are represented in the table below. They are averages of three different runs.

Table 2. Relationship between optimum cone height and the amount of enterotoxin A during Laurell electroimmunodiffusion. Antibody dilution was 1:200.

Conc. toxin μg/ml	Cone length in mm
0.5	20.5
2	26.0
5	34.0
10	43.0

Effect of 2, 4-DNP on Synthesis of Enterotoxin A

In order to develop a suitable culture medium for stimulation of low enterotoxin A producers, the metabolic properties of *Staphylococcus aureus* were first investigated. FRI strain #722 was used to study the effect of 2, 4-DNP on growth and toxin synthesis.

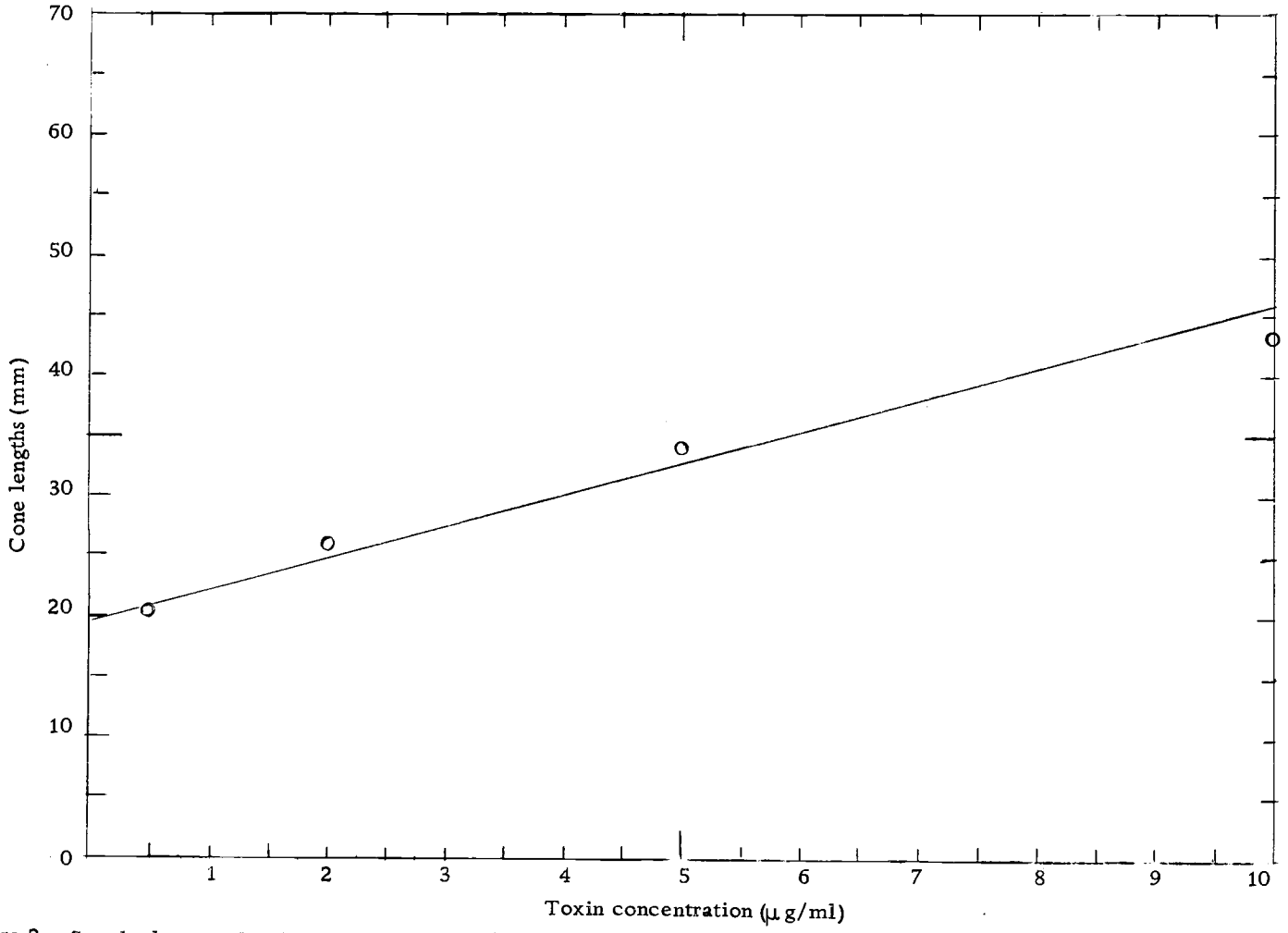


Figure 2. Standard curve showing the relationship between optimum cone height and the concentration of enterotoxin A applied to wells during Laurell electroimmunodiffusion. Antibody dilution is 1:200.

