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

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ABSTRACT

PRODUCTION OF TYPES A AND B ENTEROTOXIN
BY STAPHYLOCOCCUS AUREUS IN CHEESE AND
IN BRAIN HEART INFUSION BROTH

by Larry Lee Hood

Cheddar and colby cheese were manufactured from normal pasteurized milk with and without inoculations of strains of Staphylococcus aureus producing types A and B enterotoxin. The above varieties of cheese were also made from inoculated milk to which penicillin had been added at the rate of approximately 0.49 to 0.54 Units per milliliter of milk.

One of the Cheddar cheeses and one of the colby cheeses manufactured from milk treated with penicillin and inoculated with a strain of S. aureus producing type A enterotoxin was found to contain enterotoxin during ripening at 48 F. The toxic Cheddar cheese had a maximum S. aureus population of 110 million organisms/g of cheese after 30 days of curing. Toxin was formed in this Cheddar cheese between 20 and 30 days of ripening. The maximum amount of toxin detected in the cheese was 4 µg/100 g of cheese after 30 days of curing. The toxic colby cheese had a maximum staphylococcal population of 6.5 million organisms/g of cheese after 20 days of curing. Toxin was formed in the colby cheese between 1 and 10 days of ripening. The maximum amount of toxin detected in the cheese was 2 µg/100 g of cheese.

No toxin was detected in the cheese manufactured from milk inoculated with the strain of S. aureus producing type B enterotoxin.

However, one of the Cheddar cheeses manufactured from milk containing penicillin and inoculated with the S. aureus producing type B toxin, contained 480 million staphylococci per gram of cheese after 60 days of curing.

The type A producing strain of S. aureus produced maxima of 8, 8 and 6 µg of toxin/ml of supernatant in aerated brain heart infusion (BHI) with respective pH values of 6.0, 7.4, and 8.0. Toxin was first detected in the BHI broth at pH 7.4 when the S. aureus population was 270 million organisms/ml. This population coincides reasonably well with the maximum S. aureus population that occurred during ripening of the toxic Cheddar cheese, but is substantially greater than the 6.5 million staphylococci/g in the toxic colby cheese.

The type B toxin producing strain of S. aureus produced maxima of 382, 382, and 32 µg of toxin/ml of supernatant in aerated BHI broth with respective pH values of 6.0, 7.4 and 8.0. Toxin was first detected in the BHI broth at pH 7.4 when the S. aureus population was 40 million organism/ml. No explanation is offered as to why toxin was not detected in the Cheddar cheese made from milk inoculated with the type B enterotoxin producing strain of S. aureus and which attained 480 million staphylococci/g during ripening.

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BY

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L.L.H.

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INTRODUCTION

The occurrence of staphylococci as common residents of the skin and in the suppuration of wounds and open sores on the body is unquestioned. Also the widespread relationship between food poisoning and the growth of certain strains of Staphylococcus aureus has been thoroughly investigated by many workers (Barber, 1914; Dolman, 1934; Dack, 1956; and Casman and Bennett, 1963, 1965). Early investigations on the nature of the toxic principal resulting from the growth of S. aureus by Dolman et al. (1936) has lead to the purification of several distinct toxic proteins, which in turn has resulted in the application of serological methods in detection of staphylococcal enterotoxin.

The amount of enterotoxin necessary to cause illness in humans is not known, nor has much information been gathered on the amount of enterotoxin that can result from the growth of toxigenic strains of S. aureus in foods. Very little is known presently of the relationship between time and the population necessary for the appearance of enterotoxin in food.

Dairy products have not generally been implicated in staphylococcal food poisoning incidents, but recently ice cream, milk powder and both raw and pasteurized milk cheese have been found to contain enterotoxin. The purpose of this research was to enlarge our knowledge of the growth and production of toxin by certain strains of S. aureus in Cheddar and colby cheese. A supporting investigation was conducted to see what effects pH of the medium had upon growth and toxin production by S. aureus

in aerated brain heart infusion broth. One of the strains used in this research produces type A toxin while the other produces type B toxin.

REVIEW OF THE LITERATURE

History

Partially because of the ubiquity of staphylococci, their role in food poisoning was not recognized until 1914. However, descriptions of food poisoning cases given by medical workers, even before Pasteur found staphylococci in pus in 1880, are evidence that staphylococci undoubtedly had caused food poisoning for centuries (Dack, 1956).

Barber in 1914 (Dack, 1956) associated the etiology of an explosive, violently nauseating, selectively occurring food poisoning with the growth of a single strain of staphylococcus. Barber's report was overlooked until Dack *et al*. (1930) rediscovered the role of staphylococci in food poisoning. These workers not only isolated staphylococci from incriminated food, but also reproduced the illness by feeding culture filtrates to human volunteers.

Jordan *et al*. (1931) demonstrated the heat resistance of the toxic principle resulting from the growth of staphylococci, by boiling culture filtrates for 30 minutes and subsequently inducing food poisoning symptoms in humans by feeding them the filtrates. These early studies indicated that the toxic agent was present in culture supernatants as an exotoxin and because the predominant symptoms of the food poisoning illness were gastroenteric, the toxin was called *enterotoxin*.

Since the "modern era" of staphylococcal food poisoning investigations began in 1930, the search for an adequate enterotoxin assay

has turned from human volunteers to animal tests and more recently, to biological and chemical methods.

The earliest of the animal assays was the monkey feeding test of Jordan and McBroom (1931). However, Dolman et al. (1936) sought to circumvent the difficulty of handling and cost of the young monkeys used by Jordan and McBroom and so developed the "Dolman Kitten Test" for enterotoxin. During the development of the kitten test, Dolman found evidence that enterotoxin might be antigenic in nature, suggesting the possibility of an antigen-antibody assay for the toxin. Further proof of the antigenic nature of enterotoxin was offered by Dolman and Wilson (1938) when they demonstrated a specific flocculation reaction which appeared to involve enterotoxin and its homologous antibody. However, development of an antigen-antibody assay for enterotoxin was delayed until enterotoxin could be purified. Therefore, because of the lack of a better assay, workers were forced to depend upon animal tests for enterotoxin for many years. Such tests were inaccurate due to varying levels of susceptibility to the enterotoxin within a group of test animals, non-specific emetic responses during a given test, and increasing resistance to the enterotoxin by repeated exposure.

Scientists in the area of enterotoxin research always recognized the need for a pure enterotoxin preparation around which to build a chemical assay and for various study purposes. Early attempts by Jordan and Burrows (1933) to concentrate the toxin by dialysis were successful. Following the suggestion of Dolman that the enterotoxin was antigenic and therefore a protein, many people turned to the

application of protein fractionation techniques in order to purify the toxin.

Davidson and Dack (1939) attempted to purify enterotoxin by ammonium sulfate precipitation. Hammon (1941) suggested the use of a combination of ammonium sulfate and ethanol precipitation to concentrate the toxin. An acid precipitation technique was used by Bergdoll et al. (1951) in a successful attempt to concentrate the toxin from bacterial filtrates. While these attempts were being made to purify the toxin, bacterial physiologists were working on methods to rapidly indicate the presence of enterotoxin in food by bacteriological methods rather than depend upon inferior animal assays.

Information gathered on the physiology of the staphylococcal organism, revealed that staphylococci have the ability to ferment mannitol with acid production, produce a gelatinase, and grow well in the presence of high sodium chloride concentrations. But of greater importance was the finding that certain strains of Staphylococcus aureus associated with food poisoning incidents could also produce the enzyme coagulase. The work of Evans and Niven (1950), and Evans, Buettner, and Niven (1950) showed that the enterotoxigenic strains of S. aureus appeared to be a very homogeneous group. Of the strains tested, they found that the enterotoxigenic strains produced coagulase, but not all coagulase-positive strains produced enterotoxin. On the other hand, they reported that all coagulase-negative cultures studied failed to produce enterotoxin. They suggested that the coagulase test could be a rapid indication of staphylococcal food poisoning when

coagulase-positive strains could be isolated from a suspect food. The coagulase test has remained an important indirect determination for enterotoxin since the work by Evans, Buettner, and Niven until recently when Bergdoll (1967) proved the existence of several coagulase-negative, enterotoxin producing strains of S. aureus. The test has now assumed a somewhat less important role in the identification of enterotoxin producing strains of S. aureus in favor of the more specific serological tests.

The production of heat labile hemolysins by the growth of S. aureus was recognized (Surgalla and Hite, 1945) but not until 1953 did people learn that the organism might also produce more than one distinct enterotoxin (Surgalla et al. 1954; Matheson and Thatcher, 1955). Through the use of gel-diffusion analysis Surgalla et al. (1954) attempted to demonstrate conclusively that staphylococcal enterotoxin was a specific antigen. Although they failed to detect the specific antigen they were able to neutralize toxic material completely. However, Casman (1958) was able to demonstrate the antigenicity of enterotoxin and also in vivo protection of cats against the toxin with both heterologous and homologous strains.

A second aspect of the pathogenicity of enterotoxin other than as a causative agent in food poisoning was investigated by Surgalla and Dack in 1955. These workers were able to isolate several enterotoxigenic strains of S. aureus from fecal cultures of a few individuals experiencing gastroenteritis while on antibiotic therapy.

