EFFECT OF TEMPERATURE AND pH ON PRODUCTION OF STAPHYLOCOCCAL ENTEROTXIN IN BRAIN HEART INFUSION BROTH AND IN FOOD

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

DALE LEE SCHEUSNER

1972

THESIS

This is to certify that the

thesis entitled

Effect of Temperature and pH on Production of Staphylococcal Enterotoxin in Brain Heart Infusion Broth and in Food

presented by

Dale L. Scheusner

has been accepted towards fulfillment of the requirements for

Ph. D. degree in Food Science

Laurence G. Harmon
Major professor

Date Feb. 21, 1972

O-7639

ABSTRACT

EFFECT OF TEMPERATURE AND pH ON PRODUCTION
OF STAPHYLOCOCCAL ENTEROTOXIN IN
BRAIN HEART INFUSION BROTH AND IN FOOD

By

Dale Lee Scheusner

Growth and enterotoxin production by four stains of *Staphylococcus aureus* which produce four immunologically different types of enterotoxins were compared in food and in Brain Heart Infusion (BHI) broth. Measurement was made of the hours of incubation required before enterotoxin could be detected by the microslide gel-diffusion assay. The temperature range for enterotoxin production was established for the four strains, and the pH range for production of type B enterotoxin was established. Comparisons were made of the incubation times required to produce measureable amounts of enterotoxin in food and in BHI broth.

Strain 243 of *S. aureus*, which produces type B enterotoxin, was inoculated into BHI broth with the initial pH adjusted with and without 0.2M sodium phosphate buffer. The intial pH of the BHI broth was adjusted to several values within the range of 3.6 to 9.9. Growth occurred in the agitated broth when the initial pH was within the range of 4.7 to 9.4; whereas, production of enterotoxin was

restricted to the pH range of 5.1 to 9.0. Less incubation time was needed to produce measurable amounts of enterotoxin in the nonbuffered BHI broth than in BHI broth which contained the phosphate buffer.

Four strains of *S. aureus* were inoculated into buffered BHI broth and incubated with agitation at several selected temperatures within the range of 7 to 50 C. Only minor differences in growth and enterotoxin production were observed among the four strains. None of the cultures grew at 7 or 50 C. Growth of all four strains occurred at 13 C, but only one strain produced enterotoxin after one week of incubation. All strains produced enterotoxin in the range of 19 to 39 C. Three strains grew and produced enterotoxin at 45 C, and there was a rapid decline in the population of the other strain. The same four strains all grew and produced enterotoxin when incubated in vanilla pudding in the temperature range of 19 to 45 C.

Numerous foods were inoculated with *S. aureus*. In some foods the organisms did not grow. The lack of growth was attributed to low pH, low water activity, competition from other microorganisms, or inhibitors in the food. In some foods enterotoxin was not detected although the organism grew; whereas, in other foods both growth and toxin were detected. Growth and enterotoxin production by *S. aureus* in agitated BHI broth could not be correlated directly to growth and enterotoxin production in food.

EFFECT OF TEMPERATURE AND pH ON PRODUCTION OF STAPHYLOCOCCAL ENTEROTOXIN IN BRAIN HEART INFUSION BROTH AND IN FOOD

By

Dale Lee Scheusner

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition

1972

67-1054

ACKNOWLEDGEMENTS

The author expresses his thanks and appreciation to Dr. L. G. Harmon for his counsel and guidance during the course of the Ph.D. program.

The author is grateful to Dr. C. M. Stine, Dr. E. S. Beneke, Dr. Evelyn Sanders, Dr. R. V. Lechowich, and Dr. K. E. Stevenson for their counsel and service on the advisory committee.

Gratitude is expressed to the microbiology laboratory of the Federal Food and Drug Administration in Washington, D. C., for furnishing the purified enterotoxins and the balanced antitoxins which were used in this investigation.

For financial support, the author expresses his thanks to the United States Public Health Service and to the Michigan Agricultural Experiment Station of Michigan State University.

The author is indebted to the Department of Food Science and Human Nutrition for the use of facilities for this research project.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	ii
LIST OF TABLES.	v
LIST OF FIGURES	vii
INTRODUCTION	1
REVIEW OF LITERATURE.	3
MATERIALS AND METHODS	10
Cultures.	10
Media.	10
Foods.	11
Inoculation of Foods and BHI Broth	14
Incubation and Sampling of Foods and BHI Broth . .	14
Determination of pH	16
Determination of Population	16
Storage of Samples for Enterotoxin Analysis . . .	17
Extraction of Enterotoxin from Foods	17
Enterotoxin Assay.	18
Decontamination of Equipment	19
RESULTS	20
Estimating Concentrations of Enterotoxin	20
Growth Characteristics of _S. aureus_ in BHI Broth Buffered with Phosphate	22
Effect of pH on Growth and Enterotoxin Production by _S. aureus_ 243 in BHI Broth	24
Effect of Temperature on Growth and Enterotoxin Production by Four Strains of _S. aureus_ in Buffered BHI Broth.	31
Effect of Temperature on Enterotoxin Production in Vanilla Pudding.	34
Growth and Enterotoxin Production by _S. aureus_ in Selected Foods	37

Page

DISCUSSION 42

Estimating Concentrations of Enterotoxin 42
Effect of pH on Growth and Enterotoxin Production by *S. aureus*. 44
Effect of Temperature on Growth and Enterotoxin Production by *S. aureus* 48
Comparisons of Growth and Enterotoxin Production Among Four Strains of *S. aureus* 50
Growth and Enterotoxin Production by *S. aureus* in Selected Foods 51
Comparisons of Enterotoxin Production in BHI Broth and in Vanilla Pudding 55

SUMMARY AND CONCLUSIONS. 58

LIST OF REFERENCES 62

LIST OF TABLES

Table Page

1. Production of enterotoxin by four strains of *S. aureus* in agitated BHI broth buffered to pH 6.75 with 0.2M phosphate and incubated at 26 C 25

2. Incubation times, pH, and population associated with the samples which contained the first measurable amounts of enterotoxin B formed by *S. aureus* 243 grown at 37 C with agitation in phosphate buffered BHI broth with variable initial pH. . . . 28

3. Incubation times, pH, and population associated with the samples which contained the first measurable amounts of enterotoxin B formed by *S. aureus* 243 grown at 37 C with agitation in nonbuffered BHI broth with variable initial pH 29

4. Incubation times, pH, and population associated with the samples which contained the first measurable amounts of enterotoxin formed by four strains of *S. aureus* when incubated with agitation in BHI broth buffered at pH 7 with 0.2M phosphate 32

5. Incubation times, pH, and population associated with the samples which contained the first measurable amounts of enterotoxin formed by four strains of *S. aureus* when incubated without agitation in vanilla pudding at various temperatures 35

6. Incubation temperature and pH of selected foods in which added *S. aureus* did not grow. 38

Table | Page

7. Incubation temperature, pH, and growth response of *S. aureus* when inoculated into selected foods. 39

8. Incubation temperature, pH, and enterotoxin production of selected foods to which *S. aureus* was added and grew. 41

LIST OF FIGURES

Figure		Page
1.	Diagram of enterotoxin assay results.	21
2.	Population and pH of four strains of *S. aureus* during growth at 26 C in agitated BHI broth buffered to pH 6.75 with 0.2M phosphate	23
3.	Effect of initial pH of BHI broth on the time required for production of a detectable amount of enterotoxin B by *S. aureus* 243 incubated with agitation at 37 C	26
4.	Effect of incubation temperature on the time required for production of a detectable amount of enterotoxins A, B, C, and D by four strains of *S. aureus* incubated with agitation in BHI broth buffered at pH 7 . .	27
5.	Effect of incubation temperature on the time required for production of a detectable amount of enterotoxins A, B, C, and D by four strains of *S. aureus* incubated without agitation in vanilla pudding	36

INTRODUCTION

For many years, staphylococcal enterotoxin has been known as the cause of a common food poisoning. Nearly everyone has encountered this discomfort, although they may not have identified it as staphylococcal food poisoning. The typical symptoms of nausea, vomiting, and diarrhea usually develop 2 to 8 hr after consumption of food containing the toxin. Recovery normally occurs in 24 to 48 hr and is uneventful. Occasionally more severe symptoms such as shock, dehydration, and a drop in body temperature may occur. The intoxication is rarely fatal, but it may weaken the body thus permitting a secondary infection. Immunity in man generally is not developed, and the same person may show the symptoms of the poisoning several times.

The early research on staphylococcal food poisoning was hampered by the lack of a simple, accurate assay for the enterotoxin. The recent chemical purification of some staphylococcal enterotoxins has permitted the formation of antitoxins against these compounds. The development of immunological assays for enterotoxin followed. These assay methods, though delicate, give good reproducibility of results and can be used in nearly any laboratory. The

availability of these methods has permitted further study of the environmental conditions which lead to production of staphylococcal enterotoxin.

The early studies of staphylococcal food poisoning usually used only strains of *Staphylococcus aureus* which produced enterotoxin B. This toxin is found in relatively few outbreaks of staphylococcal food poisoning. The early investigations did show the importance of environmental conditions such as temperature, pH, and growth medium. If we know the conditions under which staphylococcal enterotoxin can be formed, then we may handle food in such a way as to reduce the incidence of staphylococcal food poisoning. With this in mind, one objective of this study was to investigate the pH range for production of enterotoxin B using Brain Heart Infusion (BHI) broth as a growth medium. Similarly, another objective was to investigate the temperature range for enterotoxin production in BHI broth. There now are available four purified enterotoxins and their antitoxins; therefore, a third objective was to compare growth and enterotoxin production by four strains of *S. aureus*, each of which produces one of the four immunologically different enterotoxins. From the practical standpoint, we are more interested in enterotoxin production in food than in BHI broth; therefore, a fourth objective was to compare results of experiments involving enterotoxin production in BHI broth and in food.

REVIEW OF LITERATURE

The problem of staphylococcal food poisoning has been studied for several years. A substantial reservoir of scientific articles has accumulated, and it does not seem necessary to review all of these publications at this time. Hood (1968) in his thesis describing work performed in this laboratory included a comprehensive literature review. Reviews have recently been published by serveral other workers in this field. The review by Hobbs (1967) points out the impracticality of a zero tolerance for *S. aureus* in foods. She suggests that a level of 100 to 1000 *S. aureus* per gram of food is a reasonable tolerance. This indicates the need for proper handling of food so that the organisms do not grow and produce toxin. Jay (1970) reviewed several articles which discussed the occurrence of *S. aureus* in many commercial foods. The cultural characteristics of the *S. aureus* organism were reviewed by Angelotti (1969) who also discussed the occurrence of this organism in various foods. In addition to enterotoxin, *S. aureus* produces several other toxins. These toxins as well as the pathogenic characteristics of *S. aureus* were discussed by Davis *et al.* (1969). The details of enterotoxin synthesis and its manner

of attack on humans is not fully understood. These topics were reviewed by Bergdoll (1970). The chemical nature of enterotoxin has been widely studied and was reviewed by Angelotti (1969), Bergdoll (1970), and Jay (1970). Several methods of assaying for enterotoxin have been attempted. Previously used animal assays recently have been replaced by several immunological methods including the microslide gel-diffusion technique of Casman and Bennett (1965). These methods for enterotoxin assay and the techniques for production and purification of relatively large amounts of enterotoxin were reviewed by Bergdoll (1970) and others. Methods for extraction of enterotoxin from food have been developed by Casman and Bennett (1965), Casman (1967), and Hall *et al*. (1965). These and other methods of extraction also were reviewed by Bergdoll (1970).