Donnelly et al. (20) have shown that the production of enterotoxin A in milk is associated with microbial growth. Markus and Silverman (42) showed that enterotoxin A secretion resembled that of a primary metabolite by being secreted during the exponential phase of growth. They also showed that non-replicating cells harvested after 3.5 hrs of growth and incubated for 4 hrs at 37°C in medium 2 (4% N-Z amine type A, 0.1% K_2HPO_4) supplemented with 2 μmoles/ml of 2, 4-DNP did not secrete any enterotoxin A. They found that although most of the enterotoxin A secretion occurred during exponential phase of growth, 20% of the total was secreted during the early stationary phase.

The energy released during the oxidation of the electrons as they move along the electron transport chain is coupled to the synthesis of ATP. Hall and Palmer (28) in their review article, stated that 2, 4-DNP when added to mitochondria in state IV (Mitochondria with endogenous ADP), increases the rate of electron flow. They suggested that the control of electron flow exerted by the phosphorylating system has been released. 2, 4-DNP may bring about this uncoupling by causing the dissipation of some high energy intermediate other than ATP (X~I and X~P). The diagrammatic representation of the respiratory chain and the phosphorylating reaction according to Hall and Palmer is shown in Figure 3:

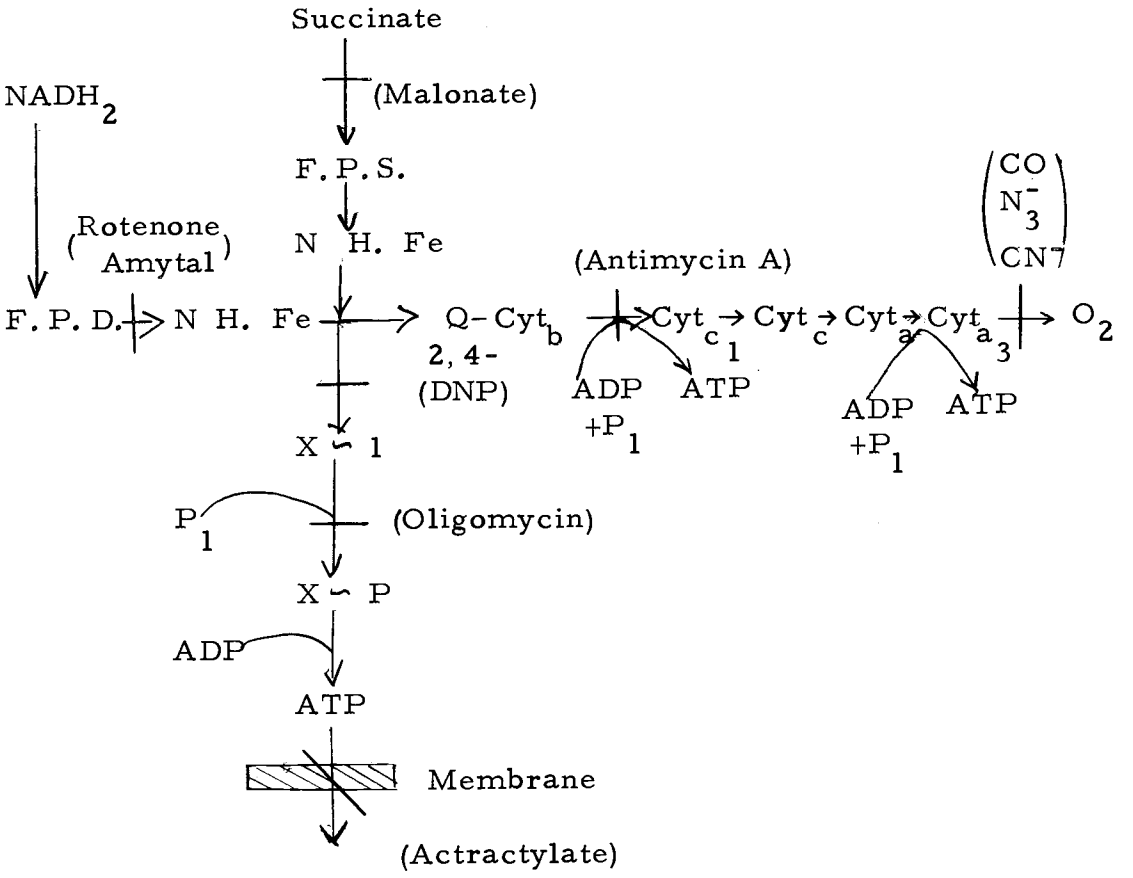


Figure 3. Diagrammatic representation of the respiratory chain and phosphorylation reaction according to Hall and Palmer. F. P. D. denotes NADH dehydrogenase; FPS, succinic dehydrogenase; N H. Fe, non heme iron; Q, ubiquinone. - denotes inhibition by the compound in parentheses.

Putting cells under physiological stress by the use of 2, 4-DNP should give us indirect evidence as to whether toxin synthesis is an essential or unessential metabolic process. The rationale being that, if with the addition of varying concentrations of 2, 4-DNP, it is found that simultaneously reduced synthesis of toxin and growth always occur, then toxin synthesis may be a necessary metabolic process. Another explanation for the observed expression of both growth and toxin production in the presence of a limiting concentration of 2, 4-DNP is that the process of growth cannot be separated from the synthesis of toxin.

The effect of 2, 4-DNP was investigated as described in the Materials and Methods and the results are as follows:

Table 3. Effect of different concentrations of 2, 4-DNP on the production of enterotoxin A and growth. Staphylococcus aureus strain FRI #722 was used.

DNP mM conc.	Enterotoxin A μg/ml	% Growth[a]
0.0	14	100
0.1	3	30
1.0	0	0
2.0	0	0

[a]% Growth measured by optical density.

In the experiment in which the effect of 2, 4-DNP on the synthesis of enterotoxin A was investigated, the results suggested the following:

1) Toxin synthesis may be a necessary process in replicating cells or

2) Toxin synthesis cannot be uncoupled from the process of growth.

If this were not so, addition of varying concentrations of 2, 4-DNP should have retarded toxin synthesis and shifted the available energy towards maintaining adequate growth. This did not happen, instead, 2, 4-DNP reduced both growth and toxin production by almost equal proportions. This also suggests that one cannot uncouple growth from toxin production by simple energy limitation. It might also be interpreted to mean that availability of energy may go to support growth and toxin production equally. This is very important in terms of designing a culture medium to spark both growth and toxin synthesis.

Development of Culture Medium for Production of Enterotoxin A

Stedman and Kravitz (49) have given evidence for a common pathway for pyruvate and acetate oxidation by Micrococcus pyogenes var. aureus. They presented data in which certain tricarboxylic acid (TCA) intermediates were used to spark the oxidation of pyruvate and acetate. Goldsmith and Powelson demonstrated the sparking of acetate by succinate but not by fumarate (26). All these present evidence that TCA cycle might be used as an energy

pathway during growth. Based on the above suggestion, different media were designed in an attempt to increase cell growth and toxin production.

The method used for the study was the sac culture technique (21). The experiment in which the effect of the metabolic inhibitor, 2, 4-DNP, on the synthesis of enterotoxin A was tested, suggested that toxin production may be coupled to growth. Growth and toxin production depend on the availability of energy. Medium 1, 2-A, 2-B, 3-A, 3-B, 4-A, 4-B were examined for their ability to increase the amount of enterotoxin A produced by S. aureus strain FRI #722.

The protocol was as described in the Materials and Methods and the results are as follows:

Table 4. Enterotoxin production in different growth media.