Improved fractionation procedures for enterotoxin from strain S-6 of S. aureus were reported by Bergdoll et al. (1959) and Hibnick

and Bergdoll (1959). Included in the procedure was a combination of acid precipitation, alumina absorption, alcohol precipitation, Amberlite IRC-50 column chromatography, and finally zone starch electrophoresis to determine purity. Single and double gel-diffusion tests were used to follow the purification process. The chemical properties of the purified preparation were also reported. They found that the toxin was a protein with no indication of the presence of carbohydrate or lipid, had a molecular weight of 24,000 ± 3,000, and an isoelectric point of 8.6. An earlier publication by Bergdoll (1956) gave evidence that the toxin was resistant to trypsin and contained a high percentage of lysine. Bergdoll *et al.* also in 1959, using gel-diffusion techniques and highly purified enterotoxin (presently called type B), demonstrated the presence of a specific precipitating antibody with an enterotoxin neutralizing property. The identification of this antibody provided a basis, for the first time, for an *in vitro* assay of staphylococcal enterotoxin.

Using the more relaible serological method for studying enterotoxin, Sugiyama *et al.* (1960) reported a difference in the levels of toxin produced by different subcultures of a single *S. aureus* strain and also gave evidence for at least one more antigenically distinct enterotoxin resulting from the growth of strain 196-E and differing from that produced by strain S-6. Casman (1958, 1960) prepared antiserum for the new type toxin (196-E) using a technique of specific absorption with certain non-enterotoxigenic cultures known to share antigens with the new type. Casman also reported that there were strains of *S. aureus* which (a) reacted with both S-6 and 196-E

antisera, (b) reacted only with S-6 antiserum-strain 243, (c) reacted only with 196-E antiserum and finally, (d) showed no reaction with either antiserum but were toxic to cats. This toxicity was lost however, when the culture filtrates were heated in boiling water for 30 minutes or when dialyzed through cellophane. Strains producing the type 196-E, heat-resistant enterotoxin were of food origin and Casman designated such enterotoxin as type "F". The heat-resistant type S-6 enterotoxin produced by strain 243 was related to strains associated with enteritis from antibiotic therapy and was designated as type "E". Bergdoll et al. (1962) reported that possibly a third type of enterotoxin existed, resulting from the growth of strain 137, since its toxin did not cross react with antisera from either strain S-6 or 196-E. He also reported an increase in the recovery and purification of type E toxin and a 70% purification of type F toxin by using Amberlite XE-64 chromatography.

Dack (1962) reviewed the staphylococcal enterotoxins with respect to types, production, resistance to heat, chemistry and mode of action in the human and animal bodies. He recognized three types of enterotoxin: one associated with food poisoning (type F); one produced by strains of S. aureus associated with enteritis (type E); one immunologically unrelated to either of the above (type 137). These were described as distinctive proteins, and unlike lysins, resistant to pepsin and trypsin.

A new method of designating the staphylococcal toxins was suggested by Casman et al. (1963) using the capital letters of the alphabet. With this nomenclature the previous type 196-E or type F would be

designated as type A with the prototype strain being 196-E. The type S-6 or type E became known as type B with the prototype strain being 243. Recently, the serological identification of type C enterotoxin from strain 137 (Bergdoll et al. (1966)) and type D enterotoxin from strain 494 (Casman et al. (1967)) has also been reported. The designation of additional enterotoxins will follow alphabetically when they are established as immunologically distinct entities.

Casman and Bennett (1963) reported that, at least in culture media, type B strains usually produced more toxin than type A strains. Because type A strains produced very low amounts of enterotoxin, they suggested growing the cultures in dialysis tubing placed in contact with the media for pure culture studies.

The growth and production of enterotoxin by the type A strain in raw and cooked meat was studied by Casman et al. in 1963. To detect the low amounts of enterotoxin produced they used carboxymethyl cellulose in column chromatography for the extraction and concentration of the toxin. The concentrated toxin was then identified with a microscope slide version of the Ouchterlony gel-diffusion assay. Other workers have since reported on the extraction and identification of either type A or B enterotoxin from foods (Hall et al., 1963; Read et al., 1964; Genigeorgis, 1966; and Zehren and Zehren, 1968) with varying success. Most methods used to detect enterotoxin in food have included blending to separate the solid material from the water soluble toxins, concentration of the toxin by dialysis, and ion-exchange chromatography and gel-diffusion methods for quantitation of the toxin

levels. Casman (1966) also reported on the frequency with which the different types of enterotoxins were produced by coagulase-positive staphylococci isolated from several different sources. Of the staphylococci isolated from foods implicated in seventy-five different food poisoning incidents, 49% produced enterotoxin A alone and 28.5% produced enterotoxin A together with other enterotoxins. Enterotoxin D was produced alone by 10% of these strains and together with enterotoxin A by approximately 25% of the strains. The incidence for each of types B and C was approximately 4%.

Thermal stability of staphylococcal enterotoxin was discussed by Jordan in 1931, by Denny et al. (1966), and Read and Bradshaw (1966). Denny reported F_{250} values (z = 48 F) of 11 minutes and 8 minutes for the inactivation of type A toxin as assayed by intraperitoneal injection of cats and the monkey feeding test, respectively. Read and Bradshaw reported F_{250} values (z = 32.4 F) of 9.9 minutes for type B enterotoxin as assayed by both gel-diffusion and cat emetic responses. They also gave some evidence for an increase in heat stability of the toxins if they were in a crude protein preparation rather than highly purified.

The increase in use of gamma radiation preservation of foods in recent years has also prompted a study of the stability of type B enterotoxin to radiation. Read and Bradshaw (1967) determined D values for inactivation of toxin in Veronal buffer and milk to be 2.7 and 9.7 Mrad, respectively. End points for inactivation were determined by parallel titrations with gel-diffusion methods and cat emetic

responses. It can be concluded on the basis of the data presented in these studies on the thermal and radiation treatments of enterotoxin that the current thermal processing times and temperatures are sufficient to inactivate any existing type A or type B enterotoxins, unless they are afforded some unknown protection by the food or occur in extremely high concentrations. However, Read and Bradshaw indicated that the irradiation processes now used for pasteurization or sterilization of foods cannot be expected to inactivate type B toxin if it were present in food before processing.

Production of Enterotoxin

Very little has been reported on the conditions under which toxigenic strains of staphylococci produce enterotoxin. Due to the lack of an easy assay for this toxin, the bulk of the reported studies deal with the growth of staphylococci in culture media or food, under a variety of environmental conditions. Appreciable levels of enterotoxin are produced only after considerable growth of the staphylococcus. Usually a population of at least several million per milliter or gram must be attained. Therefore, the conditions that favor toxin production are those best for growth of the staphylococcus (Frazier, 1967). The effect of temperature, pH, competing organisms, and the number of staphylococci needed to initiate growth and toxin production will be reviewed.

Effect of Temperature. Segalove and Dack (1941), under laboratory conditions, demonstrated enterotoxin production in a culture grown for 3 days at 18 C and in one grown for 12 hours at 37 C. Cultures grown

for shorter periods did not contain enterotoxin as assayed by the kitten test. They also found that enterotoxin was not demonstratable in cultures grown 3 and 7 days at 9 and 15 C, respectively. According to Frazier (1967), toxin production is best at 21.1 to 36.1 C, a temperature range which easily falls within that required for the best growth of S. aureus. Casman and Bennett (1963) reported maximum toxin production for type A and B enterotoxigenic strains of S. aureus when incubated at 37 C for 48 hours on Brain Heart Infusion (BHI) agar at pH 5.3. Casman et al. (1963) have also reported the production of type A enterotoxin when organisms were incubated on disks of ham held at 30 C for 72 hours. Dack (1956), Hendricks et al. (1958) and Zehren and Zehren (1968b) all have identified cheddar cheese in food poisoning outbreaks, indicating the development of toxin from the growth of S. aureus during cheese manufacture and curing. During manufacture and processing cheddar cheese is at a temperature of 80 to 102 F and during ripening or curing the cheese is usually stored at 40 to 55 F for 2 to 12 months.

Effect of pH. Lechowich et al. (1956) found staphylococci able to grow vigorously in ground pork muscle containing any combination of curing ingredients permissible and palatable. However, if the pH of the meat was lowered to 4.8 to 5.0, anaerobic growth of staphylococci was prevented. In a broth medium, growth was inhibited at pH 5.6 anaerobically, and aerobically growth was inhibited at pH 5.6 and prevented at pH 4.8.

Dack and Lippitz (1962) reported that staphylococci inoculated

into slurries of frozen pot-pies grew well at pH 5.0 and higher. They grew slightly at pH 4.5 but not at pH 4.3 to 4.0.

Casman and Bennett (1963) found that a semisolid BHI agar at pH 5.3 to 5.5 gave higher yields of type A toxin than BHI agar at pH 6.0, 6.5, 7.0 and 7.5. With 3.7% BHI broth, they found maximum toxin when the initial pH was 6.5 to 7.0. This seems to indicate that toxin production depends upon more than one environmental factor. For instance, the effect of sodium chloride and pH upon the production of type B enterotoxin was studied by Genigeorgis (1966). He found that with the same initial pH, the growth rate and toxin production by S. aureus decreased as the sodium chloride concentration increased. Enterotoxin B was detected in six day old BHI broth cultures in which the concentration of salt was 10%.

Time of incubation and toxin production in food. The shortest period of time necessary for toxin production in foods in not known. Only a small number of experiments have been conducted in the past (Dack, 1956) because of a lack of an easy assay method. Experiments with human volunteers showed that cream became toxic after 5 hours at room temperature, mashed potatoes and milk after 6.5 hours at room temperature, canned oysters after 72 hours at 37 C, canned corn after 96 hours at 37 C, and home-baked ham and lettuce sandwich after 72 hours at 37 C.