The environmental conditions for growth of *S. aureus* have been studied by several workers; however, there are relatively few studies on the environmental conditions which permit enterotoxin production. As reported by Troller (1971), enterotoxin B production by *S. aureus* is extremely sensitive to a reduction in water activity. Very small amounts of enterotoxin were produced when the water activity was decreased from 0.99 to 0.98 in one medium and to 0.97 in another medium. Growth was far less sensitive to a similar reduction in the water activity.

Less than 3% NaCl in the growth medium has essentially no effect on the amount of enterotoxin B produced as shown by McLean *et al*. (1968) and Stark and Middaugh (1969). At NaCl concentrations in the range of 3 to 10%, growth of *S. aureus* was affected only slightly, but production of enterotoxin B decreased as the NaCl concentration increased. At 10% NaCl in the growth medium, no enterotoxin was detected. The effect of NaCl concentration on the production of enterotoxin C was similar to the effect on production of enterotoxin B according to Genigeorgis *et al*. (1971) who also studied the combined effects of pH and NaCl concentration on enterotoxin production and showed that the pH range for enterotoxin production becomes narrower as the NaCl concentration increases.

Few studies have been conducted on the effect of aeration and atmospheric conditions on production of enterotoxin B by *S. aureus*. Stark and Middaugh (1969) and McLean *et al*. (1968) demonstrated that aerated cultures produce more enterotoxin and produce it in less time than do static cultures. Stark and Middaugh (1970) also found a decrease in enterotoxin production when they used Co_2 and N_2 atmospheres in growing *S. aureus*.

According to Peterson *et al*. (1964) and Walker and Harmon (1965), temperatures below 10 C inhibit growth of *S. aureus*. Between 10 and 20 C growth occurred, but a long lag phase or a long generation time was observed.

McLean *et al*. (1968) showed that lowering the temperature to 16 C decreased the total amount of entertoxin B produced even though total growth approached that observed at 37 C. The incubation time required for production of a detectable amount of enterotoxin A in milk incubated at different temperatures was determined by Donnelly *et al*.(1968). They also found that the amount of time needed for enterotoxin to be produced was dependent on the size of the *S. aureus* inoculum. Stark and Middaugh (1969) obtained growth of *S. aureus* at 12 C, but they did not detect enterotoxin B even after six days of incubation. The review by Angelotti (1969) contains a complete discussion of the effect of temperature on growth and enterotoxin production by *S. aureus*.

Several investigators, including McLean *et al*. (1968) and Morse *et al*. (1969), have noted the change in the pH of the growth medium during growth of *S. aureus*, and Peterson *et al*. (1964) reported that growth of *S. aureus* was inhibited when the initial pH of the growth medium was 5 or 9. Kato *et al*. (1966) obtained growth as well as production of enterotoxin A in 24 hr at 37 C in the pH range of 5.0 to 8.0. Morse *et al*. (1969) obtained little or no enterotoxin B when the initial pH of the growth medium was less than 5.0. Using very large inocula, Genigeorgis *et al*. (1971a) obtained growth and production of enterotoxin C throughout the pH range of 4.00 to 9.83.

At this time thre are four immunologically different staphylococcal enterotoxins which have been purified and for which antitoxins are available. Some strains of S. aureus produce no enterotoxins, some produce only one enterotoxin, and others may produce a combination of enterotoxins. Casman et al. (1967) investigated the incidence of each type of enterotoxin as produced by S. aureus strains from various sources. They found that 49% of the staphylococcal food poisoning outbreaks involved only enterotoxin A and an additional 25% involved enterotoxins A and D. Enterotoxins D, B, and C were found alone in 7.6, 3.8, and 2.5%, respectively, of the staphylococcal food poisoning outbreaks. Other combinations were involved in 2.5% or less of the outbreaks. In 3.8% of the staphylococcal food poisoning outbreaks, none of the four known enterotoxins were found. In studies of growth initiation, Genigeorgis et al. (1971b) used five strains of S. aureus which produce four different types of enterotoxins. They found statistically significant differences between the growth initiation characteristics of the five strains used under various conditions of pH and NaCl concentration.

The substrate in which S. aureus is grown has a major effect on the amount of enterotoxin produced. Kato et al. (1966) reported wide variations in the amounts of enterotoxins A, B, and C produced in different growth media. They concluded that the conditions for production

of each enterotoxin should be studied separately. Friedman (1966) found that certain chemicals such as K_2HPO_4 and streptomycin when added to the growth medium may delay enterotoxin production beyond 16 hr. According to Reiser and Weiss (1969), the maximum amounts of enterotoxins A, B, and C produced are affected by the nature of the growth medium. These workers found that the maximum concentrations they obtained were 5 to 6 μg/ml of enterotoxin A, about 350 μg/ml of enterotoxin B, and over 60 μg/ml of enterotoxin C under their experimental conditions. The work of Stark and Middaugh (1969) showed that little or no difference in enterotoxin production could be detected between BHI broth and shrimp slurry. Genigeorgis _et al_. (1971c), however, found that data derived from broth studies could not be used to reliably predict initiation of growth of _S. aureus_ processed meats. The meat was more conducive to growth of _S. aureus_ than was BHI broth.

Numerous foods such as cheese and meat salads have been implicated in staphylococcal food poisoning. Jay (1970) reviewed several articles on the topic. The presence or absence of other microorganisms in food may have a major effect on enterotoxin production. As reported by Zehren and Zehren (1968), enterotoxin in cheese is often associated with lack of good growth of the normal lactic cultures. As reported by Peterson _et al_. (1962), substantial growth of _S. aureus_ was not possible under the conditions which

permitted defrost of chicken pot pies. At temperatures which permitted growth of *S. aureus* in the pot pies, spoilage occurred more rapidly than growth of *S. aureus*. Troller and Frazier (1963) found that other bacteria in food grew most rapidly under conditions which they speculated were similar to those for maximum production of staphylococcal enterotoxin. McCoy and Farber (1966) showed that in the presence of other microorganisms good growth of *S. aureus* may occur without detectable amounts of enterotoxin being formed. Genigeorgis *et al.* (1971c) used meat in their experiments and found that all the meat samples which contained enterotoxin also contained high populations of *S. aureus*; however, some samples which did contain high populations of *S. aureus* did not contain enterotoxin. The work of Read and Bradshaw (1966) on thermal inactivation of enterotoxin B in milk showed a D value of 9.4 min at 250 F and 68.5 min at 210 F. Since enterotoxin is heat stable, normal cooking procedures will not inactivate the toxin.

MATERIAL AND METHODS

Cultures

Lyophilized cultures of *S. aureus* were obtained from Dr. E. P. Casman of the Food and Drug Administration Laboratory in Washington, D. C. The four strains which were used were known to produce four immunologically different types of staphylococcal enterotoxin. Strains 265, 243, 493, and 315 produced enterotoxin types A, B, C, and D, respectively. Each strain produced only one type of enterotoxin. The lyophilized cultures were activated by incubation in BHI broth at 37 C. Following 24 hr incubation at 37 C, the cultures were stored temporarily on BHI agar slants in screw cap test tubes held at 5 C.

Media

Media used for propagating and enumerating organisms were prepared from the dehydrated forms according to the manufacturer's directions. Staphylococcus Medium 110 (S-110), Mannitol Salt Agar (MSA), Plate Count Agar (PCA), and BHI broth were obtained from Difco Laboratory (Detroit). The BHI agar was prepared by adding 1.5% Bacto-Agar (Difco) to the BHI broth. The phosphate buffered BHI broth was

prepared by adding varying amounts of $NaH_2PO_4 \cdot H_2O$ (Malinckrodt) and $Na_2HPO_4 \cdot 7H_2O$ (Baker). The total phosphate concentration was maintained at 0.2M in all trials. The amount of each salt was estimated using a buffer table and varied depending on the desired pH. When necessary, the pH was adjusted using 6N H_3PO_4 (Matheson, Coleman and Bell) or 6N NaOH (Mallinckrodt).

All media were sterilized at 121 C for 15 min. Media not used immediately were stored at room temperature and later melted in a steam bath. The medium for pour plates was tempered to 47 C before use.

Foods

Several foods were used to ascertain growth and enterotoxin production by added *S. aureus*. The foods were inoculated with *S. aureus* to determine if the results of growth and enterotoxin production by *S. aureus* added to the food could be predicted from the results of growth and enterotoxin production by *S. aureus* grown in BHI broth. Two kinds of "home-made" poultry dressing were prepared. One contained 12 cups cubed dry bread, ½ cup chopped onion, ½ cup chopped celery, ¼ cup chopped apple, ¼ cup chopped raisins, 1 teaspoon salt, ¼ teaspoon pepper, 1 teaspoon sage, 1 teaspoon poultry seasoning, 2 beaten eggs, ½ cup melted butter, 1 cup milk, 1 lb chopped giblets, and water for proper consistency. The giblets, celery, and onion

were cooked until tender. All the ingredients were mixed thoroughly, but gently, and baked in a 375 F oven for 75 min. The other dressing contained 10 cups cubed dry bread, 5 cups crushed saltine crackers, ½ cup chopped celery, 1 teaspoon poultry seasoning, 2 beaten eggs, 1 cup milk, ½ cup melted butter, 1 lb chopped giblets, and water for proper consistency. The giblets and celery were cooked until tender. All the ingredients were mixed thoroughly, but gently, and baked in a 300 F oven for 35 min.

All of the other foods used were commercial products purchased in local stores and handled normally until the experiment began. A prepared sandwich spread and a potato salad were bought at a local delicatessen (Schmidt's brand). Donut cream and Bavarian cream were removed from fresh cream-filled donuts (Dunkin Donuts brand). A prepared frozen breakfast (Swanson) contained egg, potato, and sausage each of which was used separately. Pork, chicken, and brown gravy mixes (French's) were prepared as directed on the package. The gravy mixes also were prepared with steaming times of up to an hour.

Other foods used were strained sweet potato (Gerber), strained vegetables with liver (Gerber), toddler's beef stew (Gerber), canned chili hot dog sauce (Gebhardt's), canned turkey salad sandwich spread (Carnation), canned ham salad sandwich spread (Carnation), salad dressing

(Gaylord), shrimp cheese spread (Buko), tofu (Hinode), creamed herring (Noon Hour), canned tuna (Chicken of the Sea), canned beef gravy (Franco-American), frozen tuna pot pie (Top Frost), frozen turkey pot pie (Top Frost), frozen chicken pot pie (Top Frost), frozen beef pot pie (Top Frost), frozen banana cream pie (Banquet), frozen coconut cream pie (Banquet), frozen chocolate cream pie (Banquet), berry fruit pie (Hostess), cherry fruit pie (Hostess), canned apple sauce (Food Club), imitation whipped cream (Presto Whip), polynesian parfait containing lactic culture (Michigan Cottage Cheese), canned ready-to-use vanilla frosting (Pillsbury), canned ready-to-use chocolate frosting (Pillsbury), rice pudding with raisins (Bill Stern's brand), canned rice pudding (Thank You), canned chocolate pudding (Thank You), canned butterscotch pudding (Thank You), and canned vanilla pudding (Thank You).

According to the label, Thank You brand canned, homogenized vanilla pudding contains water, sugar, nonfat dry milk, modified food starch, corn starch, vegetable shortening, mono- and diglycerides, salt, natural and artificial flavoring, and artificial color. Thank You brand is from Consolidated Foods and is packed by Michigan Fruit Canners Inc. of Benton Harbor, Michigan.