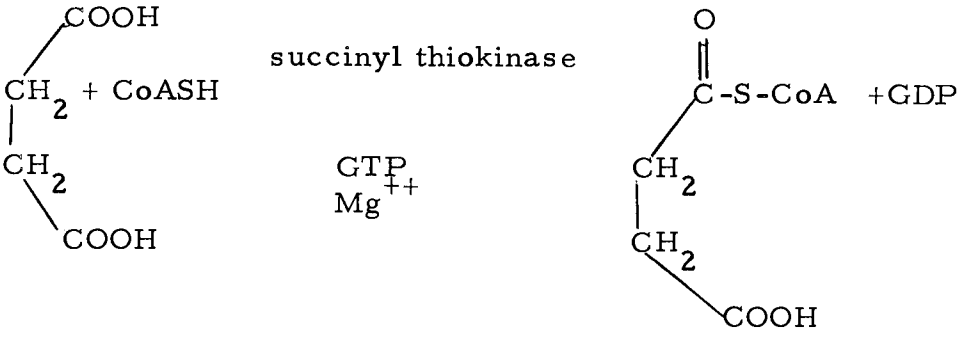
Medium	Enterotoxin μg/ml[a]
1	60 ± 4
2-A	60 ± 7
2-B	60 ± 8
3-A	50 ± 2
3-B	60 ± 8
4 -A	75 ± 5
4 -B	80 ± 5

[a] / Results are averages of three different trials.

The effect of different culture media on the production of enterotoxin A by S. aureus strain FRI #722 was tested as shown in this experiment. This was an attempt to shift the metabolism of the cells towards aerobic respiration. The idea was to generate more energy to support both growth and toxin synthesis.

The highest amount of toxin was obtained in medium 4-B, which was 33.3% more than obtained in medium 1. The result suggests that the cells may have been generating most of the energy necessary for growth and toxin production using aerobic respiration.

Medium 3-A when compared to medium 3-B showed that an increase in the amount of succinate from 1.85 gms to 3.70 gms decreased toxin synthesis by as much as 10 μg/ml. This can be explained in terms of succinate entering as succinyl-SCoA which then requires 1 mole of ATP for every mole of succinate. The reaction is represented:



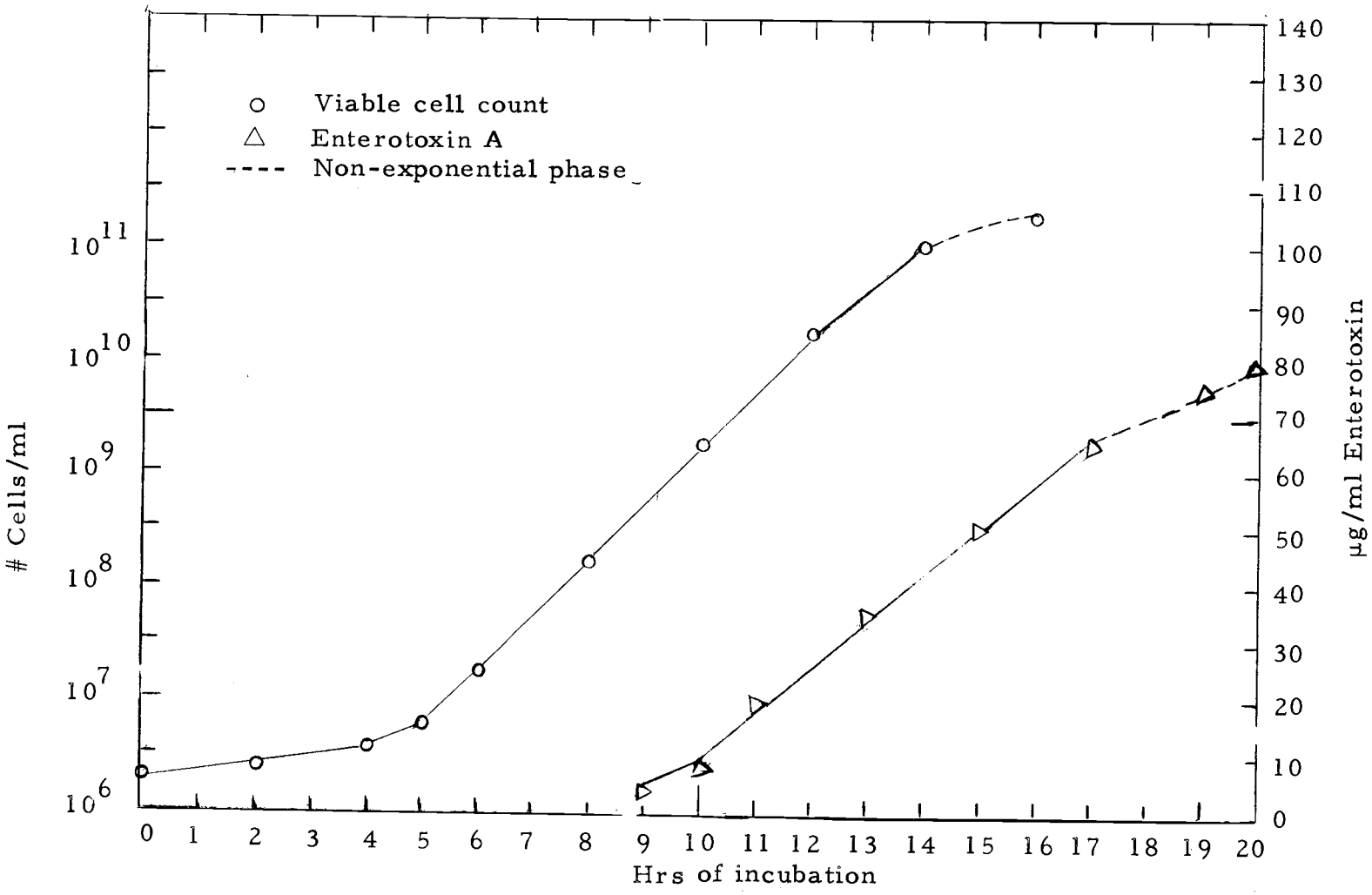
Even when succinate enters as succinyl-SCoA, there is no assurance that the succinyl-SCoA will be utilized via the Krebs cycle. It could be used in many other ways. A typical example is the diaminopimelic acid and L-lysine pathway in bacteria (40). In this pathway, starting with pyruvate, tetrahydropicolinic acid is made. This condenses with succinyl-SCoA and via a series of reactions ends up as L-lysine in gram positive organisms. In gram negative organisms, diaminopimelic acid is made instead. Another reaction that succinyl-SCoA may be used is in the synthesis of protoporphyrin IX. The function of protoporphyrin is very well known in the synthesis of cytochromes.