Competition of staphylococci with other bacteria. The poor ability of staphylococci to compete with other organisms present in foods has long been recognized (Casman and Bennett, 1963). Bacterial

inhibition of staphylococcal growth and toxin production is mainly due to a competition for essential nutrients, or production of antibiotic or other inhibitory substances. Dack and Lippitz (1962) inoculated slurries of frozen pot-pies with varying numbers of staphylococci. In the presence of the natural flora, staphylococci usually did not grow sufficiently to suggest a hazard in such foods from staphylococcal food poisoning. The predominant organism, a lactobacillus, produced considerable acid in the medium. Such was also the case in a recent, rather large outbreak of staphylococcal food poisoning occurring in cheddar cheese. Epidemiology of the cheese revealed toxin production only in those cases where there was low acid production during cheese manufacture (Zehren and Zehren, 1968b). Normal acid production in cheese manufacture is the result of good growth of the starter organisms in the milk. Staphylococcal food poisoning appears to occur in foods which have been treated to drastically reduce the bacterial population or in foods which selectively favor the growth of staphylococci; for example, cured hams which are pasteurized and contain a high salt concentration. With such a reduction of natural flora and inhibition of non-salt tolerant bacteria, staphylococci grow luxuriously and produce toxin. This may explain why ham is the most commonly implicated food item in staphylococcal food poisoning (Brandly, 1965). Casman and Bennett (1963), working with raw and cooked meat inoculated with a type A enterotoxin producing strain of S. aureus, have determined that there was no growth of S. aureus in the raw meat but good growth and toxin production in the cooked. This shows

again the inhibitory effect of the competitive bacteria in the raw meat.

Finally, staphylococcal enteritis which appears in patients under oral antibiotic treatment can result from a lack of microbial competition. Antibiotic resistant staphylococci in this case survive the antibiotic treatment and grow and produce toxin after the antibiotic sensitive competitors have been eliminated from the digestive tract.

Amount of enterotoxin and number of staphylococci necessary to induce food poisoning. The amount of enterotoxin necessary to induce illness in man is not known. Purified (50%) type A enterotoxin was found by Casman and Bennett (1965) to have a toxicity level of 1 to 2 µg in one cat test. A highly purified type B enterotoxin (Bergdoll, 1961) produced emesis in rhesus monkeys when administered orally in amounts of 1 µg of nitrogen.

Casman and Bennett (1965) reported the following foods and numbers of staphylococci responsible for food poisoning out breaks: coconut cream pie and banana cream pie with 200 million and 72 to 73 million staphylococci/g producing type A enterotoxin, respectively; and macaroni salad with 1 million organisms/g producing type A toxin. Experimentally, they found the production of 0.2, 14 and 8-9 µg of type A toxin per gram of food in coconut custard, shrimp paste, and turkey paste with 2.5, 15 and 13.5 billion staphylococci/g of food, respectively.

Hall et al. (1963) found detectable levels of type B enterotoxin (4 to 68 µg/ml) were produced consistently in slurries of shrimp,

scallops, lobster, and crabmeat containing 110 million to 1 billion organisms/ml. This number was reached after 24 to 48 hours incubation at 35 C following inoculation with one milliter of an 18 hour culture.

Staphylococcal food poisoning from cheese. Staphylococcal food poisoning is presently considered to be the most common type of food-bourne disease in the United States (Dauer, 1961). Even though food poisoning in general is poorly reported, many different types of food have been incriminated in staphylococcal food intoxications. Very few food poisoning cases have been attributed to milk and dairy products in recent years due to improved supervision in sanitary production and processing of milk and dairy products. Furthermore, of the cases due to dairy products, those reported as being due to cheese are uncommon (Hendricks et al., 1959). There have been a few, however, and some of these will be reviewed.

Vaughn (1884) reviewed 300 cases of poisoning that supposedly resulted from Michigan cheese of a cheddar type that was eaten by many persons over a six month period. Staphylococcal food poisoning was not recognized at that time and he concluded that the causative agent was a chemical poison; however, he did state that the chemical poison might have been generated by the agency of bacteria. Also in 1884, Dr. Sternberg at the Johns Hopkins University was able to recover micrococci from samples of the suspect Michigan cheese investigated by Dr. Vaughn. Sternberg concluded that the food poisonings were probably the result of the production of a poisonous "ptomaine" as the result of the growth of the micrococci. Dack (1956)

describes the work of a man named Barber who, in 1914, attributed several acute gastro-intestinal upsets on a certain farm in the Philippine Islands to the growth of a white staphylococcus in one of the udders of the family cow. In 1930, three separate outbreaks of food poisoning involving 18 persons who had eaten cheese occurred in Puerto Rico. Staphylococci were recovered from the cheese in each outbreak. Filtrates of the organisms isolated from two of the outbreaks were given to human volunteers and produced symptoms similar to those of the original cases (Hendricks, et al., 1959). MacDonald (1945) described four severe cases of staphylococcal food poisoning in Great Britain from home made goat's milk cheese. S. aureus was recovered from the cheese and from freshly drawn milk from one of the goats. Hendricks et al. (1959) reported an outbreak of food poisoning occurring in 200 persons as the result of eating Cheddar cheese manufactured from raw milk. Coagulase-positive S. aureus organisms were isolated from both the cheese and from the milk of some of the herds that supplied milk to the factory that produced the cheese. The most recent instance of staphylococcal poisoning resulting from cheese is the case discussed by Zehren and Zehren (1968a) in which 2112 vats of Cheddar, Monterey and Kuminost cheese were implicated in an unknown number of widespread food poisonings. Examination showed 56 vats of Cheddar, two vats of Monterey and one vat of Kuminost contained type A enterotoxin and were destroyed. The remainder of the cheese implicated was released for sale and no reports of additional illnesses have occurred.

Detection of Enterotoxin

Because staphylococcal food poisoning is an intoxication, researchers have worked to develop a reliable, sensitive and inexpensive test for the detection of enterotoxin in food poisoning outbreaks and in culture media.

The first attempts were directed toward methods involving the use of sensitive animals which respond in a characteristic and reproducible way to the enterotoxin. When the nature of enterotoxin was further elucidated and more data was obtained abouts its properties, a new area in the methodology of enterotoxin detection opened. This was mainly due to the demonstration of its antigenicity and precipitation by specific antibodies. The techniques tried and developed through the years can be divided into three major groups. The first group involves purely biolobical methods in which live animals are used. The second group involves immunological methods based on the antigenicity of enterotoxin and the reaction with its antibody. The third group involves methods which demonstrate certain staphylococcal characteristics which later are correlated with enterotoxicity. These three major groups involve biological, immunological and indirect bacteriological methods.

Biological methods. Testing with human volunteers is considered best because humans are highly sensitive to the enterotoxin. However, variations in the response of different individuals, naturally occurring resistance against enterotoxin, and the difficulty in recruiting volunteers complicates tests using humans.

The monkey feeding test was developed by Jordan and McBroom (1931) and is still in use. Culture filtrates or food extracts are made to 50 ml volume and are fed by stomach tube to young Rhesus monkeys (Macaca mullata). The animals are then observed continuously for 5 hours. Of the symptoms caused by ingestion of enterotoxin, only vomiting is accepted as a positive reaction. Because monkeys develop tolerance to enterotoxin, it is recommended that six animals be used per sample of which at least two should react to consider the sample positive for enterotoxin. Although alpha- and beta-hemolysins have been shown to provoke emesis when injected into monkeys and other animals, both toxins are sensitive to the proteolytic enzymes of the gastro-intestinal tract and no special treatment is needed to eliminate these toxins from the sample. However, should the test be performed by intravenous injection it is necessary to destroy the hemolysins by boiling the filtrates from the S. aureus culture for 30 minutes, or by neutralization with specific antiserum or by adding a proteolytic enzyme such as trypsin which does not affect enterotoxin.

The kitten test was developed by Dolman et al. (1936) who suggested the use of kittens from 6 weeks to 3 months of age and weighing between 350-700 grams. The toxic material, culture filtrate, or food extract is boiled for 30 minutes to destroy hemolysins and centrifuged to remove precipitates. The clear supernatant is injected intraperitoneally in amounts of 1 to 3 ml. For reliable results at least 3 kittens per sample should be used. Strong peristaltic movements may be noted which, after 15 minutes to 1.5 hours, culminate in the first of a series of attacks of retching and vomiting. The same

kitten may be used for several tests within a 7 to 10 day period provided the injections are so spaced as to permit complete recovery from each preceding dose.

Hammon (1941) has described a kitten test wherein the toxic material is injected intravenously. Usually 0.5 to 5.0 ml of culture filtrate that has been properly treated to remove hemolysins, is injected into a kitten weighing about 800 grams. With this intravenous injection, only vomiting occurring after 15 minutes should be considered as a positive reaction. A moderate meal eaten shortly before the inoculation of the toxic material increases the effectiveness of the vomiting stimulus. All toxic samples should be first inoculated into cats not previously used. Positive samples can then be confirmed on twice-used or thrice-used kittens.

The parenteral administration of enterotoxin to cats is probably less desirable than intravenous administration, since it may be complicated by the hemolysins, rapid development of increased tolerance to the enterotoxin, and by a considerable variation in susceptibility of test animals (Casman, 1958). The kitten test, however, is more sensitive, less expensive and more convenient than the monkey feeding test.

The frog test developed by Robinton (1950), involves feeding toxic filtrates to frogs (Rana pipiens) and observing for reverse peristaltic waves in the stomach and intestine. It was found however, that the reaction of frogs to enterotoxin was inconsistent and apparently unrelated to dosage. Positive reactions (reverse peristaltic motion) have been seen in frogs fed saline and other nonenterotoxic

materials (Surgalla, 1953).