Inoculation of Foods and BHI Broth

The *S. aureus* inoculum was grown at 37 C in BHI broth and was transferred daily for at least three days before use. A culture which was in the stationary phase, 12 to 24 hr of incubation, was used for inoculation. The culture was diluted in standard phosphate buffer before inoculation when relatively few *S. aureus* cells were desired in the inoculated food or BHI broth. The inoculum was added to the growth medium in 0.5 to 5.0 ml of liquid depending on the population desired. Immediately after inoculation, the growth substrate contained between 10^4 to 10^6 *S. aureus* per gram of medium. The poultry dressings were inoculated and placed into covered metal containers before baking. The other growth media were tempered to the incubation temperature before inoculation, except for the frozen foods which were tempered at 5 C prior to inoculation. With the exception of the turkey pot pies, the frozen foods were held at 5 C until they were thawed. The turkey pot pies were held at 5 C for 24 hr after they were thawed. This permitted some growth of the bacterial flora already in the turkey pot pie, thus there was more competition for the *S. aureus* culture which was added.

Incubation and Sampling of Foods and BHI Broth

The inoculated BHI broth was incubated in a one liter erlenmeyer flask in a rotary shaker (Gyrotory shaker,

New Brunswick Scientific Company). The rotation was constant at approximately 175 rpm for all broth experiments. The temperature was controlled with the heating unit of the shaker and/or placing the shaker in a refrigerated room. At selected times, samples were taken aseptically for pH, population, and enterotoxin determinations.

The inoculated foods were incubated without agitation. Temperature was controlled by placing the food in a constant temperature chamber. The food was placed in an appropriate size sterile, covered glass beaker, inoculated, mixed thoroughly, and incubated. At scheduled times, portions were weighed aseptically for population determinations and pH measurement. After the terminal sample was taken for determination of population and pH, the remainder was frozen and stored at -30 C for an enterotoxin determination at a later time.

Samples for analysis for the presence of enterotoxin were collected periodically during incubation of the vanilla pudding. The vanilla pudding was weighed into small, sterile screw cap bottles which were incubated without agitation at constant temperatures in the range of 19 to 45 C. At each sampling time, a bottle of pudding was removed, the pH was determined, the S. aureus population was measured, and the remainder of the pudding in that bottle was frozen and stored at -30 C for use in an enterotoxin assay at a later time.

Determination of pH

A small portion of the BHI broth or food was collected at each sampling interval and placed in a 5 ml glass beaker or a disposable medicine cup. A Corning single electrode on a Beckman Research pH Meter was used to measure the pH. The broth was centrifuged before the pH was measured (see methods for Storage of Samples for Enterotoxin Analysis).

Determination of Population

The initial dilution of the food samples was by weight. All other samples were diluted volumetrically. Ten-fold and hundred-fold dilutions were made using the standard phosphate buffer described by the American Public Health Association (1960). Disposable plastic petri dishes (100 x 15 mm) were used for all platings.

Spread plates containing MSA or S-110 were employed for the enumeration of *S. aureus*. The plates were poured less than one day before use. A 0.1 ml volume of the desired dilution was planted on the plate and spread evenly with a sterile, bent glass rod. The plates were incubated at 37 C for about 48 hr, and typical colonies were counted.

The initial bacterial load of the food and a measure of the mixed populations were determined using PCA. The

pour plate method was employed with 0.1 or 1.0 ml of the diluted sample being planted. The plates were incubated at 37 C for about 48 hr. All colonies were counted.

Storage of Samples for Enterotoxin Analysis

The broth samples used for analysis for enterotoxin were centrifuged immediately after collection. Approximately 10 ml of broth was placed in a 50 ml centrifuge tube. Centrifugation was at 12,000 g (10,000 rpm with a SC-3856 head) for 15 min at 2 C in a Sorvall Superspeed RC-2 centrifuge. The pellet was discarded. The supernatant liquid was placed in a screw cap bottle and stored at -30 C until the enterotoxin assay was performed.

The food samples were collected and stored at -30 C with no preliminary treatment.

Extraction of Enterotoxin from Foods

The food samples were extracted according to the method of Casman and Bennett (1965) and Casman (1967). No extraction was done on any of the samples of BHI broth. Although some broth samples were concentrated by dialysis or diluted with physiological saline before assay, most of the BHI broth was assayed directly.

The vanilla pudding was extracted using a shortened version of the Casman and Bennett (1965) method. The food was blended, adjusted to pH 7.5, centrifuged, and dialyzed

following the beginning portion of the procedure of Casman and Bennett (1965). The extract thus obtained was used for the enterotoxin assay.

After the first assay, the extracted material which remained was stored at -30 C for future use.

Enterotoxin Assay

The microslide gel-diffusion method of Casman and Bennett (1965) was used to determine presence of staphylococcal enterotoxin. Purified staphylococcal enterotoxins A, B, C, and D and balanced antitoxins for the A, B, C, and D toxins were obtained from Dr. Casman of the FDA laboratory in Washington, D. C. This assay was used as a semi-quantitative determination. The minimum amount of enterotoxin which can be detected by the microslide technique is about 1 μg/ml according to Casman and Bennett (1965).

The stored extracted samples were thawed and assayed. Freezing and thawing caused precipitation of a starchy material in some of the food samples. These samples were not stirred, and only the liquid portion was used in the assay. Samples with large amounts of the starchy material could not be assayed accurately without precipitation by freezing of starchy material.

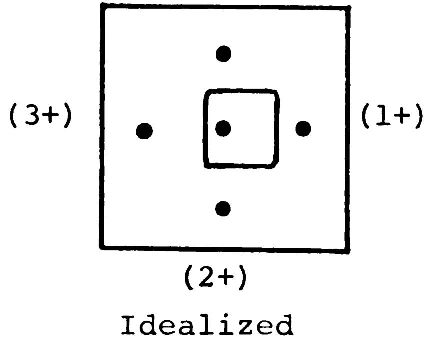
Decontamination of Equipment

All used media, food, petri dishes, and equipment which came in contact with the *S. aureus* organisms or the enterotoxins were autoclaved 45 min at 121 C. The equipment then was washed with hot water and detergent. The pipettes were held for at least 4 hr in an acid cleaning solution also. The templates and slides from the microslide assay were not autoclaved but were washed vigorously by hand in a detergent solution. The slides were kept in boiling water for several minutes. The plexiglass templates could not be boiled or heated because heating would cause the plexiglass to warp and thus ruin the template.

RESULTS

Estimating Concentrations of Enterotoxin

In the microslide gel-diffusion assay, the reaction between the reference antitoxin and the extract from the sample formed a precipitation line which was evaluated according to its location in respect to the precipitation line formed by the reaction between the reference antitoxin and the standardized enterotoxin. Samples which did not produce a precipitation line were given a negative (-) rating. The locations of precipitation lines associated with 1+, 2+, or 3+ ratings are illustrated in Figure 1. A 2+ rating indicates that the sample had an enterotoxin concentration approximately equal to the amount of enterotoxin in the standard reference, which was 1 μg/ml. A 1+ rating indicates less toxin in the sample than in the reference; whereas, a 3+ indicates more toxin in the sample than in the reference. A 4+ rating was given to a sample which formed a precipitation line at the original location of the antitoxin. The 4+ rating indicates a high toxin concentration. Some samples with very high concentrations of enterotoxin did not form a precipitation line because the antitoxin concentration was not high enough to balance



center well contains an antitoxin which is balanced with the reference enterotoxin in the upper well.

standard reference in upper well; antitoxin in center well

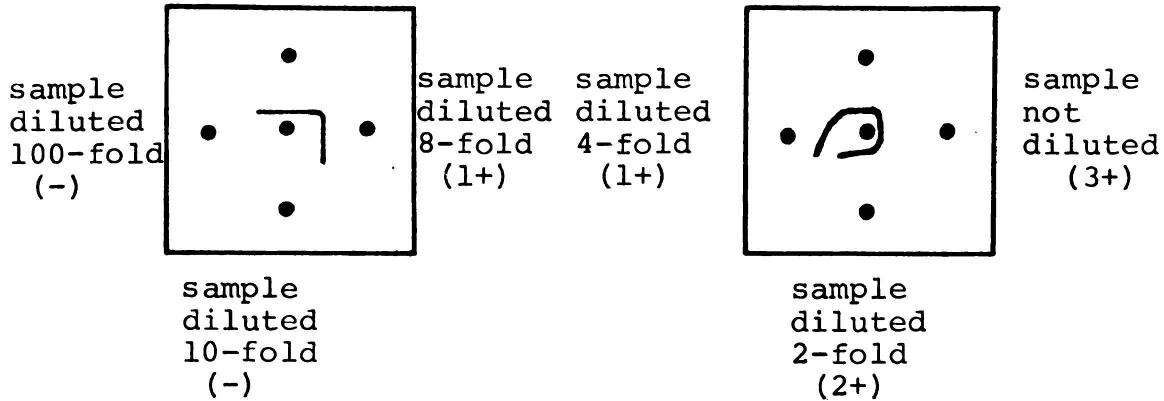


FIGURE 1.--Diagram of enterotoxin assay results.

with the toxin concentration; however, they did form a precipitation line when diluted.

A 1+ or 2+ sample when diluted five-fold or greater did not form a precipitation line. A 3+ sample formed a precipitation line at an eight-fold dilution, but not at a ten-fold dilution (Figure 1). When diluted, the 4+ samples gave varying results depending on the intensity of the 4+. In some cases, the hundred-fold dilutions of 4+ samples gave 2+ ratings. In other cases, the hundred-fold dilution of a 4+ sample did not form a precipitation line.

Growth Characteristics of *S. aureus* in BHI Broth Buffered with Phosphate

The data presented in this work were obtained through a series of experiments designed to yield data that could be used to produce growth curves for any one strain or for the four strains of *S. aureus* used. The data illustrated in Figure 2 show typical pH values and populations for the four strains when grown with agitation at 26 C in BHI broth with 0.2M phosphate at pH 6.75. There is little difference in the populations or the pH values among the four strains at any one sampling time. The initial population of *S. aureus* in the BHI broth was appoximately 10^6 cells/ml. A lag phase was not detected, and the stationary phase began at about 27 hr. Enterotoxin was first detected after 18 hr, and 3+ quantities of toxin were