Growth and Toxin Production Kinetics in Medium 1

Taking into consideration the cost of production of each medium, further investigation on the growth and toxin production kinetics was carried out with medium 1 only. The experimental protocol was as described in Materials and Methods and the results are represented in a graphical form.

The growth and toxin production kinetics revealed several things about the sac culture method of toxin production. Results suggest that for the first 5 hrs, the system was establishing an equilibrium condition between phosphate flow and the flow of nutrients. The equilibrium condition was probably necessary for the exponential growth which started at the fifth hour. Toxin

Figure 4. Enterotoxin A production by cells FRI #722 in BHI broth supplemented with 0.5 mg/l thiamine-HCl and 50 mg/l nicotinic acid. The method used was the sac culture technique. Cells were harvested at different time periods and supernatants assayed for enterotoxin A using Laurell electroimmunodiffusion. Antiserum dilution was 1:200.


○ Viable cell count
△ Enterotoxin A
---- Non-exponential phase
Cells/ml
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Hrs of incubation
140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
µg/ml Enterotoxin

production followed in 3.5 to 4 hours after commencement of the exponential growth. Before the commencement of toxin production, viable cell count was about $8x10^8$ cells per ml. The secretion of enterotoxin A by the sac culture method occurred mostly during the exponential phase of growth and directly related to the cell growth. This suggests that enterotoxin A may be a primary metabolite. A question now arises, Can cell density directly trigger the production of enterotoxin A? The data in this report is not sufficient to answer this question.

Effect of Trypsin on Mobility and Recovery of Enterotoxin A

Enterotoxins in their active state are resistant to proteolytic enzymes such as trypsin, chymotrypsin, renin, and papain (4). Since S. aureus produces considerable quantity of proteins in the medium, which affects the rate of mobility of enterotoxin A during electroimmunodiffusion and the recovery of enterotoxin in PM-10 membrane, it was necessary to trypsinize the extraneous proteins for best recovery. Such treatment does not affect the the toxin.

The experimental protocol is given in the Materials and Methods and the results are as follows:

Table 5. Effect of trypsin treatment of supernatant fluids from S. aureus growth medium on the recovery of enterotoxin A from PM-10 membrane (25 mm).