Nematodes were once thought to show certain coiling reactions when in contact with solutions containing enterotoxin. However, it is now known that such coiling reactions were not specific for enterotoxin, and the test is invalid (Bergdoll, 1962, 1963).

Milone (1961) reported the cytopathogenic effects on tissue cultures by the A and B enterotoxins in various states of purity, in an attempt to adapt tissue culture methods to a bioassay of enterotoxin. This test was not reliable because not all of the presently known enterotoxins have cytopathogenic effects on tissue, and because impurities may cause non-specific effects (Bergdoll, 1963).

Tropical fish of Tilapia spp. were used by Raj and Liston (1962) to demonstrate enterotoxin. Bergdoll (1963) concluded from tests performed with purified enterotoxin that the observed reaction of distress and death of fish was due to some substance other than enterotoxin.

Immunological methods. The demonstration of the antigenicity of enterotoxin and the identification by gel-diffusion methods of a specific precipitating antibody against enterotoxin opened a new area for which to study the properties of the toxin.

Dolman and Wilson (1938) using horse antiserum and crude toxins reported 3 flocculation zones in Ouchterlony plates. The Oudin (1952) tube gel diffusion test has also been used in the study of enterotoxin homogeniety, purification and production. Surgalla et al. (1952) applied this technique to follow the purification of staphylococcal

enterotoxin.

Immunological techniques, are considered more specific than the biological tests since they are based on the reaction of enterotoxin (antigen) with its specific antisera. The main disadvantage of these techniques is that antisera prepared against highly purified enterotoxin are necessary and thus only the known antigenic types of enterotoxins can be detected.

Crowle (1961) described the principles and details of all the tests based on gel-diffusion. A brief description of such tests is included here. Gel-diffusion can either be single or double:

Single gel-diffusion, also known as Oudin's technique, involves the layering of an agar column containing antiserum with a solution of antigen. Under favorable conditions, a band of precipitate forms in the agar for each antigen-antibody system present. In the case of enterotoxin, as it diffuses the front of the band formed moves down the agar column at a rate corresponding to the concentration of enterotoxin and the concentration of antibody. This method has been adapted for the determination of the concentration of type B enterotoxin, at levels as low as 1 µg/ml (Bergdoll, 1962).

In double gel-diffusion, a thin glass tube is prepared by placing a layer of agar containing the antisera in the bottom, then a layer of neutral agar, and finally a layer of antigen, also in agar, over the neutral zone. As both the reagents diffuse into the neutral zone, a band of precipitate is formed where the two reagents meet in optimal proportions. The test as described above is known as

Oakley's double diffusion tube method (Oakley and Fulthorpe, 1955) and has been used by Hall et al. (1963) to detect enterotoxin concentrations as low as 0.05 µg/ml.

Double diffusion can also be performed in a petri dish layered with agar. This technique is known as Ouchterlony's method (Ouchterlony, 1949, 1953) and is an excellent method for detecting soluble antigens and antibodies. To conduct the test, one first layers a petri dish with a buffered agar and cuts a central hole in the agar after solidification. Several more holes are also cut so that they form a circle around the center one. Antiserum is added to the centrally located well in the agar and antigen is added to the peripheral wells. Antigens and antibodies diffuse through the agar to form zones or lines of precipitation where they combine in optimal proportion. The method permits separation of multiple precipitating systems into their individual components and, in addition, permits comparison of two antigens or antibodies for identification. A more sensitive modification of the above method was developed by Wadsworth (1957) and described in detail by Crowle (1961). This method involves layering a microscope slide with a thin film of agar and then placing a plastic template in complete contact with the agar. Reagents are placed in funnel-like wells drilled in the plastic template. This method, known as the slide gel-diffusion test or microdiffusion test, involves the same principles and method of interpretation as the Ouchterlony test. Measurement of the amount of enterotoxin consists in the determination of the highest dilution giving a line of precipitation which could

be identified through its coalescence with a reference line of precipitation produced by known samples of pure enterotoxin. The product of the volume of toxic food extract or toxic broth used and the reciprocal of its dilution giving such a line of precipitation is used to indicate the amount, in µg, of enterotoxin since it is estimated that a concentration of 1 µg/ml represents the limit of sensitivity of the test (Casman and Bennett, 1965).

Hopper (1963) used a flotation system to extract and detect staphylococcal enterotoxin from food. Ham salad was chosen as a food to which enterotoxin type B was added. The toxin was extracted by grinding and centrifugation. Rhodamine conjugated antiserum was added to the clear supernatant liquid. The treated supernatant was then passed through Sephadex C-25 and the eluent was mixed with a wetting agent. Through the use of compressed air, foam was formed and the conjugate-toxin complex, located on the layer of foam was separated to give a distinctly red-colored fraction. The toxin was identified by mixing one drop of the foam fraction, one drop of phosphate buffer at pH 4.5 and one drop of latex polyester suspension. Within one minute, a heavy agglutination occurred. Concentrations of 1 µg/ml from aqueous solutions were detected by this technique within 2 to 3 hours.

Robinson and Thatcher (1965) have reported an indirect hemagglutination inhibition procedure for detecting staphylococcal enterotoxin. Erythrocytes were sensitized with purified enterotoxin agglutinated in the presence of specific antiserum. The hemag-

glutination reaction was inhibited by prior incubation of a specific dilution of immune serum with graded amounts of enterotoxin in the presence or absence of impurities. By comparing the inhibitory effect of this reaction of known and unknown preparation of enterotoxin, Robinson and Thatcher were able to detect as little as 0.04 µg/ml of enterotoxin type A in 2 to 3 hours. Since other than enterotoxic substances may nonspecifically agglutinate sensitized red blood cells it seems that only highly purified food extracts will be identified as containing enterotoxin. Therefore, the problem of adequate extraction and purification methods must be solved first.

Labeling of antibodies by coupling with fluorescent dyes to permit their detection by direct microscopic examination was suggested by Coons et al. in 1941. Briefly, the method consists of attaching a compound such as fluorescein to the antibody molecule. The fluorescein-antibody conjugate is then mixed with antigen and the excess conjugate washed away, leaving behind an antigen-antibody complex which will fluoresce under ultra-violet light. The detection of small amounts of antigen-antibody complex is possible when the test is conducted on a microscope slide and the slide viewed under a U.V. microscope. Such an immunofluorescent technique has been applied to the detection of type B enterotoxin in foods by Genigeorgis (1966). Specific staphylococcal enterotoxin B antiserum was conjugated with fluorescein isothiocynate and used to detect amounts of enterotoxin less than 0.05 µg/ml of food extract without involving any purification procedures.

The demonstration of enterotoxin in food involves two main problems, extraction and concentration when small quantities are present. Experiments have been conducted by adding known amounts of toxin to foods and then recovering them by extraction. The amount of recovery varies. For example, Casman and Bennett (1965) reported recovery of 68% of enterotoxin type A and 48 to 72% of enterotoxin type B in such experiments. Hall et al. (1963, 1965) reported recoveries of over 90% of type B. Zehren and Zehren (1968a) have reported recoveries of 16 to 35% for type A enterotoxin added to Cheddar cheese.

The strain of S. aureus producing enterotoxin necessitates some consideration of the method of analysis used. For instance, those producing type B toxin characteristically do so in large amounts, often over 100 µg/ml in broth cultures. Conversely, those producing type A toxin do so in very low amounts, only 2 to 4 µg/ml in aerated culture as reported by Casman and Bennett (1965). Much lower amounts of toxin are expected to occur in food, and a concentration procedure is required when gel-diffusion techniques are used to detect the toxin.

Indirect bacteriological methods. As a result of intensive studies during the past 30 years, a variety of metabolic characteristics of staphylococci have been studied and correlated with enterotoxin production. The production of the enzyme coagulase is the most notable of those studied.

The presence of coagulase may be demonstrated directly by mixing about 0.1 ml of a broth culture of S. aureus with 0.5 to 1.0 ml of a

1/10 dilution of citrated human or rabbit plasma and incubating at 37 C. Clotting of the plasma in 3 to 6 hours is a positive indication of coagulase.

Many indirect assays for coagulase production are also available. The use of tellurite-glycine agar as described by Zebovitz et al. (1955) is a common method. To conduct this test one streaks a suspension of S. aureus onto a petri plate of tellurite-glycine agar and incubates it 24 hours at 37 C. The production of black colonies on the plate is considered a positive indication of coagulase production.

Other characteristics of S. aureus that have been studied as possible indicators of enterotoxin production include phosphatase, lipase, hemolysins, gelatinase, golden pigment and phage typing. However, it appears that no single one of these characteristics can be correlated completely with enterotoxicity since most of them have also been found to be characteristic of nonenterotoxic strains (Genigeorgis, 1966).

MATERIALS AND METHODS

Gel-diffusion reagents. Antisera for types A and B enterotoxin and highly purified A and B reference toxins were obtained through the courtesy of Dr. E. P. Casman, U. S. Food and Drug Administration Laboratories, Washington, D. C. The antisera and reference toxins were held at -30 C until used.

Staphylococcal strains. Two different strains of Staphylococcus aureus have been used throughout this study. They are identified as (1) strain 265-1 which produces type A enterotoxin and (2) strain 243 which produces type B enterotoxin. The toxin producing strains of S. aureus were also provided by Dr. Casman and were maintained on porcelain beads held at 4 C until reactivated by rolling the beads onto nutrient agar slants and incubating at 37 C. The cultures were maintained in an actively growing condition by transferring them to a fresh nutrient agar slant 24 hours before they were needed for inoculation into brain heart infusion broth used in toxin production investigations or into milk manufactured into cheese.