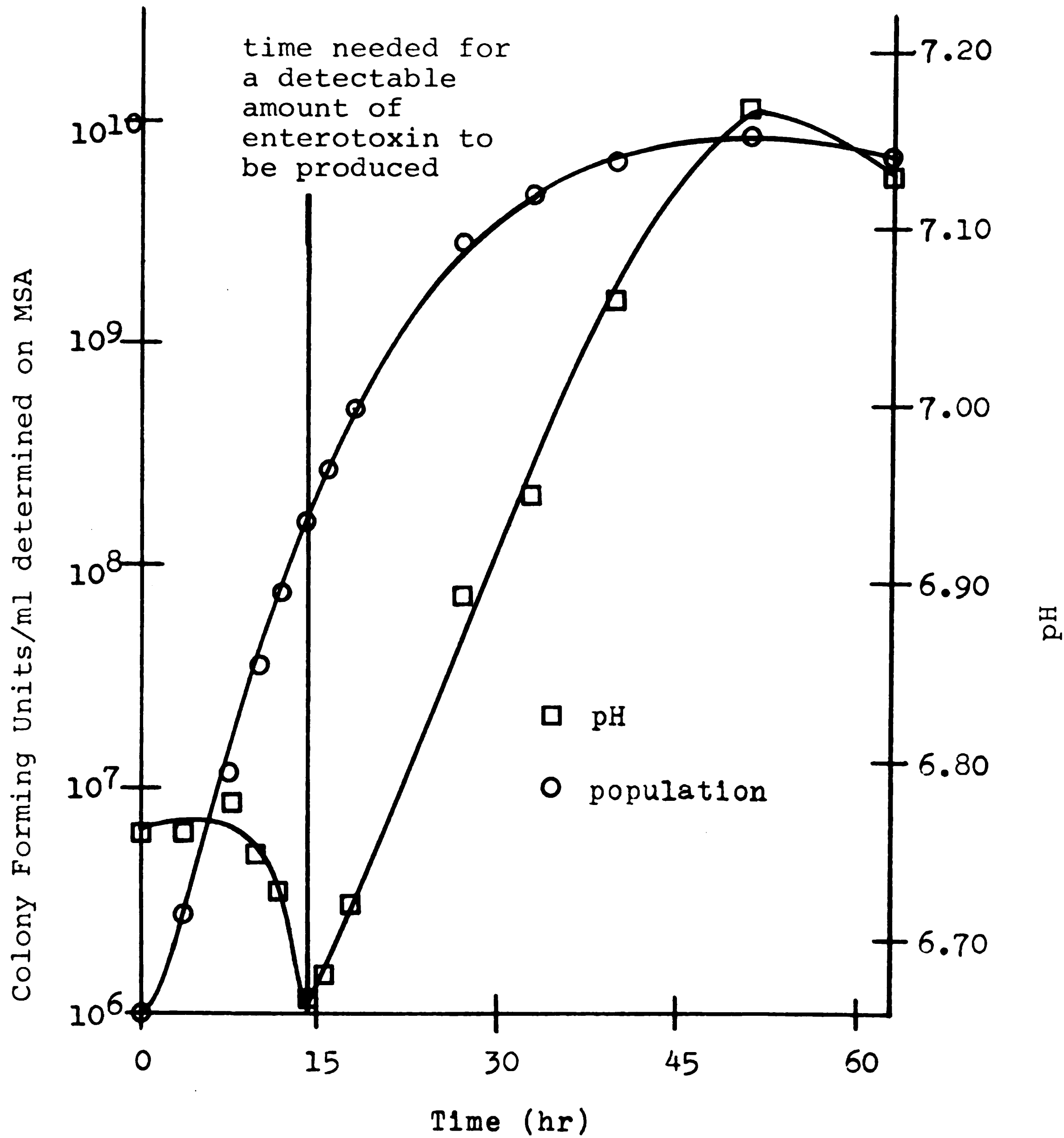


FIGURE 2.--Population and pH of four strains of *S. aureus* during growth at 26 C in agitated BHI broth buffered at pH 6.75 with 0.2M phosphate.

detected at the next sampling time which was 27 hr. The data in Table 1 represent the results of entertoxin assays associated with the data depicted in Figure 2. The variations observed between strains was greater at temperatures above or below 26 C than it was at 26 C. When the pH or temperature was raised or lowered from the optimum conditions, (Figures 3 and 4), growth and enterotoxin production were delayed.

Effect of pH on Growth and Enterotoxin Production by *S. aureus* 243 in BHI Broth

When the initial pH was in the range of 4.7 to 9.4, growth of *S. aureus* 243 occurred in agitated BHI broth buffered with phosphate (Table 2). Growth of *S. aureus* 243 appeared to be similar in BHI broth which was not buffered and in BHI broth which contained the phosphate buffer (Table 3). Detectable amounts of enterotoxin B were produced in phosphate buffered BHI broth with an initial pH in the range of 5.1 to 9.0. The pH range for enterotoxin production is narrower than the pH range for growth of *S. aureus*. The pH range for enterotoxin production appeared to be similar in both nonbuffered BHI broth and in BHI broth buffered with phosphate, although fewer experiments were performed in the nonbuffered broth. With the nonbuffered BHI broth, entertoxin appeared as early as 4 hr after inoculation; whereas with the buffered broth,

TABLE 1.--Production of enterotoxin by four strains of S. aureus in agitated BHI broth buffered to pH 6.75 with 0.2M phosphate and incubated at 26 C.

Time (hrs)	S. aureus 265 (A toxin)	S. aureus 243 (B toxin)	S. aureus 493 (C toxin)	S. aureus 315 (D toxin)
0	NR*	NR	NR	NR
4	-**	-	-	-
8	-	-	-	-
10	NR	NR	NR	NR
12	-	-	-	-
14	-	-	1+	1+
16	1+	1+	1+	1+
18	2+	2+	1+	2+
27	3+	3+	2+	3+
33	NR	NR	NR	NR
40	NR	NR	NR	NR
51	NR	NR	NR	NR
63	NR	NR	NR	3+

*NR indicates that an enterotoxin assay was not performed.

**- indicates that the enterotoxin assay was performed but that no enterotoxin was detected.

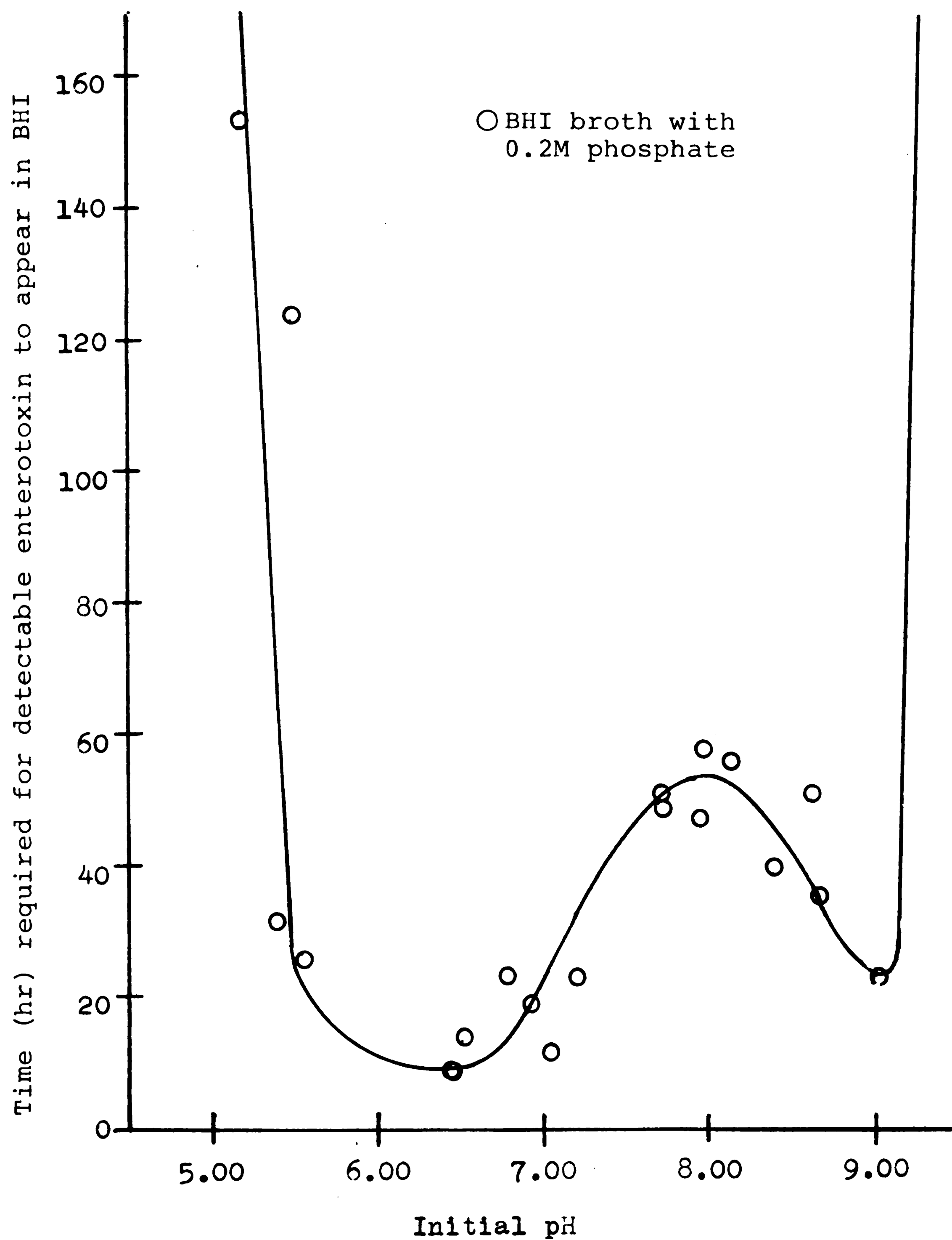


FIGURE 3.--Effect of initial pH of BHI broth on the time required for production of a detectable amount of enterotoxin B by S. aureus 243 incubated with agitation at 37 C.

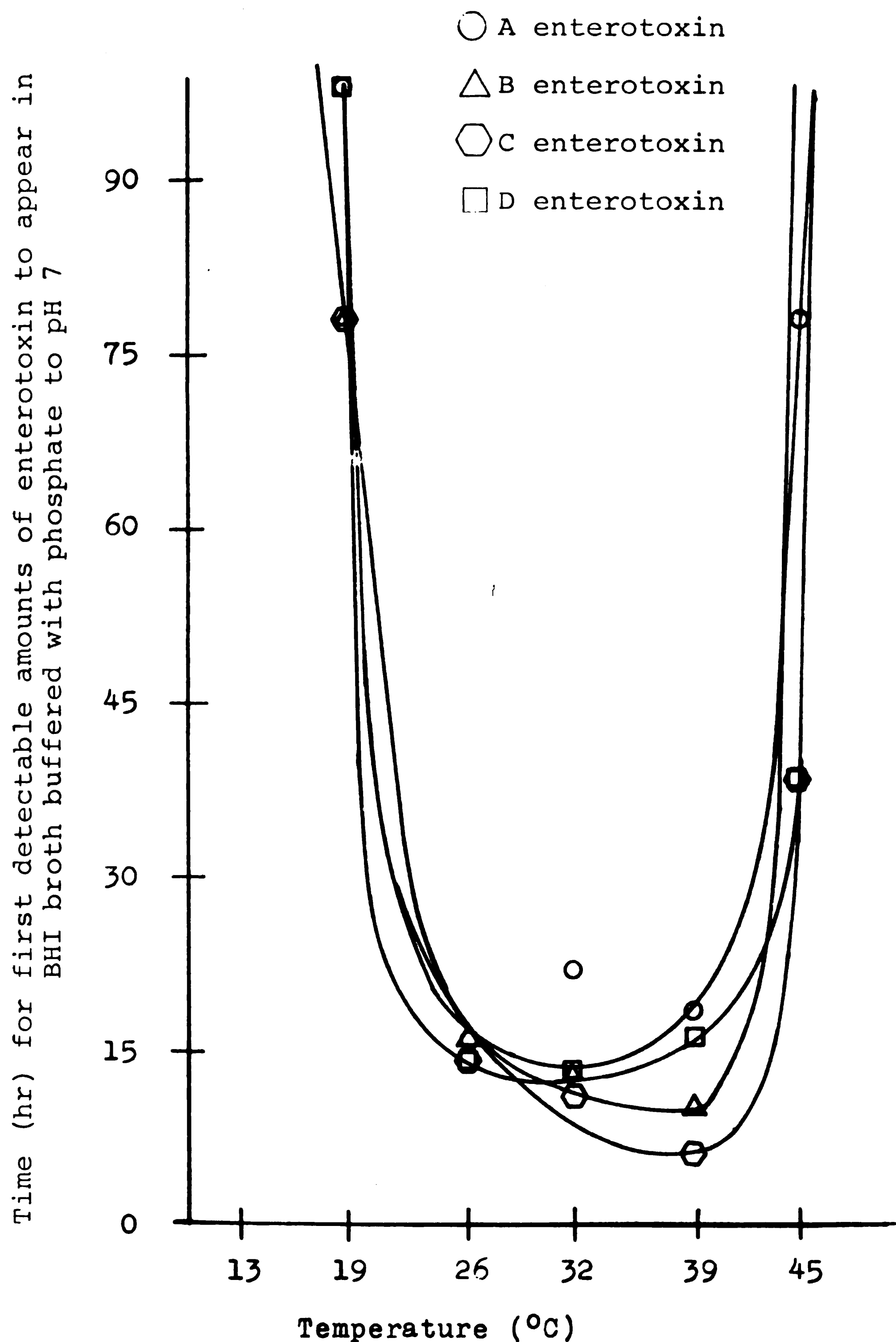


FIGURE 4.--Effect of incubation temperature on the time required for production of a detectable amount of enterotoxins A, B, C, and D by four strains of *S. aureus* incubated with agitation in BHI broth buffered at pH 7.