Sample	% Trypsin in sample	% Recovery	Electrophoresis time for optimum cone length (hrs)
1	0	95	9.00
2	0.01	100	8.50
3	0.08	100	8.25
4	0.16	100	8.25

[a]Samples were diluted accordingly to fit the standard curve and % recovery estimated relative to the highest cone.

Detection of Enterotoxin A Using Test Strains

In summary, an excellent comparison for enterotoxin A production using different test strains could be achieved if the following steps were taken:

1) Cells are cultured by the sac culture method, using as the nutrient medium, BHI broth supplemented with 50 mg/l nicotinic acid and 0.5 mg/l thiamine-HCl, pH adjusted to 7.4, and surrounded by cells suspended in 0.02 M phosphate buffer pH 7.4.

2) Cells are grown in 37°C shaken at the rate of 120 rpm to aerate for 17 hours.

3) Growth in centrifuged at 12, 000 g for 30 mins and the extraneous proteins digested with 0.08% trypsin at 40°C for 30 min.

4) Supernatants are assayed for enterotoxin A.

5) Negative cultures are subjected to membrane ultrafiltration using PM-10 membrane at 55 psi nitrogen atmosphere.

6) Concentrated supernatants are re-assayed for the presence or absence of enterotoxin A.

Having established these parameters, the usefulness of the method was tested for the detection of enterotoxin A in culture supernatants. Three million cells were used as inoculum and 20 mls of 0.02M phosphate buffer in physiological saline pH 7.4 was used to suspend the cells. Results are represented as follows:

Table 6. Enterotoxin production by different strains of S. aureus.

S. aureus strain	Enterotoxin A (μg/ml)
FDA 209	a
ATCC 25923	a
SDA 247A	a
SDA 402 (1)	a
" 402 (2)	a
" 402 (3)	a
" 246 A	a
" 249 A	a
FRI 265-1	22
" 722	65
" 888	2.5
" 100	20
" 196E	16
" 184	a
" L-19	0.15[b]

[a]No toxin was detected even after concentration 4X using PM-10 membrane.

[b]Positive after 4X concentration using PM-10 Diaflo membrane.

The criteria used in our laboratory for labelling strains positive for type-A enterotoxin is by a combination of PM-10 Diaflo membrane ultrafiltration followed by Laurell immunoelectrodiffusion. The sensitivity described here is sufficient to detect enterotoxins obtained from strains producing small concentrations as shown in the case of FRI #888 and L-19, the quantities most commonly encountered in food poisoning.

SUMMARY

In order to compare the amount of enterotoxin A produced using different strains of S. aureus, it was found that a measurement had to be made during the first 17 hours. A study of toxin production kinetics using S. aureus strain FRI #722 shows that the exponential phase of production also ends about the seventeenth hour.

Enterotoxin A appears during the exponential phase of growth. This is different from enterotoxin B which appears during early stationary phase (41). This may explain the greater occurrence of type A intoxication in food poisoning outbreaks.

Trypsinization of the supernatant fluid after centrifugation decreased both the time required for electroimmunodiffusion and for PM-10 membrane ultrafiltration. 0.08% trypsin was the minimal concentration required to effectively digest most of the interfering proteins and free the enterotoxin. The interfering proteins were probably produced by the cells and some contributed by the nutrient media.

It is hoped that the procedure laid out in this paper can be standardized for use in most Public Health Laboratories which deal with routine isolation of Staphylococcus aureus from food samples and infections.

BIBLIOGRAPHY

1. Angelotti, R. 1969. Staphylococcal intoxications, p. 359-393. In H. Riemann (ed.), Food-borne infections and intoxications. Academic Press, New York, 698 p.

2. Aronsson, T. 1961. Concentrating solutions of macromolecular substances by sample ultrafiltration. Science Tools 8:1.

3. Avena, R. M., M. S. Bergdoll. 1967. Purification and some physicochemical properties of enterotoxin C, Staphylococcal aureus strain 361. Biochemistry 6:1474-1480.

4. Bergdoll, M. S. 1972. The nature of bacterial toxins. Clinical Toxicology 5 (4)441-451.

5. Bergdoll, M. S., C. R. Borja, and R. M. Avena. 1965. Identification of a new enterotoxin as enterotoxin C. J. Bacteriol 90:1481-1485.

6. Bergdoll, M. S., C. R. Borja, R. N. Robbins, and K. F. Weiss. 1971. Identification of enterotoxin E. Infect. Immunity 4:593-595.