Culture media. Nutrient agar slants were used for reactivation of the S. aureus strains as mentioned. Nutrient broth at pH 7.4 (Difco), was used for growing the strains of S. aureus prior to inoculation into milk used to make the cheese in this investigation. Brain heart infusion (BHI) broth (Difco) was used for growing the strains to obtain enterotoxin. Casman (1967) has reported this is an excellent medium for obtaining maximum toxin production for several strains of S. aureus.

Staphylococcus 110 (Difco) and Standard Plate Count agar (Cudahy) have been used for enumeration of bacterial populations. The Staphylococcus 110 (S-110) medium was used as a selective growth substrate for the determination of the number of staphylococci occurring during the manufacture and ripening of the cheese made in this investigation and in the cultures of S. aureus grown in BHI broth. Plate Count agar (PCA) was used to determine total populations of microorganisms during the manufacture and ripening of the cheese.

Pilot plant experiments. Approximately 2500 pounds of pasteurized milk was made into Cheddar and colby cheese by a standard commercial process (Van Slyke and Price, 1952) in the Michigan State University dairy plant. A total of twelve vats of cheese were manufactured - six Cheddar and six colby. Each vat initially contained 210-230 pounds of milk which was made into a cheese weighing 20-25 pounds. Following manufacture the cheese was stored in a 48 F curing room until termination of the study at six months. After 60 days of ripening, the cheese was removed from its paper wrapping material because of mold growth on the cheese surface. The moldy areas were trimmed off and the remaining cheese coated with wax and returned to the curing room. The problem of mold on the cheese did not occur again during the remainder of the ripening period.

During manufacture of the cheese, certain vats of milk were treated in the following manner: (a) inoculated with 24 hour cultures of type A or type B toxigenic strains of S. aureus, (b) added penicillin to a concentration of 0.49 to 0.54 U/ml of milk or (c) added both

penicillin and the S. aureus inoculum. Other vats of milk received no special additions during manufacture and served as control vats. The staphylococcal inoculum and the penicillin were added 45 minutes after the addition of a 1% lactic starter culture to the milk. A summary of the treatments of the twelve vats during manufacture of the Cheddar and colby cheese used in this research is illustrated by the data in Table 1. Samples for determination of total and staphylococcal populations, pH and of enterotoxin were taken from, (a) milk before addition of the starter, (b) milk after inoculation with S. aureus, (c) whey-at-cutting, (d) curd-at-cutting, (e) curd-at-pressing, and (f) the cheese after 1, 10, 20, 30, 60, 90, and 180 days of ripening at 48 F.

Whey and curd samples were taken as aseptically as possible using a clean ladle and held at 4 C in sterile milk dilution bottles until bacterial populations could be determined. The curd-at-pressing and cheese samples, were held at 4 C in sterile glass beakers until bacterial populations could be determined. The 1, 10, and 20 day cheese samples were mistakenly frozen at -30 C before populations were determined. Freezing of these samples probably accounts for some of the erratic populations observed in the cheeses during the first 20 days of ripening.

The pH of the samples was determined by inserting the electrodes of a Corning Model 7 pH meter directly into the whey and curd. For the curd-at-pressing and cheese samples, pH was determined on a 50% (w/v) slurry made with distilled water.

Table 1: Amounts of penicillin and toxibenic strains of Staphylococcus aureus added to vats of milk made into Cheddar and colby cheese.

Vat number	Penicillin added (U/ml milk)	Staphylococcus aureus (organisms/ml milk)
Cheddar		
1	none	none
2	0.54	none
3	none	480,000 type A
4	0.54	480,000 type A
5	none	450,000 type B
6	0.49	450,000 type B
Colby		
7	none	none
8	0.49	none
9	none	440,000 type A
10	0.49	440,000 type A
11	none	450,000 type A

Following the enumeration of bacteria and measurement of pH, all samples were frozen at -30 C until enterotoxin assays could be conducted.

Enumeration of organisms. Total bacterial populations were determined with PCA medium, and the plates were incubated at 32 C for 72 hours. Staphylococcal populations were determined by surface streaking on pre-poured S-110 medium. A separate, sterile glass rod, bent into the shape of an "L," was used to spread the inoculum over the surface of the medium. For zero dilutions one ml of inoculum was spread over the surface of the medium in three plates while for other dilutions one-tenth ml of the appropriate dilution was used to inoculate each plate. Incubation was at 37 C for 48 hours. Dilutions for both total and staphylococcal populations were plated in triplicate. The populations of each sample were recorded as the average number of organisms in the three plates at each dilution.

Whey samples required no special treatment before enumeration. Samples of curd and cheese required blending of 11 grams of the sample with 99 ml of sterile 0.2% sodium citrate before dilutions could be made.

Analysis of cheese for enterotoxin. Casman (1966) has reported an extraction procedure which selectively removes and concentrates trace amounts of enterotoxin resulting from the growth of toxigenic strains of S. aureus in food. Casman's procedure employs two principles in the recovery of enterotoxin from food. These are, (a) the use of physical and chemical procedures for the selective removal of

enterotoxin from food constituents, leaving the toxin in solution and (b) the selective absorption of the enterotoxin from the soluble food extractives onto a carboxymethyl cellulose column. A description of the technique used to detect enterotoxin in the cheese manufactured in this study is illustrated in Figure 1. To conduct the assay, 100 grams of cheese were blended with 500 ml of 0.2 M NaCl at high speed in a Waring blender for 3 minutes and the slurry adjusted to pH 7.5 with 1.0 N NaOH. The slurry was then allowed to stand 15 minutes after which the pH was checked and re-adjusted to 7.5 if necessary. The slurry was then centrifuged at 29,700 x g for 15 minutes in a Sorvall RC-2B refrigerated centrifuge. The supernatant was strained through a 4 x 4 inch piece of screen-type wire to remove any floating lipoidal material and held at 4 C during which time the sediment was re-extracted with 125 ml of 0.2 M NaCl by blending as before and centrifuging for 15 minutes at 29,700 x g. The two supernatants were pooled, placed in dialysis tubing and concentrated to 40-50 ml by immersion in 30% polyethylene glycol (PEG) 20,000 (Matheson, Coleman and Bell). This step was very conveniently accomplished overnight since usually 7-9 hours were required to obtain the desired reduction in volume. Following dialysis, the tubing was washed in tap water to remove the PEG and rehydrated momentarily in distilled water and then placed in a beaker of 0.2 M NaCl for 3 minutes, to further rehydrate the contents. The dialysis tubing was then emptied of its contents and each section of tubing carefully washed with 0.2 M NaCl to remove any traces of extract containing

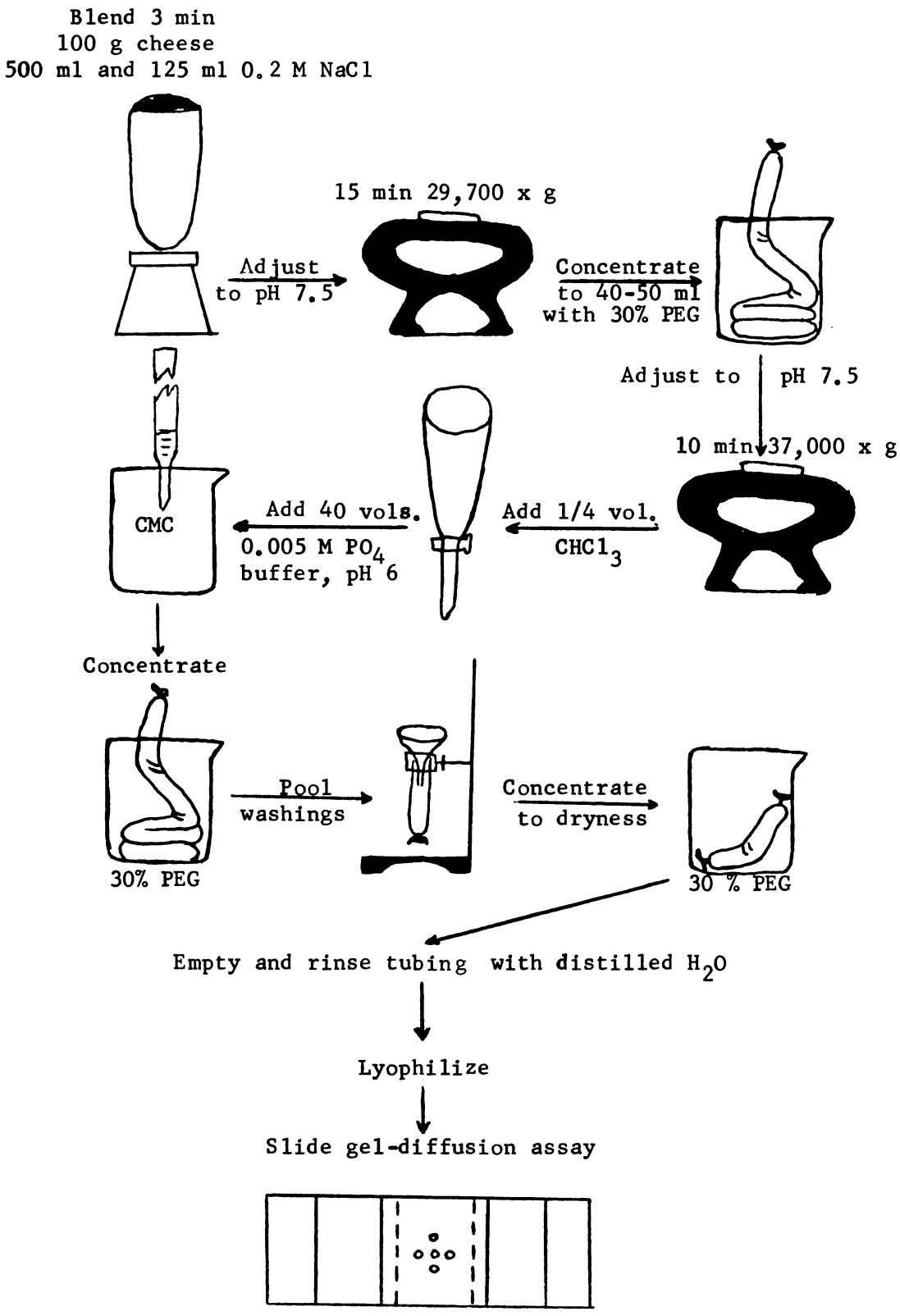

Figure 1: Extraction of enterotoxin from cheese.