TABLE 2.--Incubation times, pH, and population associated with the samples which contained the first measurable amounts of enterotoxin B formed by <u>S. aureus</u> 243 grown at 37 C with agitation in phosphate buffered BHI broth with variable initial pH.

Initial pH	First Detectable Enterotoxin		
	Time (hr)	pH	Count on S-110* X 10^6
3.62	no toxin		no growth
4.48	no toxin		no growth
4.76	no toxin		growth
4.96	no toxin		growth
5.15	154	5.66	350
5.38	32	5.42	57
5.48	124	6.08	220
5.56	26	5.58	90
6.44	9	6.48	32
6.45	9	6.44	30
6.53	14	6.42	720
6.79	23	6.78	1700
6.94	19	6.79	570
7.03	12	6.97	25
7.20	23	7.17	1600
7.72	52	7.71	0.66
7.73	48	7.61	4.6
7.96	47	7.80	3000
7.97	58	7.75	800
8.14	56	7.89	1500
8.40	40	7.86	1200
8.61	52	7.71	400
8.67	36	8.02	680
9.02	24	8.89	0.2
9.40	no toxin		growth
9.84	no toxin		no growth

*Initial counts on S-110 were approximately 10^6 cells/ml.

TABLE 3.--Incubation times, pH, and population associated with the samples which contained the first measurable amounts of enterotoxin B formed by *S. aureus* 243 grown at 37 C with agitation in nonbuffered BHI broth with variable initial pH.

Initial pH	First Detectable Enterotoxin		
	Time (hr)	pH	Count on S-110* X 10^6
5.02	no toxin		growth
6.14	6	5.34	500
6.62	6	5.70	750
7.13	4	6.78	7.5
7.55	6	6.62	1200
7.95	4	7.63	76
9.08	no toxin		growth
9.86	no toxin		no growth

*Initial counts on S-110 were approximately 10^6 cells/ml.

the earlist that enterotoxin was detected was 9 hr after inoculation (Table 2 and 3). In nonbuffered BHI broth, the incubation time required for production of a detectable amount of enterotoxin varied little within the pH range in which toxin production did occur. In buffered BHI broth, the incubation time required for production of a detectable amount of enterotxin varied considerably as the initial pH of the growth medium varied. Two- to six-fold longer incubation times were needed before enterotoxin could be detected when the initial pH of the growth medium was 7.7 to 8.6 rather than 6.5 to 7.2 or 9.0. The initial population of S. aureus in the BHI broth was approximately 10^6 cells/ml. When the initial pH of the BHI broth was below 4.8 or above 7.7, the population declined and subsequently an increase in population occurred in some samples and production of enterotoxin occurred in some samples. In the first samples which contained a detectable amount of enterotoxin, the populations varied from 2.0×10^5 to 3.0×10^9 cells/ml in buffered BHI broth and from 7.5×10^6 to 1.2×10^9 cells/ml in nonbuffered BHI broth. Little correlation between population and enterotoxin production was observed over the pH range.

Effect of Temperature on Growth and Enterotoxin Production by Four Strains of *S. aureus* in Buffered BHI Broth

When inoculated into buffered BHI broth with an initial pH of approximately 7, all four strains of *S. aureus* grew throughout the range of 13 to 45 C, except for strain 243 which did not grow in BHI broth at 45 C. No growth of any of the four strains was detected when incubation was at 7 or 50 C (Table 4). The initial population of *S. aureus* was approximately 10^6 cells/ml.

Enterotoxins A, C, and D were produced by strains 265, 493, and 315, respectively, throughout the range of 19 to 45 C. At 13 and 50 C none of these strains produced measurable amounts of enterotoxin. Strain 243 produced enterotoxin B throughout the range of 13 to 39 C, but it did not produce measurable amounts of enterotoxin in BHI broth at 7 or 45 C. At 13 C strain 243 produced a measurable amount of enterotoxin in 158 hr but not in 132 hr. The other strains did not produce measurable amounts of enterotoxin at 13 C in 158 hr (Figure 4). At 19 C strains 265 and 315 produced measurable amounts of enterotoxin in 98 hr but not in 78 hr; whereas, strains 243 and 493 produced measurable amounts of enterotoxin in 78 hr but not in 54 hr. At 26 C all four strains produced measurable amounts of enterotoxin in 14 to 16 hr, but none produced measurable amounts of toxin in 12 hr. At 32 C strain 265 produced a

TABLE 4.--Incubation times, pH, and population associated with the samples which contained the first measurable amounts of enterotoxin formed by four strains of *S. aureus* when incubated with agitation in BHI broth buffered at pH 7 with 0.2M phosphate.

°C	*S. aureus* 265 (A toxin)			*S. aureus* 243 (B toxin)			*S. aureus* 493 (C toxin)			*S. aureus* 315 (D toxin)		
	time (hr)	pH	count* X 10^6	time (hr)	pH	count* X 10^6	time (hr)	pH	count* X 10^6	time (hr)	pH	count* X 10^6
7	no growth			no growth			no growth			no growth		
13	no toxin,growth			158	6.77	3.2	no toxin,growth			no toxin,growth		
19	98	6.96	16	78	6.94	8.0	78	6.91	130	98	6.94	3200
26	16	6.66	360	16	6.66	190	14	6.62	450	14	6.66	110
32	22	6.85	550	13	6.82	50	11	6.83	69	13	6.86	50
39	18	6.82	440	10	6.96	2	6	6.98	1.3	16	6.79	80
45	78	6.83	20	no growth			38	6.78	120	38	6.82	15
50	no growth			no growth			no growth			no growth		

*Counts were made on MSA. The initial counts were about 10^6 cells/ml.

measurable amount of enterotoxin in 22 hr but not in 19 hr; whereas, strains 243 and 315 produced measurable amounts of toxin in 13 hr but not in 11 hr. Strain 493 at 32 C produced a measurable amount of enterotoxin in 11 hr but not in 8 hr. At 39 C strains 265, 243, and 315 produced measurable amounts of enterotoxin in 18, 10 and 16 hr but not in 16, 6, and 14 hr, respectively. Strain 493 at 39 C produced a measurable amount of enterotoxin in 6 hr which was the first assay time. At 45 C strains 493 and 315 produced measurable amounts of enterotoxin in 38 hr but not in 26 hr. Strain 265 at 45 C produced a measurable amount of enterotoxin in 78 hr but not in 54 hr. Strain 243 did not grow in BHI broth at 45 C.

The population of the first samples which contained a detectable amount of enterotoxin varied from about 10^6 to 3.2×10^9 cells/ml. The initial population of *S. aureus* in the BHI broth was about 10^6 cells/ml, and the initial enterotoxin assays were negative. The pH of the first samples which contained a detectable amount of enterotoxin varied from about 6.6 to 7.0. The initial pH of the phosphate buffered BHI broth was about 7. Little correlation between pH, population, and enterotoxin production was observed over the incubation temperature range.

Effect of Temperature on Enterotoxin Production in Vanilla Pudding

Over the temperature range of 19 to 45 C, enterotoxin was produced by all four strains of *S. aureus* when incubated without agitation in vanilla pudding (Table 5 and Figure 5). At 19 C all four strains produced measurable amounts of enterotoxin in 84 hr but not in 50 hr. At 26 C strain 315 produced a measurable amount of enterotoxin in 25 hr but not in 20 hr. The other strains at 26 C produced measurable amounts of enterotoxin in 48 hr but not in 25 hr. At 37 C all four strains produced measurable amounts of enterotoxin in 22 hr but not in 15 hr. At 45 C strains 243 and 493 produced measurable amounts of enterotoxin in 72 hr but not in 48 hr; whereas, strains 265 and 315 produced measurable amounts of enterotoxin in 31 hr but not in 24 hr.

The bacterial load before inoculation was less than 10^2 organisms per gram of pudding and was considered insignificant. After inoculation the *S. aureus* population was about 10^5 cells/g of pudding. There was little variation between counts on PCA and MSA. The populations of the first samples which contained a detectable amount of enterotoxin varied from about 7×10^6 to 4×10^8 cells/g. The initial pH of the pudding was about 6.3. The pH of the first samples which contained a detectable amount of enterotoxin varied from about 5.6 to 6.4. Little correlation between

TABLE 5.--Incubation times, pH, and population associated with the samples which contained the first measurable amounts of enterotoxin formed by four strains of *S. aureus* when incubated without agitation in vanilla pudding at various temperatures.

°C	*S. aureus* 265 (A toxin)			*S. aureus* 243 (B toxin)			*S. aureus* 493 (C toxin)			*S. aureus* 315 (D toxin)		
	time (hr)	pH	count* X 10^6	time (hr)	pH	count* X 10^6	time (hr)	pH	count* X 10^6	time (hr)	pH	count* X 10^6
19	84	6.26	81	84	6.45	240	84	6.32	410	84	6.17	270
26	48	6.21	48	48	6.27	190	48	5.74	390	25	6.12	420
37	22	5.92	16	22	6.07	400	22	6.06	41	22	5.99	350
45	31	5.90	30	72	6.23	7	72	5.56	9.6	31	6.00	90

*Counts on MSA and PCA were essentially the same. The bacterial load in the vanilla pudding before inoculation was low and was considered insignificant. Sufficient *S. aureus* cells were added to give an initial population of approximately 10^5 cells/g of pudding.