7. Bergdoll, M. S., Ruth Robbins, and Sara Gould. 1973. Staphylococcal aureus. Methods for enterotoxin detection. Address in Fourth International Conference on Global Impacts of Applied Microbiology Sao Paulo, Brazil.

8. Bergdoll, M. S., M. J. Surgalla, and G. M. Dack. 1959. Staphylococcal enterotoxin. Identification of a specific precipitating antibody with enterotoxin neutralizing property. J. Immunol. 83:334-338.

9. Bergdoll, M. S., K. F. Weiss, and M. J. Muster. 1967. The production of staphylococcal enterotoxin by a coagulase-negative micro-organism. Bacteriol. Proc. 1967:12.

10. Bergstrom, B. 1973. The ExaPhor Ready-Made Electro-Immuno-Assay Plate. A new tool for the clinical immunologist. Science tools 20 (2-3) 21, 36.

11. Blatt, W. F., M. P. Feinberg, H. B. Hopfernberg, and C. A. Saravis. 1965. Protein solutions: Concentration by a rapid method. Science 150:224-226.

12. Blatt, W. F., B. G. Hudson, S. M. Robinson and E. M. Zipilivan. 1967. Fractionation of protein solutions by membrane partition chromatography. Nature 216:511-513.

13. Borja, C. R. and M. S. Bergdoll. 1967. Purification and partial characterization of enterotoxin C produced by Staphylococcal strain 137. Biochemistry 6:1467-1473.

14. Casman, E. P. 1960. Further serological studies of staphylococcal enterotoxin. J. Bacteriol. 79:849-856.

15. Casman, E. P., and R. W. Bennett. 1963. Culture medium for the production of staphylococcal enterotoxin A. J. Bacteriol. 86:18-23.

16. Casman, E. P., R. W. Bennett, A. E. Dorsey, and J. A. Issa. 1967. Identification of a fourth staphylococcal enterotoxin, enterotoxin D. J. Bacteriol. 94:1875-1882.

17. Cheslon, W. R., and K. Auburn. 1967. Enzymatic detection of the growth of Staphylococcus aureus in foods. Appl. Microbiol. 15:1150-1157.

18. Chugg, L. R. 1972. Rapid, sensitive methods for enumeration and enterotoxin assay for Staphylococcus aureus. Ph. D. thesis, 82 p. Oregon State University, Corvallis, Oregon 97331.

19. Coulter, J. R. 1961. Production, purification, and composition of staphylococcal α-toxin. J. Bacteriol. 92:1655-1662.

20. Donnelly, C. B., J. E. Leslie, and L. A. Black. 1968. Production of enterotoxin A in milk. Appl. Microbiol. 16: 917-924.

21. Donnelly, C. B., J. E. Leslie, L. A. Black and K. H. Lewis. 1967. Serological identification of enterotoxigenic staphylococci from cheese. Appl. Microbiol. 15:1382-1387.

22. Fish, D. C., B. G. Mahlandt, J. P. Dobbs and R. E. Lincoln. 1968. Purification and properties of In vitro-produced

anthrax toxin components. J. Bacteriol. 95:907.

23. Fung, D. Y. C. and J. Wagner. 1971. Capillary tube assay for staphylococcal enterotoxin A, B and C. Appl. Microbiol. 21:559-561.

24. Gandhi, N. R., and G. H. Richardson. 1971. Capillary tube immunological assay for staphylococcal enterotoxins. Appl. Microbiol. 21:626-627.

25. Gasper, E. 1973. Detection of Staphylococcal enterotoxin A by Laurell electroimmunodiffusion. Ph. D. thesis, 71 p. Oregon State University, Corvallis, Oregon 97331.

26. Goldschmidt, M. C., Dorothy M. Powelson. 1953. Effect of culture medium on the oxidation of Acetate by Micrococcus pyogenes var. aureus. Arch. Biochem. and Biophys. 46: 154-163.

27. Hall, H. E. R., Angelotti, and K. H. Lewis. 1965. Detection of Staphylococcal enterotoxins in food. Health Lab. Sci. 2: 179-191.

28. Hall, D. O., J. M. Palmer. 1969. Mitochondrial Research today. Nature 221:717-723.

29. Hallander, H. O. 1965a. Production of large quantities of enterotoxin B and other Staphylococcal toxins on solid media. Acta Pathol. Microbiol. 63:299-305.