toxin. The volume of the extract at this time should be 50-70 ml. The extract was then adjusted to pH 7.5 with 0.1 N NaOH and centrifuged at 37,000 x g for 10 minutes at 10 C. The volume of supernatant was measured, combined with one-fourth volume of chloroform, added to a separatory funnel and shaken vigorously ten times through an arc of 90 degrees. The mixture was centrifuged at 37,000 x g at 5 C for 10 minutes. The chloroform layer and any denatured protein was discarded, leaving a light brown, opaque liquid with a volume of 30-50 ml. If this final extract was overly cloudy as judged by experience, the chloroform fractionation could be repeated. The volume of extract was measured and subsequently diluted with 40 volumes of 0.005 M phosphate buffer of pH 6.0, and adjusted to pH 6.0 with 0.005 M H_3PO_4. The diluted and adjusted extract was placed in an erlenmyer flask large enough to accommodate the volume, which was usually 1500 to 3000 ml. The flask was then sealed with a rubber stopper through which passed a piece of glass tubing, 7-10 cm in length and 5-7 mm in diameter, which was placed flush with the inside surface of the stopper inside the flask. A length of rubber tubing, 5-10 cm in length, and of a size to fit the glass tubing tightly was fitted with a screw clamp and placed onto the projecting end of the glass tubing. The whole assembly was then placed in a cold room at 4 C.

A glass column 2 x 40 cm was plugged with glass wool and clamped onto a metal ring stand. One gram of carboxymethyl cellulose (Biorad) was mixed with 100 ml of 0.005 M phosphate buffer at pH 6.0. The pH

of the suspension was adjusted to 6.0 with 0.005 M phosphate buffer of pH 7.5 and allowed to stand with intermittent mixing for 15 minutes. After this time the pH was readjusted to 6.0 with either 0.005 M phosphate buffer of pH 7.5 or 0.005 M H_3PO_4 as required. The suspension of CMC was then poured into the column and allowed to settle for one hour. The suspending buffer was drawn off at the rate of 1-2 ml per minute and the column fines were washed out with 200 ml of 0.005 M phosphate buffer of pH 6.0 at the rate of 1-2 ml per minute. The pH of the buffer coming off the column was checked when the last of the washing buffer had passed. If the pH was not 6.0, washing was continued with 50 ml aliquots of the 0.005 M phosphate buffer of pH 6.0 until the eluate buffer pH was 6.0. A ring clamp of sufficient size to pass over the neck of the flask containing the diluted extract was placed above the column. The clamp on the rubber tubing projecting from the sealed flask was closed and the flask inverted through the ring clamp. The rubber tubing was passed into the column and the clamp released to permit the flow of extract from the flask. This arrangement permits the passage of extract from the flask at the same rate as it passes through the column and maintains a relatively constant "head" pressure on the column. The entire arrangement was kept in a 4 C cooler during the time required for the entire volume of extract to percolate through the column at the rate of 1-2 ml per minute. The required time for this percolation varies with sample volume and ranged from 18 to 48 hours throughout this investigation. After the passage of the toxic extract, the column was washed with 100 ml of 0.005 M phosphate buffer

at pH 6.0 to remove any material left in the resin but not bound to it. The toxin was then eluted off the column by washing it with 150 ml of 0.2 M NaCl in 0.2 M phosphate buffer at pH 7.4. The eluate was collected and placed in a single length of dialysis tubing and concentrated to near dryness in 30% PEG. The tubing was removed, washed in tap water, and rehydrated 3 minutes in distilled water. The liquid inside was pushed to one end of the tubing and the last 6 inches of tubing containing the liquid was cut off. A small funnel was inserted into the open end of the short tubing and clamped to a metal ring stand as indicated in Figure 1. The longer length of tubing was washed carefully with 0.5 ml aliquots of distilled water until no longer cloudy, which usually required 7-8 washings. The washings were pooled in the shorter piece of tubing, the tubing tied off and re-immersed in 30% PEG until dryness was attained. The dialysis tubing was washed in tap water and rehydrated for 3 minutes in distilled water and the contents removed to a 5 ml lyophilizing vial with a disposable Pasteur pipet. The tubing was washed 8-10 times with 0.1 ml aliquots of distilled water or until the washings were no longer cloudy. The final volume of concentrated extract should not exceed 1.5 ml to insure proper lyophilization. The sample was lyophilized for 24 hours at which time it was rehydrated with 1.0 ml of 0.15 M NaCl and titrated for the presence of toxin using the slide gel-diffusion assay.

Casman and Bennett (1963, 1965) and Zehren and Zehren (1968) employed a microscope slide modification of the Ouchterlony gel-diffusion assay to detect enterotoxin in food extracts. In this test the antibody

and enterotoxin combine to form a line of precipitation in a thin layer of agar gel between a microscope slide and a square of Plexiglas containing funnel-shaped holes into which the reactants are introduced. The presence of enterotoxin in the sample is verified by the coalescence of its line of precipitation with the reference line.

A modification of this test as described by Crowle (1958) was used. This modification consists of the incorporation of 0.8% sodium barbital (pH 7.4) and 0.01% thimersal (merthiolate) in the agar gel, and the use of plastic squares in which the distance between the centers of the peripheral wells and central well was 4.5 mm instead of 4.0 mm. The agar gel was prepared by dissolving 1.2% Special Noble agar (Difco) in an aqueous fluid base containing 0.85% NaCl, 1:10,000 thimersal, 0.8% sodium barbital and having the pH adjusted to 7.4 with 1.0 N HCl. The agar was dissolved by boiling and the solution filtered while hot through a double layer of Whatman no. 1 filter paper. The agar was then held at 4 C until used.

The plastic squares, called templates, were made from Plexiglas 0.32 cm thick and 2.54 cm square. Location of the holes is shown in Figure 2. The holes were prepared by drilling to a depth of 0.238 cm with a 3.6 mm drill and then drilling through the remaining thickness with a 1.6 mm drill. The surface of the template containing the smaller holes rests on the agar and to facilitate easy removal, is coated with silicone grease (Dow Corning) before being placed on the agar.

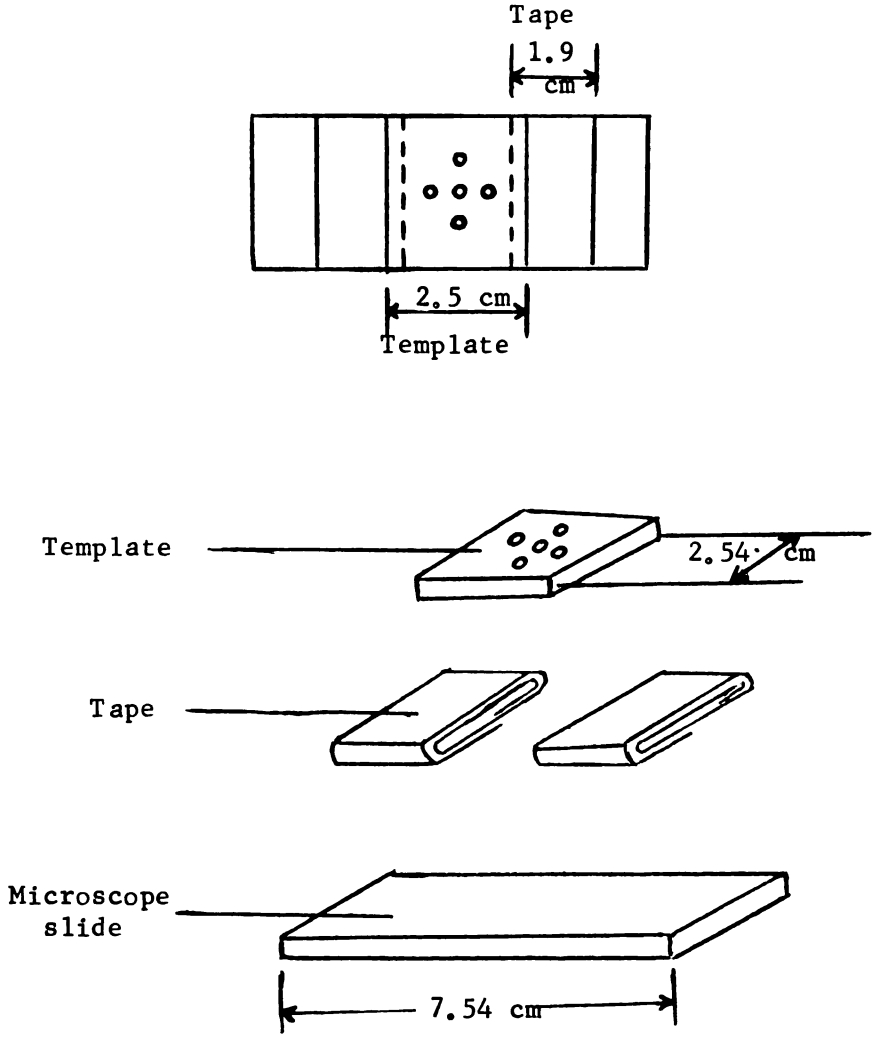

Figure 2: Diagram of slide and accessories for gel-diffusion (redrawn from Zehren and Zehren, 1968a).