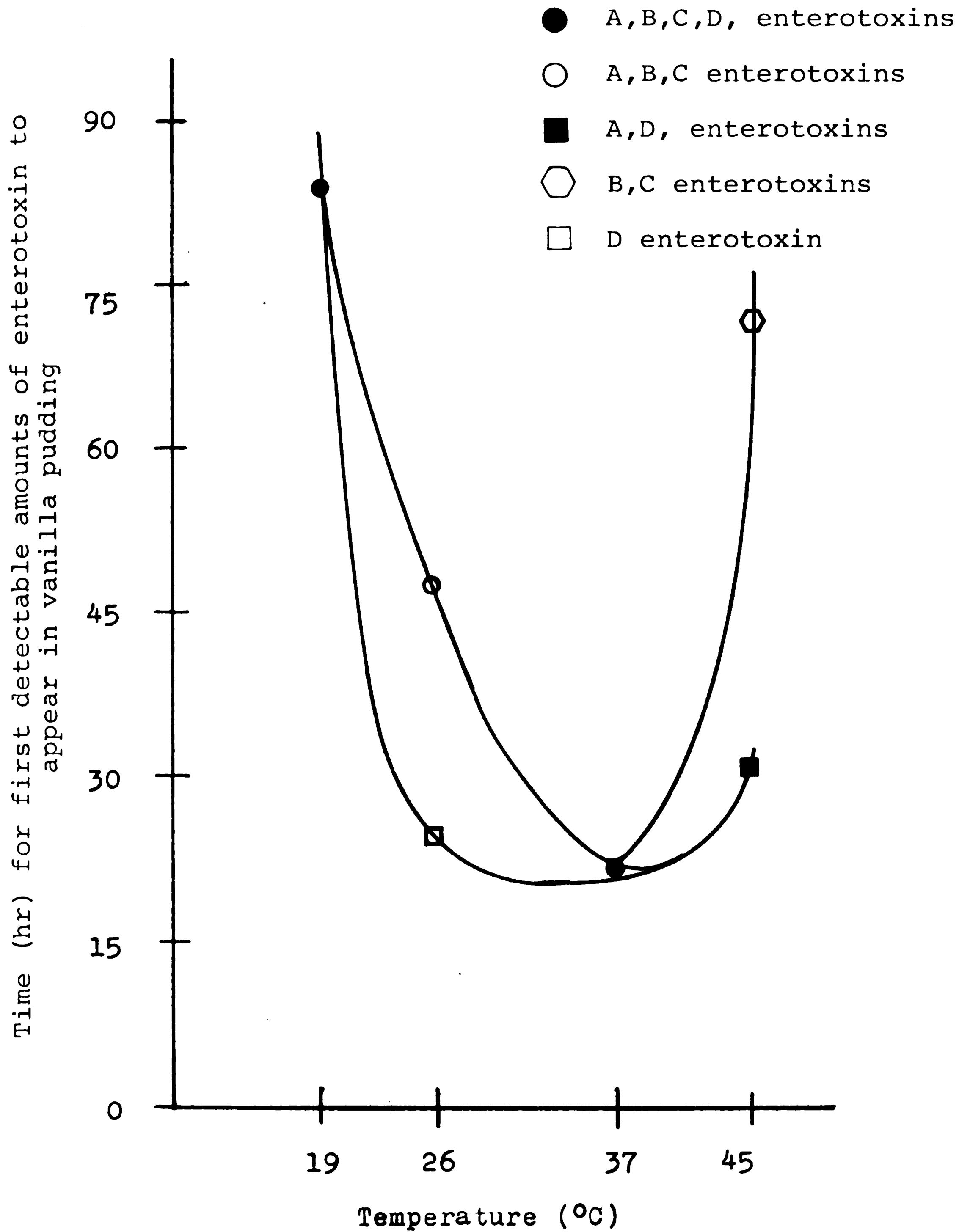


FIGURE 5.--Effect of incubation temperature on the time required for production of a detectable amount of enterotoxins A, B, C, and D by four strains of *S. aureus* incubated without agitation in vanilla pudding.

enterotoxin production, population, and pH was observed over the temperature range.

Growth and Enterotoxin Production by *S. aureus* in Selected Foods

The pH of some foods was measured, but the foods were not inoculated because the data on pH from the broth studies indicated that *S. aureus* would not grow. The foods tested were fruit pies and turkey salad. Two foods with a low pH were inoculated to verify the data obtained from growth of the cultures in BHI broth, and the *S. aureus* did not grow. These foods were creamed herring and apple sauce. Some foods which seemed to pose potential food poisoning hazards were inoculated, but the *S. aureus* cultures did not grow (Table 6). An enterotoxin assay was not performed. These foods included dressings, canned beef gravy, chili sauce, salad dressing, donut cream fillings, and frostings. Certain foods were inoculated, and growth of *S. aureus* was inhibited (Table 7). These foods included gravies, pot pies, beef stew, sandwich spreads, potato salad, and polynesian parfait. In some cases, the food had a low pH. In some foods, other bacteria present in the food grew rapidly and inhibited the growth of *S. aureus*. When the dried gravy mixes were rehydrated as directed by the manufacturer, other organisms in the gravies grew faster than did the *S. aureus*. The rehydrated gravies were

TABLE 6.--Incubation temperature and pH of selected foods in which added *S. aureus* did not grow.

Food	Incubation temperature	Initial pH	*S. aureus* strain added*
Poultry Dressing	37	4.89	265,243,493,315
Cracker Dressing	43	--	265,243,493,315
Beef Gravy (canned)	43	5.91	243
Chili Hot Dog Sauce	43	5.57	243
Creamed Herring	21	3.90	493
Turkey Salad	--	4.78	none
Salad Dressing	21	3.24	493
Donut Cream Filling	21	3.5	265,243,493,315
Bavarian Cream Filling	21	4.75	265,243,493,315
Vanilla Frosting	25	4.59	315
Chocolate Frosting	25	5.67	315
Berry Fruit Pie	--	3.25	none
Cherry Fruit Pie	--	3.91	none
Apple Sauce	25	3.34	315

*Except for the noninoculated foods, approximately 10^4 to 10^6 cells were added per gram of food depending on the particular food. Only one strain was added to a sample.

TABLE 7.--Incubation temperature, pH, and growth response of _S. aureus_ when inoculated into selected foods.

Food	Incubation temperature	Initial pH	_S. aureus_ strain added*	Growth response
Brown Gravy	45	5.93	243	growth of 7 to 10 generations in 24 hr; then overgrown by normal flora.
Pork Gravy	45	5.93	243	
Chicken Gravy	45	6.15	243	
Chicken Pot Pie	21	5.56	none, 315	" " "
Tuna Pot Pie	21	6.10	none, 265	" " "
Beef Pot Pie	21	6.04	none, 265 243, 493	" " "
Turkey Pot Pie	21	6.07	none, 265 243, 315	overgrown by normal flora
Beef Stew	21	5.81	243	good growth, became gassy
Ham Salad	21	4.81	493	slow decline
Sandwich Spread	25	4.76	243	slow decline
Potato Salad	25	4.67	243	slow decline
Polynesian Parfait	21	4.05	493	slow decline

*Except for the noninoculated foods, approximately 10^4 to 10^6 cells were added per gram of food depending on the particular food. Only one strain was added to a sample.

steamed intermittently to eliminate the organisms in the gravies before inoculation. The steaming caused coagulation of the gravy. Other foods were inoculated with S. aureus, enterotoxin was extracted, and an enterotoxin assay was performed (Table 8). These foods included several puddings, cream pies, tuna, tofu, cheese spread, imitation whipped cream, baby food, potato, egg, and sausage patty. With samples of the egg and the cheese spread, problems developed in the extraction procedure and with the assay; thus the negative results may be misleading. Little correlation between the kind of food and enterotoxin production was observed with the foods used in this study.

TABLE 8.--Incubation temperature, pH, and enterotoxin production of selected foods to which *S. aureus* was added and grew.

Food*	Incubation temperature	Initial pH	*S. aureus* strain added** 10^5	or 10^6	Enterotoxin
Tuna	25	5.72		315	2+
Sausage Patty	21	6.2		243	1+
Vegetables with Liver	21	5.74		243	1+
Sweet Potato	21	5.31		243	4+
Potato	21	5.7		243	1+
Egg	21	7.4		243	-
Tofu	21	4.65		493	-
Cheese Spread with Shrimp	21	6.11	493		-
Imitation Whipped Cream	21	6.46		493	1+
Banana Cream Pie	21	6.13		265	2+
Chocolate Cream Pie	21	5.91		265	1+
Coconut Cream Pie	21	6.60		265	2+
Rice Pudding with Raisins	21	6.30	493		1+
Rice Pudding	25	6.28	315		1+
Chocolate Pudding	25	6.56	315		-
Butterscotch Pudding	25	6.34	315		-
Vanilla Pudding	25	6.37	315		2+

*The incubation time varied from 68 to 119 hr depending on the individual food. We would expect that many consumers would accept a food of the apparent quality of these foods at the time that the sample was collected for the enterotoxin analysis.

DISCUSSION

Estimating Concentrations of Enterotoxin

The microslide gel-diffusion assay used in this study is delicate, but with this method one has the advantage of being able to distinguish precipitation lines denoting enterotoxin from lines of interference. The presence of the known enterotoxin in one well permits the formation of a continuous diffusion line with the toxin from a sample well. When using extracts from food samples, which often contain interfering proteins, this continuous diffusion line may be very helpful in distinguishing positive from negative results. The microslide gel-diffusion assay is a semi-quantitative method in which titers can be determined. Other methods are more accurate for a quantitative assay. The immunological assay methods, which are easier and more reliable than the animal assay methods for staphylococcal enterotoxin, have enabled investigators to assay many more samples for enterotoxin and to obtain greater accuracy of results. Methods for assay of enterotoxin were reviewed by Bergdoll (1970).

The principal measurement for the work reported in this thesis was the incubation time required under

certain conditions for a culture of *S. aureus* to produce a measurable amount of enterotoxin. Presence or absence of measurable enterotoxin was considered as more important than actual quantity of toxin. Relative quantitative values for enterotoxin were estimated for certain samples when it was thought that such information would be helpful in selecting sampling times in later experiments. The concentration of the standard reference enterotoxin was 1 μg/ml. Samples which formed a precipitation line equidistant with the reference line presumably had an enterotoxin concentration of approximately 1 μg/ml. Because of the loss of detectability of enterotoxin when samples were diluted, we can estimate enterotoxin concentrations of approximately 0.5, 1.0, and 5 μg/ml in samples labeled 1+, 2+, and 3+, respectively. The samples of main concern were those which contained a detectable amount of enterotoxin in the shortest incubation time. Thus the samples of main concern would contain enterotoxin concentrations in the range of 1 μg/ml.

The exact dose of staphylococcal enterotoxin which is toxic to man is not known. It is known that the susceptibility of individuals varies widely. It is estimated by Raj and Bergdoll (1969) that about 20 μg of staphylococcal enterotoxin B, and much less of enterotoxin A, is sufficient to cause the typical symptoms of staphylococcal food poisoning in man. Considering the size of an average serving

of the foods used in this study and considering the estimated toxicity of staphylococcal enterotoxin, it seems obvious that an enterotoxin concentration of 1 μg/g of food is sufficient to cause concern. In this study the foods which were reported as positive for enterotoxin contained approximately 1 μg of enterotoxin per gram of food. If a person were to consume a food containing enterotoxin in the concentration present in the foods reported positive in this study, there is a high probability that the person would exhibit the symptoms of staphylococcal food poisoning. The severity of illness is dependent on individual susceptibility and on the quantity of food consumed. From the food safety aspect, we are not primarily concerned with the total amount of enterotoxin present in a food if at least a toxic dose is present. The principal concern is the time needed under certain conditions for the enterotoxin concentration to reach a toxic dose.

Effect of pH on Growth and Enterotoxin Production by *S. aureus*

The pH of the growth substrate has an effect on the incubation time needed for production of a detectable amount of staphylococcal enterotoxin. Foods within the pH range of 5.1 to 9.0 may be considered, in general, to have a potential for staphylococcal food poisoning if other environmental conditions are favorable. It is

theoretically possible to adjust the pH of food to above or below the pH range for enterotoxin production. This, however, is not feasibile. Very few foods would be accepted by the consumer if the pH were above 9; although, a number of foods which are acceptable have a pH below 5. Each food must be considered individually, however, for its potential for causing staphylococcal food poisoning. The data accumulated in this study indicate that the information concerning environmental conditions for enterotoxin production in BHI broth can not be applied directly to food substrates.