30. Hallander, H. O. 1965b. Production of large quantities of enterotoxin B and other Staphylococcal toxins on solid media. Acta Pathol. Microbiol. Scand. 67:117-132.

31. Heidelberger, M. 1939. Quantitative absolute methods in the study of antigen-antibody reactions. Bact. Rev. 3:49.

32. Jarvis, A. W. and K. C. Lawrence. 1970. Production of high titers of enterotoxin for the routine testing of Staphylococci. Appl. Microbiol. 19:698-699.

33. Johnson, H. M., J. A. Bukovic, P. E. Kauffman and J. T. Peder. 1971. Staphylococcal enterotoxin B. Solid-phase radioimmunoassay. Appl. Microbiol. 22:837-841.

34. Kabat, E. A. 1961. Dialysis and Ultrafiltration, p. 429-436. In Kabat, E. A. (ed.), Kabat and Mayer's Experimental Immunochemistry. Thomas, Springfield, Illinois.

35. Kato, E., M. Khan, L. Kajovich, and M. S. Bergdoll. 1966. Production of enterotoxin A. Appl. Microbiol. 14:966-972.

36. Kimble, C. E. 1970. Improved detection methods and physiological studies of Staphylococcal enterotoxin. Ph. D. thesis, 63 p. Oregon State University, Corvallis, Oregon 97331.

37. Lachica, R. V., K. F. Weiss, and R. H. Deibel. 1969. Relationships among coagulase, enterotoxin, and heat-stable deoxyribonuclease production by Staphylococcal aureus. Appl. Microbiol. 18:126-127.

38. Laurell, C. B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal. Biochem. 15:45-52.

39. Laurell, C. B. 1972. Electroimmuno assay. Scand. J. Clin. Lab. Invest. 29. Suppl. 124, p. 21-23.

40. Lehninger, A. L. 1970. Biochemistry. Worth Publishers, Inc., New York. 833 p.

41. Markus, Z., Gerald J. Silverman. 1968. Enterotoxin B production by non-growing cells of Staphylococcus aureus. Journal of Bacteriol. 96:1446-1447.

42. Markus, Z. H., G. J. Silverman. 1970. Factors affecting the secretion of Staphylococcal enterotoxin A. Appl. Microbiol. 20:492-496.

43. Morse, S. A., and R. A. Mah. 1967. Microtiter hemagglutination-inhibition assay for Staphylococcal enterotoxin B. Appl. Microbiol. 15:58-61.

44. Orth, D. S., and A. W. Anderson. 1970. Polymyxin-Coagulase-Mannitol Agar 1. A selective isolation medium for coagulase-positive staphylococci. Appl. Microbiol. 19: 73-75.

45. Orth, D. S. and A. W. Anderson. 1970. Polymyxin-Coagulase-deoxyribonuclease Agar: A selective isolation medium for Staphylococcus aureus. Appl. Microbiol. 20: 508-509.

46. Ouchterlony, O. 1953. Antigen-antibody reactions in gels IV. Types of reactions in co-ordinated systems of diffusion. Acta Pathol. Microbiol. Scand. 32:231.

47. Robbins, R., Sara Gould, Merlin Bergdoll. Detecting the enterotoxigenicity of Staphylococcal aureus strains. Food Research Institute, University of Wisconsin, 2115 Herrick Drive, Madison, Wisconsin 53706. FDA Contract 69-36 publication.

48. Stark, R. L., and P. R. Middaugh. 1969. Immunofluorescent detection of enterotoxin B in food and culture medium. Appl. Microbiol. 18:631-635.

49. Stedman, R. L., E. Kravitz. 1955. Evidence for a common pathway for pyruvate and acetate oxidation by Micrococcus pyogenes var. aureus. Arch. of Biochem and Biophys. 59: 260-268.

50. Silverman, S. J., A. R. Knott and M. Howard. 1968. Rapid sensitive assay for Staphylococcal enterotoxin and a comparison of serological methods. Appl. Microbiol. 16: 1019-1023.

51. U.S. Department of Health, Education and Welfare. Annual summary of food borne outbreaks. 1971. Center for Disease Control, Atlanta, Georgia. 28 p.