A microscope slide 2.54 x 7.62 cm was taped with a double layer of plastic electric insulating tape (Scotch Brand) as shown in Figure 2. After taping, the slides were boiled in a weak detergent solution (Alconox) for 3 minutes, rinsed several times in tap and distilled water, placed in 95% ethanol for 3 minutes and wiped dry with clean cheese cloth. Slides that were clean but not used immediately were stored in a flat pan and covered with several layers of cheese cloth. Before placing the gel-diffusion agar on the slides, the surface between the tape was coated with 0.2% Bacto agar (Difco) at 60 C. The excess agar was removed by tilting the slide over a beaker. It is important to have clean slides to insure that the agar precoat adheres to the slide. Precoating prevents leakage between the slide and the 1.2% agar gel and also prevents movement of the diffusion agar. A small volume, 0.35-0.50 ml, of the 1.2% agar at 60 C was pipetted onto the precoated area, and the silicone-coated template immediately placed on the melted agar with the edges on the tapes. After the agar solidified, the slides were placed in 15 cm petri dishes containing strips of absorbent cotton saturated with water. This is necessary to prevent the agar from drying out during incubation.

The reactants were introduced into the funnel-shaped wells with a capillary pipette drawn from Pasteur pipets 9 cm in length. About 0.03 ml of reactant was required to fill each well to convexity. Two slides were prepared for each sample and each slide always contained reference toxin in one of the wells. A control slide containing only the reference reagents was prepared for each series of tests.

The antiserum was placed in the center well. The known enterotoxin was placed in the top well and the food extract titers placed in the two side wells. Serological dilutions of the food extract were prepared by dissolving the lyophilized sample in 1.0 ml of physiological saline and transferring 0.1 ml aliquots of this suspension to small vials containing an amount of physiological saline to make the desired dilution. After the wells were filled, they were probed with flame-sealed glass capillary tubes to remove bubbles which may be trapped in the holes. This step is best carried out against a dark background. It is important that "bubbling" be done on each slide since the reagents must be in contact with the agar or there will be no reaction.

The slides were stored at room temperature and examined after three days. The template was removed without disturbing the agar gel and the slides placed in 0.15 M NaCl overnight. The slides were then rinsed with distilled water and stained for ten minutes in 1.0% acetic acid containing 0.1% thiazine red. The slides were removed to another 1.0% acetic acid bath for ten minutes and then examined for lines of precipitation by holding over a light source and against a dark background. The presence of enterotoxin in the sample extract was verified by the coalescence of its line of precipitation with the reference line of precipitation. The toxin titer was determined by observing the highest dilution of the extract which gave a distinct coalescence with the reference line of precipitation. The product of the volume of toxic food extract or toxic broth used and the reciprocal of its dilution giving coalescence with the reference is used to

indicate the amount of enterotoxin in µg, since it is estimated that a concentration of 1 µg/ml represents the limit of sensitivity of the test (Casman and Bennett, 1965).

Recovery of enterotoxin added to non-toxic cheese. To determine the efficiency of the extraction procedure in detecting enterotoxin in cheese, known amounts of toxin were added to 100 gram samples of non-toxic colby cheese and the cheese examined for the presence of toxin. The toxin used in the recovery studies was obtained from the culture supernatants resulting from the growth of the enterotoxigenic strains of S. aureus in 3.7% BHI incubated at 37 C for 48 hours. The supernatant was obtained by centrifuging the culture broth for 10 minutes at 37,000 x g. Following centrifugation, the supernatant was concentrated to approximately one-tenth its original volume by dialysis against 30% PEG for several hours. The concentrated supernatant was removed from the dialysis tubing and its volume measured. The supernatant was then combined with one-quarter volume of chloroform, added to a separatory funnel and shaken vigorously ten times through an arc of 90 degrees. The mixture was centrifuged at 37,000 x g for 10 minutes and the chloroform and denatured protein layers discarded. The aqueous layer was titrated for the concentration of toxin and the preparation held frozen at -30 C until used.

To conduct the recovery tests, 576 to 640 µg of type A enterotoxin or 6,114 µg of type B enterotoxin, was added to six 100 gram samples of non-toxic colby cheese. These cheese samples were then analyzed by the extraction procedure described herein. Recovery was expressed as the percentage of the amount of toxin added to the original cheese

sample that was detected in the final concentrated extract.

Relationship of growth and toxin production by toxigenic strains of S. aureus in 3.7% BHI with different pH values. Brain Heart Infusion (BHI) broth was prepared from the following fluid bases and the final pH adjusted with 6.0 N HCl or 1.0 N NaOH before sterilization:

a) 0.2 M sodium phosphate buffer-pH 5.4

b) 0.2 M sodium phosphate buffer-pH 6.0

c) Distilled water (unbuffered)-pH 7.4

d) 0.2 M tris (hydroxymethyl) aminomethane buffer-pH 8.0

A total volume of 500 ml of each of the BHI suspensions was added to a 2 liter erlenmeyer flask, the flask sealed with a cotton plug and sterilized for 15 minutes at 120 C. Toxigenic strains of S. aureus were streaked onto nutrient agar slants and incubated at 37 C for 24 hours. These slant cultures were transferred with a sterile inoculating loop to tubes of sterile 3.7% BHI, the tubes mixed, and 0.5-2.0 ml of this suspension added to the prepared flasks. Initial populations of staphylococci in the culture flasks were determined on S-110 medium as previously described. Initial populations ranged from 8,000 to 300,000 organisms/ml. Following inoculation, the flasks were incubated at 37 C on a gyrotory shaker set at 150-175 rotations per minute.

Growth of the organisms was determined by optical density readings at 620 mμ on a Baush and Lomb Spectronic 20 Spectrophotometer. The relationship of $O.D._{620}$ and population of staphylococci was determined for both strains studied. To determine this relationship,

flasks of non-buffered BHI at pH 7.4 were inoculated as described, incubated at 37 C on the shaker and periodic samples removed for determining the optical density and population on S-110 medium. Estimated populations of the two strains of S. aureus used in this investigation, when grown in BHI broth at pH 6.0 and 8.0, were obtained from standard curves plotted from the data on population versus O.D. obtained during the growth of the two strains in non-buffered BHI broth at pH 7.4.

To determine the effect of pH of the medium on growth and toxin production, BHI broth preparations with four different pH values were inoculated and incubated as described previously. Samples of the S. aureus cultures were taken periodically during the lag, log and stationary phases of growth, checked for $O.D._{620}$, centrifuged for 15 minutes at 37,000 x g, and the pH of the supernatant determined. The supernatant was then held frozen at -30 C until toxin content could be determined.

Toxin in the supernatants was assayed by the slide gel-diffusion test. Undiluted samples that gave no indication of toxin were concentrated and re-titrated since they often contained toxin but at a concentration not detectable by the slide gel-diffusion test. Before re-titrating these samples, 20 ml of the supernatant was placed in a 15 cm piece of dialysis tubing and concentrated to dryness in 30% PEG. The tubing was then removed from the PEG, washed in tap water and rehydrated for 3 minutes in distilled water. The liquid was removed with a Pasteur pipet and placed in a lyophilizing vial. The tubing was washed several times with 0.1 ml aliquots of distilled water, the

washings pooled and also added to the lyophilizing vial. The sample was lyophilized for 24 hours and rehydrated with sufficient 0.15 M NaCl to yield either a 50-fold or a 100-fold concentration. The concentrated supernatant was then titrated for the presence of toxin.

RESULTS AND DISCUSSION

Recovery of enterotoxin added to non-toxic cheese. Data in Table 2 show the results of a series of tests conducted to determine the efficiency of the enterotoxin extraction procedure used in this investigation. Type A toxin was added at a concentration of 576 to 640 µg per 100 grams in six non-toxic colby cheese samples. The cheese samples were then analyzed for toxin by the extraction procedure described previously. Data show that 11.1 to 45.0% of the toxin added to the cheese samples was detected after extraction. The average recovery for the six samples was 27%. These results compare reasonably well with those of Zehren and Zehren (1968a) who used a similar procedure and recovered 16 to 35% of type A toxin added to 100 gram samples of Cheddar cheese. These workers did not state the total number of trials or the percent recovery of each trial so no comparison for average recovery can be made.

Casman and Bennett (1965) used an extraction procedure similar to the one in this investigation and recovered 43% of type A toxin added to Feta cheese. Their recovery was higher than the average found in the investigation reported herein, but represents the result of only one trial. Casman and Bennett (1965) also reported on the recovery of type A enterotoxin added to several other foods. They recovered 32% from coconut custard, 41% from cooked beef, 23% from cooked shrimp and 48% from cooked turkey.

To determine the efficiency of the procedure to detect type B toxin, 6114 µg of toxin was blended with six colby cheese samples each

Table 2: Recovery of types A and B enterotoxin added to 100 g samples of non-toxic colby cheese.

Amount of type A enterotoxin

µg added/100 g cheese	µg detected/100 g cheese	% Recovery
640	290	45.0
640	256	40.0
640	192	30.0
576	96	16.7
576	96	16.7
576	64	11.1

Amount of type B enterotoxin

µg added/100 g cheese	µg detected/100 g cheese	% Recovery
6114	4090	66.9
6114	2764	45.2
6114	2048	33.5
6114	1530	25.1
6114	614	10.0

weighing 100 grams, and the toxin extracted as previously described. During the extraction procedure several of the samples did not lyophilize properly and it is possible that some of the toxin was lost when the samples foamed over into the vacuum chamber. One of the samples was lost in a laboratory accident prior to lyophilization. The data from the remaining five samples is presented in Table 2. The type B toxin recovered ranged from 10.0 to 66.9% and averaged 36.1%, which agrees with a report by Casman and Bennett (1965) where 30% of type B toxin added to Feta cheese was recovered.