In both buffered and nonbuffered BHI broth, growth of S. aureus occurred in a wider pH range than did enterotoxin production. It was not determined in this investigation if the pH ranges for growth and enterotoxin production by S. aureus were similar in food; however, the work of Genigeorgis et al. (1971c) showed that growth of S. aureus did occur in meat without enterotoxin production in the meat. According to Reiser and Weiss (1969), the growth medium is known to affect the total amount of enterotoxin produced. It could be expected that the nature of the growth medium would also affect the incubation time needed for a measurable amount of enterotoxin to be produced.

There was a close relationship between the initial pH of the growth medium and the incubation time required for production of a measurable amount of enterotoxin. With

the data depicted in Figure 3, it is possible to predict the incubation times needed before a detectable amount of enterotoxin is produced if the initial pH of the growth medium is known and if the growth conditions are as described in Figure 3. From the initial pH of the growth medium, it is not possible to predict the S. aureus population associated with the toxic sample having the shortest incubation time. Similarly, it is not possible to predict the presence of enterotoxin in a particular sample by the population of S. aureus in that sample or by the pH of that sample.

Other investigators including Peterson et al. (1964) and Morse et al. (1969) have observed that the pH of the growth medium changes while the S. aureus culture is growing. The strains of S. aureus used in the work reported in this thesis produced in the growth medium a change of pH similar to the changes reported by others. From the data depicted in Figure 2 we see that the toxic samples with the shortest incubation times were the same samples which showed the initial rise in pH. The sampling times were not sufficiently close and the enterotoxin assay is not sufficiently sensitive to determine if the increase in pH and the production of enterotoxin coincide exactly. Morse et al. (1969) studied the control mechanism for enterotoxin production by S. aureus. It is possible to speculate from the results reported herein as well as the results published by Morse et al. (1969) and others that several metabolic

changes occur in the *S. aureus* cells shortly before the change of pH of the growth medium occurs. Besides a change in pH, a change in the odor of the culture and a change in the color of the pellet of centrifuged cells often occurs at about the same time as the rise in pH of the growth medium and the production of enterotoxin (data not included). Certainly one of the metabolic changes involves a shift from carbohydrate utilization and acid production to utilization of the organic acids. A metabolic shift of this nature explains the change observed in the pH. It is possible that the production of enterotoxin is directly related to this metabolic shift although the available data do not prove the relationship. Also it is possible that enterotoxin is produced throughout the growing stages of the culture but that the enterotoxin can not be detected by current methods until the toxin has accumulated. The appearance of enterotoxin at the same time as the appearance of a major increase in pH would then be only coincidence. Another possibility is that enterotoxin is not released into the growth medium until a drastic change in pH has occurred. The goals of this investigation were not to answer these questions or even to raise the questions. The observations were made while obtaining other data and may be the basis for further studies in this area.

Since the incubation time for the production of a detectable amount of enterotoxin was shorter in nonbuffered

BHI broth than in buffered BHI broth (Figure 3 and Tables 2 and 3), we would suspect that the phosphate affected enterotoxin production. The 0.2M phosphate buffer delayed but did not prevent the change of the pH of the growth medium. Phosphate buffer has a high buffering capacity in the range of pH 8. At approximately pH 8 the incubation times required before enterotoxin could be detected in BHI broth buffered with phosphate were considerably longer than were the incubation times at higher or lower pH values. The differences in incubation times needed for measurable amounts of enterotoxin to be produced in BHI broth buffered at different pH values as well as the difference between incubation times required for enterotoxin production in buffered and non-buffered BHI broth may be due to stabilization of the pH. This suggests that a change of pH is necessary for elaboration of staphylococcal exterotoxin into the growth medium.

Effect of Temperature on Growth and Enterotoxin Production by *S. aureus*

The temperature of the growth substrate has an effect on the incubation time needed for production of a detectable amount of staphyloccal enterotoxin. Generally, growth of *S. aureus* occurred within the range of 13 to 44 C which is in agreement with the work of Walker and Harmon (1965) and Tatini *et al*. (1971). The temperature range for enterotoxin production generally was restricted to the

range of 19 to 45 C with the exception of _S. aureus_ 243 which produced enterotoxin B in the range of 13 to 45 C. As expected, the incubation time needed for enterotoxin production increases as the temperature varies above or below 26 to 39 C, which according to the data in Figures 4 and 5 is the optimum temperature range. The incubation times needed to produce detectable amounts of enterotoxins A, B, C, and D in buffered BHI broth are similar to the times Donnelly _et al_. (1968) found for production of enterotoxin A in milk. A close relationship existed between the incubation temperature and the incubation time required for production of a measurable amount of enterotoxin. With the data depicted in Figure 4, it is possible to predict the incubation times needed before detectable amounts of enterotoxin are produced in buffered BHI broth if the incubation temperature is known and if the growth conditions are as described in Figure 4. With the data in Figure 5, a similar prediction could be made for enterotoxin production in vanilla pudding. The information concerning the environmental conditions for enterotoxin production in BHI broth can not be applied directly to a food system. Using either BHI broth or vanilla pudding as a growth medium the incubation temperature can not be used to predict the _S. aureus_ population associated with the sample in which enterotoxin was produced in the shortest incubation time. Similarly the population of _S. aureus_ in a sample

or the pH of the sample can not be used to predict the presence of enterotoxin in a particular sample.

Comparisons of Growth and Enterotoxin Production Among Four Strains of *S. aureus*

At a particular incubation temperature, the incubation times required for production of measurable amounts of staphylococcal enterotoxin were similar although not identical for the four strains of *S. aureus* used. At 39 C the relatively large differences in incubation times required for production of detectable amounts of enterotoxin in BHI broth may be an experimental oddity. As shown in Figure 5, the incubation times needed for production of detectable amounts of enterotoxin in vanilla pudding at 37 C were identical for all four strains. At 45 C, the *S. aureus* 243 population decreased rapidly in BHI broth; whereas, in vanilla pudding at 45 C, this strain grew and produced enterotoxin. The upper limit of the temperature range for growth and enterotoxin production by *S. aureus* is probably near 45 C. The work of Tatini *et al.* (1971) indicated a similar temperature limit. Incubation near this upper temperature limit may account for the differences in growth as well as the differences in incubation times required to produce detectable amounts of enterotoxin at 45 C. The curves shown in Figure 4 rise sharply around 45 C which indicates that in future studies at this

temperature wide variations could be expected in incubation times needed for measurable amounts of enterotoxin to be produced. Similarly the curves rise sharply at the lower limit of the temperature range for enterotoxin production. The 19 C temperature chosen for this study may not be the extreme lower limit. Since *S. aureus* 243 produced enterotoxin at 13 C, the other strains could be expected to produce enterotoxin somewhere in the range of 13 to 19 C. Due to the shape of the curves, all the points shown in Figure 4 could be drawn on a common curve even though there is relatively wide variation in the results obtained at the extremes of the temperature range for enterotoxin production. The curves shown in Figure 4 rise sharply around 19 C, which indicates that in future studies at this temperature wide variations could be expected in incubation times needed for measurable amounts of enterotoxin to be produced.

Growth and Enterotoxin Production by S. aureus in Selected Foods

The ubiquitous nature of *S. aureus* makes it a common contaminant in many foods; thus there is the possibility of staphylococcal food poisoning if the conditions are right for enterotoxin production. The occurrence of *S. aureus* in foods was reviewed by Jay (1970). Many commercial foods were inoculated with *S. aureus* to determine

if growth would occur and to determine if enterotoxin would be produced. Before inoculation the bacterial load of most of these foods was less than 10^2 cells/g and was considered as insignificant. The initial bacterial load in the tofu and turkey pot pies was greater than 10^5 cells/g and was considered as significant as a deterent to growth of S. aureus. The chicken, beef, and tuna pot pies as well as the gravy mixes had relatively low initial bacterial loads, but these organisms were important because the *S. aureus* which was added was overgrown by the initial bacterial flora. Generally, the foods in which *S. aureus* did not grow had a pH below 5. Although no water activity measurements were made, we might suspect from the work of Troller (1971) that low water activity may have been a cause of the lack of growth of the *S. aureus* added to the canned frostings. We might suspect that low water activity was a factor in the lack of *S. aureus* growth in other foods. We might expect also that both NaCl and nitrites may have been present in certain of these foods in concentrations which, when in combination with the low pH, may have been inhibitory to *S. aureus*. In the foods in which *S. aureus* persisted, even though growth did not occur, we would suspect the low pH to be the main inhibitory factor although low water activity may have been involved. With the polynesian parfait, the lactic culture which was present may have produced nisin, lactic acid, and other products

which according to Kao and Frazier (1966) would inhibit growth of *S. aureus*. In foods in which the initial bacterial load was relatively high, the rapid growth of the bacteria present before inoculation inhibited good growth of the *S. aureus* which was added. This inhibition of *S. aureus* has been observed by other researchers including Peterson *et al.* (1962) who found that spoilage of chicken pot pies occurred before *S. aureus* had a chance to grow in them. As in the work of Peterson *et al.* (1962), in all the pot pies used in the research reported in this thesis, spoilage occurred before good growth of *S. aureus* developed. Among the group of foods in which the bacteria present before inoculation overgrew the *S. aureus*, tofu was selected for extraction of enterotoxin. No enterotoxin was detected in the tofu, suggesting the probability that there was no enterotoxin in the other foods in which good growth of *S. aureus* did not occur.

Since enterotoxin was detected in only 12 of the 16 foods in which good growth of *S. aureus* occurred, probably environmental factors other than pH and temperature are involved in enterotoxin production. In the chocolate and the butterscotch puddings, there is no apparent reason for the lack of enterotoxin production, especially since measurable amounts of enterotoxin were produced in a similar vanilla pudding. According to Busta and Speck (1968), components of cocoa inhibited growth of salmonella. Perhaps

components of the butterscotch and the chocolate acted as inhibitors of enterotoxin production although there are no data to prove the inhibition. Under the conditions of the experiment, enterotoxin production could be expected in the inoculated and incubated samples of egg and cheese spread. The toxin may not have been produced, or it may have been produced and not detected due to loss in the extraction procedure. With samples such as egg and cheese, the enterotoxin extraction is very delicate and a large loss of toxin may occur.

All of the foods used in this research were "mishandled" in some way, and S. aureus was added to all the foods in relatively large amounts. The "mishandling", however, often was of a type which could possibly occur in a home or food store. Under the experimental conditions, it is not surprising that enterotoxin was produced in many of the foods listed in Table 8. Only a terminal sample for enterotoxin analysis was collected from these foods; thus the hours of incubation needed to produce a measurable amount of enterotoxin could not be determined. Few of the foods had undergone obvious spoilage during the 68 to 119 hr of incubation. The foods, except for the turkey pot pies, were fresh when they were inoculated. The quality of the foods deteriorated somewhat during incubation. We would expect, however, that many consumers would accept a food

of the apparent quality of these foods at the time that the sample for enterotoxin analysis was collected.

Comparison of Enterotoxin Production in BHI Broth and in Vanilla Pudding

The vanilla pudding was chosen for further study of enterotoxin production for comparison with enterotoxin production in BHI broth. Vanilla pudding supported good growth of *S. aureus* and permitted good enterotoxin production. Since the pudding was commercially sterile, the *S. aureus* added did not have competition from other microorganisms.

From the temperature range for enterotoxin production in BHI broth, four temperatures were chosen which appeared to be at critical points. Inoculated vanilla pudding was incubated at the selected temperatures so that enterotoxin production could be compared in BHI broth and in pudding. The relationship between incubation temperature and incubation time required to produce measurable amounts of enterotoxin in vanilla pudding is shown in Figure 5 and is similar to the relationship observed in BHI broth. The incubation times needed for production of measurable amounts of enterotoxin in vanilla pudding at the two extreme temperatures of 19 and 45 C are similar to the incubation times needed in BHI broth. The variation between the incubation times needed to produce measurable amounts of enterotoxin in

pudding samples at 26 C may not be as large as the data in Figure 5 indicate because there was no sample collected between 25 and 48 hr. By comparing the data depicted in Figures 4 and 5 some differences between the incubation times required for a measurable amount of enterotoxin to be produced in BHI broth and in vanilla pudding were observed.

The differences between the incubation times required in pudding and in BHI broth may be caused by several factors. One factor may be the initial pH of the growth medium. The pudding was slightly more acidic than the BHI broth; however, considering the data obtained concerning the pH effect on enterotoxin production, this should be only a minor factor. Another factor may be the population of *S. aureus* present after inoculation. The BHI broth received about a ten-fold higher inoculation than did the pudding, but considering the results published by Donnelly *et al.* (1968) the differences in inoculation also should be only a minor factor. The most obvious factor is that the pudding is semi-solid whereas the broth is liquid. The broth was agitated which created a macroenvironment condition with high aeration and constant dispersion of cells, nutrients, and metabolic by-products. The pudding was not agitated during incubation. Thus the *S. aureus* were growing in a microenvironment with limited oxygen and the possibility of nutrient depletion and accumulation of metabolic by-products. Generally, it could be expected that growth and enterotoxin production

would be faster under the macroenvironment conditions than under the microenvironment conditions. It appears that experiments using BHI broth may be used to give an indication of the behavior of *S. aureus* in food, but the same experiments should not be applied directly to a food system.

SUMMARY AND CONCLUSIONS

Strains 265, 243, 493, and 315 of *S. aureus* which produce enterotoxins A, B, C, and D, respectively, were used in growth curve studies. Determinations of population, pH, and enterotoxin were made. The initial pH of the growth medium, the incubation temperature, and the nature of the growth medium were varied. The principal measurements were the incubation times required to produce detectable amounts of enterotoxin under the various conditions. Attempts were made to correlate other measurements with incubation times needed to produce detectable amounts of enterotoxin. Comparisons were made of incubation times needed to produce measurable amounts of staphylococcal enterotoxin in BHI broth and in vanilla pudding.

The microslide gel-diffusion assay of Casman and Bennett (1965) was used for detection of enterotoxin. Although the method is delicate, a person may distinguish with relative ease the toxin precipitation lines from interference lines. With this method enterotoxin concentrations as low as 1 μg/ml can be detected, and the method was used at the lower detection limit. This concentration of enterotoxin is a reasonable amount to cause concern

considering the toxicity of enterotoxin and the size of an average serving of food.

The pH range for growth of S. aureus 243 in agitated BHI broth was 4.7 to 9.4. The pH range for production of enterotoxin B in agitated BHI broth was 5.1 to 9.0. The ranges are practically the same in BHI broth with or without 0.2M phosphate buffer. However, in the broth containing phosphate, more incubation time was required than in non-buffered BHI broth before a detectable amount of enterotoxin was produced. Over the pH range, little correlation was observed between enterotoxin production and population.

The temperature range for growth of the four strains of S. aureus was 13 to 45 C with no growth at 7 or 50 C. The temperature range for production of four immunologically different enterotoxins was 19 to 45 C, except for enterotoxin B which was produced at 13 C also. Over the incubation temperature range, little correlation was observed between enterotoxin production, population, and pH.

Each of the four strains of S. aureus used in this study produced one of four immunologically different types of enterotoxin. The growth and enterotoxin production characteristics of these strains were compared when incubated at various temperatures in agitated BHI broth and in vanilla pudding. Under the experimental conditions used, only minor differences were detected among the four strains.

Several foods were inoculated with S. aureus, and growth and enterotoxin production were determined. Responses varied from no growth, to growth but no enterotoxin production, to growth with enterotoxin production. All the foods were "mishandled" in some way in addition to being inoculated with S. aureus. At the time that samples for enterotoxin analysis were collected, however, the foods did not appear to be spoiled and probably would have been accepted by the average consumer.

The incubation times required for production of detectable amounts of enterotoxin in BHI broth and in vanilla pudding were compared. The information concerning enterotoxin production in BHI broth could be used to give an indication of enterotoxin production in food; however, the data from one system could not be applied directly to the other. The major reason probably is that in the agitated broth a macroenvironment existed; whereas, in the semi-solid pudding a microenvironment existed.

Several conclusions were derived from this study. The pH range for production of enterotoxin B in agitated BHI broth at 37 C is 5.1 to 9.0. The growth range of S. aureus 243 under similar conditions is 4.7 to 9.4. The temperature range for production of staphylococcal enterotoxins A, C, and D by S. aureus strains 265, 493, and 315, respectively, was 19 to 45 C, and the temperature range for production of staphylococcal enterotoxin B by S. aureus

243 was 13 to 45 C. Growth of all four strains occurred in the range of 13 to 45 C. The four strains of S. aureus had similar, although not identical, characteristics for growth and enterotoxin production under the experimental conditions. The information concerning enterotoxin produdction in BHI broth, a common laboratory medium, can be used to give an indication of enterotoxin production in a food substrate; however, the information can not be applied directly to a food system.

LIST OF REFERENCES

LIST OF REFERENCES

American Public Health Association. 1960. Standard Methods for the Examination of Dairy Products (11th ed.). American Public Health Association, New York.

Angelotti, R. 1969. Staphylococcal Intoxications, p. 359-393. In H. Riemann (ed.), Food-Borne Infections and Intoxication. Academic Press, New York.

Bergdoll, M. S. 1970. Enterotoxins, p. 265-326. In T. C. Montie, S. Kadis, and S. J. Ajl (eds.), Bacterial Protein Toxins. Academic Press, New York.

Busta, F. F., and M. L. Speck. 1968. Antimicrobial Effect of Cocoa on Salmonellae. Appl. Microbiol. 16:424-425.

Casman, E. P. 1967. Staphylococcal Food Poisoning. Health Lab. Sci. 4:199-206.

Casman, E. P., and R. W. Bennett. 1965. Detection of Staphylococcal Enterotoxin in Food. Appl. Microbiol. 13:181-189.

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.

Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and W. B. Wood. 1969. Microbiology. Hoeber Medical Division; Harper and Row, New York, p. 727-740.

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

Friedman, M. E. 1966. Inhibition of Staphylococcal Enterotoxin B Formation in Broth Cultures. J. Bacteriol. 92:277-278.

Genigeorgis, C., M. S. Foda, A. Mantis, and W. S. Sadler. 1971a. Effect of Sodium Chloride and pH on Enterotoxin C Production. Appl. Microbiol. 21:862-866.

Genigeorgis, C., S. Martin, C. E. Franti, and H. Riemann. 1971b. Initiation of Staphylococcal Growth in Laboratory Media. Appl. Microbiol. 21:934-939.

Genigeorgis, C., M. Savoukidis, and S. Martin. 1971c. Initiation of Staphylococcal Growth in Processed Meat Environments. Appl. Microbiol. 21:940-942.

Hall, H. E., R. Angelotti, and K. H. Lewis. 1965. Detection of the Staphylococcal Enterotoxins in Food. Health Lab. Sci. 2:179-191.

Hobbs, Betty. 1967. Health Problems in Quality Control: Microbiological Aspects, p. 85-90. In S. M. Herschdoerfer (ed.), Quality Control in the Food Industry Volume I. Academic Press, New York.

Hood, L. L. 1968. A Study on the Production of Types A and B Enterotoxin Resulting from the Growth of Staphylococcus aureus in Cheese and Brain Heart Infusion Broth. M.S. thesis, Michigan State University.

Jay, J. M. 1970. Modern Food Microbiology, Van Nostrand Reinhold Co., Cincinnati, p. 194-214.

Kao, C. T., and W. C. Frazier. 1966. Effect of Lactic Acid Bacteria on Growth of Staphylococcus aureus. Appl. Microbiol. 14:251-255.

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

McCoy, D. W., and J. E. Farber. 1966. Influence of Food Microorganisms on Staphylococcal Growth and Enterotoxin Production in Meat. Appl. Microbiol. 14:372-377.

McLean, R. A., H. D. Lilly, and J. A. Alford. 1968. Effects of Meat-curing Salts and Temperature on Production of Staphylococcal Enterotoxin B. J. Bacteriol. 95:1207-1211.

Morse, S. A., R. A. Mah, and W. J. Dobrogosz. 1969. Regulation of Staphylococcal Enterotoxin B. J. Bacteriol. 98:4-9.

Peterson, A. C., J. J. Black, and M. F. Gunderson. 1962. Staphylococci in Competition I. Growth of Naturally Occurring Mixed Populations in Precooked Frozen Foods during Defrost. Appl. Microbiol. 10:16-22.

Peterson, A. C., J. J. Black, and M. F. Gunderson. 1964. Staphylococci in Competition III. Influence of pH and Salt on Staphylococcal Growth in Mixed Populations. Appl. Microbiol. 12:70-76.

Raj, H. D., and M. S. Bergdoll. 1969. Effect of Enterotoxin B on Human Volunteers. J. Bacteriol. 98: 833-834.

Read, R. B. Jr., and J. G. Bradshaw. 1966. Staphylococcal Enterotoxin B Thermal Inactivation in Milk. J. Dairy Sci. 49:202-203.

Reiser, R. F., and K. F. Weiss. 1969. Production of Staphylococcal Enterotoxins A, B, and C in Various Media. Appl. Microbiol. 18:1041-1043.

Stark, R. L., and P. R. Middaugh. 1969. Immunofluorescent Detection of Enterotoxin B in Food and a Culture Medium. Appl. Microbiol. 18:631-635.

Stark, R. L., and P. R. Middaugh. 1970. Immunofluorescence Detection of Enterotoxin B Produced Under CO_2 and N_2 Atmospheres. Appl. Microbiol. 20:519-520.

Tatini, S. R., B. R. Cords, L. L. McKay, and R. W. Bennett. 1971. Effect of Growth Temperatures Higher than Optimal on Production of Staphylococcal Enterotoxins and Deoxyribonuclease. Bacteriol. Proc. A101 (Abstr).

Troller, J. A. 1971. Effect of Water Activity on Enterotoxin B Production and Growth of _Staphylococcus aureus_. Appl. Microbiol. 21:435-439.

Troller, J. A., and W. C. Frazier. 1963. Repression of _Staphylococcus aureus_ by Food Bacteria I. Effect of Environmental Factors on Inhibition. Appl. Microbiol. 11:11-14.

Walker, G. C., and L. G. Harmon. 1965. The Growth and Persistence of *Staphylococcus aureus* in Milk and Broth Substrates. *J. Food Sci.* 30:351-358.

Zehren, V. L., and V. F. Zehren. 1968. Relation of Acid Development During Cheesemaking to Development of Staphylococcal Enterotoxin A. J. Dairy Sci. 51: 645-649.

MICHIGAN STATE UNIVERSITY LIBRARIES
3 1293 03169 4155