Several people working with enterotoxin extraction have evaluated their procedures experimentally to determine the lower limit of toxin detectable in food extracts. For instance, Zehren and Zehren (1968a) found the technique they used could detect as little as 0.003 µg of type A toxin added per gram of cheese. Read, et al. (1964) reported that 0.02 µg of type A and 0.05 µg of type B toxin per gram of cheese was the lower limit of their extraction procedure. In another investigation, Read, et al. (1964) reported the minimum limit of detection for types A and B toxin added to milk was 0.15 and 0.03 µg/ml, respectively.

In the work reported herein there was no attempt to determine the lower limit of the amount of toxin detectable with the extraction procedure used. The lower limit of toxin that is detectable by any procedure depends upon the average percentage recovery that is possible by that procedure. For instance, assuming a requirement of 1 µg of toxin in the final concentrate for a positive gel-diffusion test and a recovery of 27% of type A toxin, then 3.7 µg of toxin must be present

in the original 100 gram sample of food for detection. This corresponds to a minimum detection of type A toxin for the extraction procedure used in this investigation of 0.037 µg/g of sample. By the same reasoning the minimum amount of type B toxin that could be detected is 0.027 µg/g of sample.

Pilot plant experiments. Cheese manufacture depends upon good growth and acid production by the lactic starter organisms (Van Slyke and Price, 1956). On certain occasions the starter culture may be destroyed by bacteriophage or inhibited by antibiotics in milk produced by cows receiving therapy for mastitis. Such milk, when used in cheese manufacture, results in "dead vats" and staphylococci, present either as part of the flora in the raw milk or as post-pasteurization contaminants, are able to grow because of lack of competition (Harmon, 1967). These strains of staphylococci, if toxigenic, could produce toxin during manufacture or curing of the cheese.

To approximate a commercial manufacturing situation cheese was prepared as described previously and aqueous suspensions of penicillin added to certain vats of milk to produce dead vats. The lactic acid bacteria normally used in making cultured dairy products are usually inhibited by penicillin at a concentration of 0.5 Units/ml of milk. On the other hand most strains of staphylococci, including those that are toxigenic, secrete a penicillinase which permits them to over-grow the lactic acid bacteria in low concentrations of penicillin. The concentration of penicillin added to the vats ranged from 0.49 to 0.54 U/ml of milk as shown in Table 1. Some of the vats containing the penicillin were also inoculated with toxigenic strains of S. aureus. Other

vats of milk lacking the penicillin were also inoculated with these strains of staphylococci and served as standards for observing the effect of lactic acid bacteria growth and acid production upon staphylococcal growth and toxin formation.

Effect of penicillin upon pH, lactic acid bacteria and staphylococcal populations in Cheddar cheese. Figures 3, 4 and 5 show the total and staphylococcal populations and pH of Cheddar cheese during storage at 48 F for 180 days. Samples of the curd at pressing represent zero time.

The data in Figure 3A are from samples of cheese made from milk (vat 1) that contained neither penicillin nor a staphylococcus inoculum and represents the production of normal Cheddar cheese. The percent acidity of the whey-at-milling during Cheddar cheese manufacture is indicative of lactic acid microorganism development and should be between 0.40 and 0.60% to facilitate normal pH and organism development during ripening. The results of tests made on the Cheddar cheese made in this investigation are shown in Table 3. The control Cheddar cheese made in vat 1 had a whey-acid of 0.50% when the curd was milled. The pH of normal Cheddar cheese should be about 5.2 when first made and should gradually increase to about 5.6 as soluble nitrogenous compounds resulting from proteolysis accumulate during ripening. Figure 3A (vat 1) shows that the pH of this cheese varied from 5.2 to 5.6 during 180 days of ripening, which is normal.

The curve representing total population of the cheese manufactured in vat 1 (Figure 3A) indicates that the maximum population was attained after 90 days of ripening. All cheese samples taken in this investi-

Table 3: Percent acidity of the whey-at-milling during the manufacture of Cheddar cheese.

Vat number	Acidity (% lactic acid)
1	0.500
2	0.165
3	0.630
4	0.125
5	0.510
6	0.125

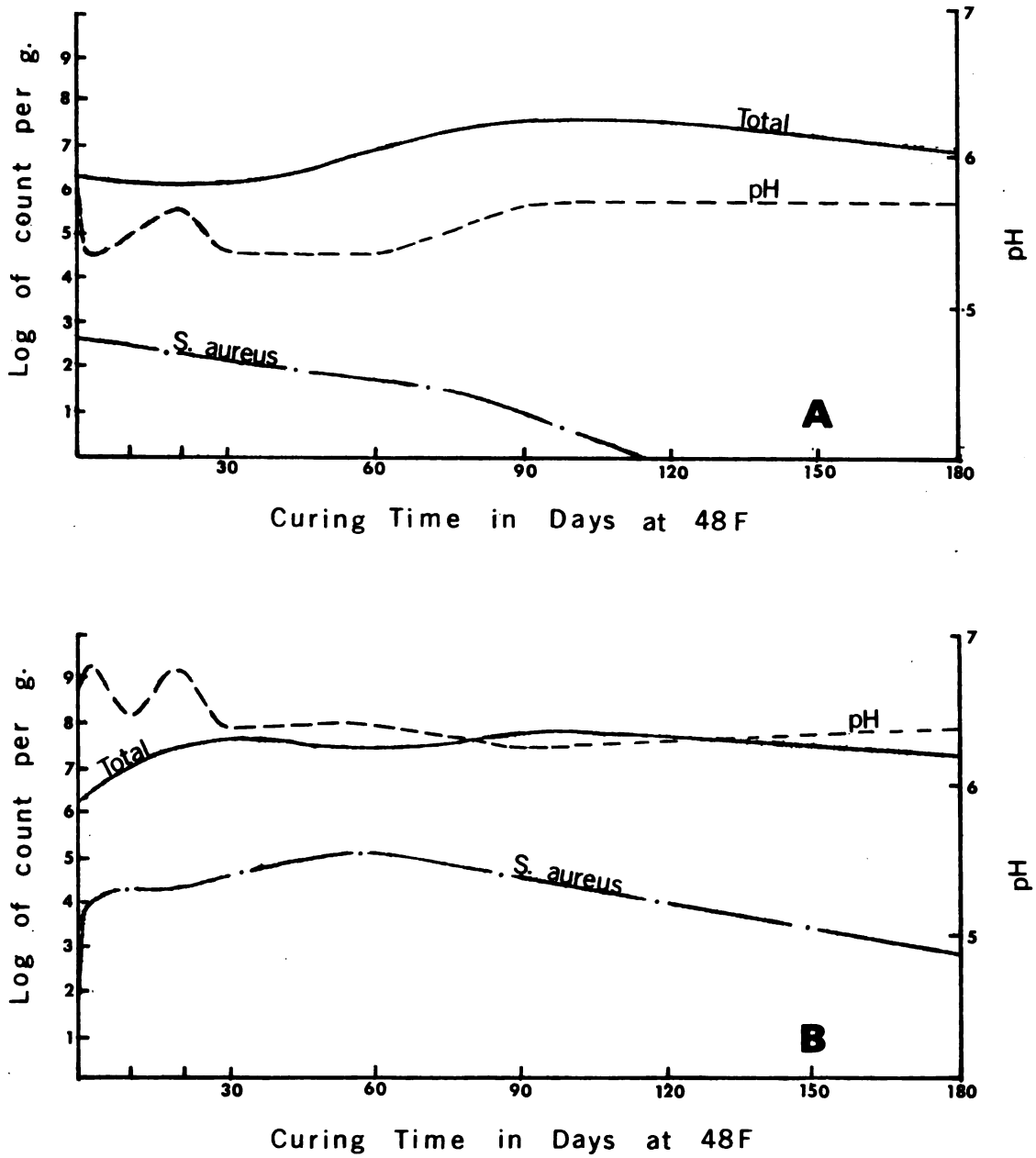

Figure 3: Total and S. aureus populations and pH of Cheddar cheese manufactured from pasteurized milk containing (A) no penicillin or (B) 0.54 Units of penicillin/ml (cured at 48 F).

gation at 1, 10 and 20 days of curing were frozen before enumeration of microorganisms, which accounts for erratic counts on these samples. The cheese manufactured in vat 1 also contained a low population of S. aureus which attained a maximum of 500 organisms/g of curd at pressing and gradually declined to less than 1 organism/g at 120 days. The milk used to manufacture the cheese was not inoculated with staphylococci but was probably contaminated by equipment used in adjacent vats of milk which were inoculated with staphylococci. There are other instances where contamination of non-inoculated vats of milk was suspected.

The data in Figure 3B are from samples of cheese made from milk in vat 2 which contained 0.54 Units of penicillin per milliliter of milk. Table 3 indicates that the acidity of the whey-at-milling for the cheese from vat 2 was 0.165%, suggesting little acid production by the lactic acid starter microorganisms. The pH of this cheese was abnormally high and ranged from 6.3 to 6.8 during ripening (Figure 3B). The maximum total population was 50 million organisms/g after 90 days of curing. The staphylococcal population, probably from contamination as suggested above, was 1300 organisms/g on the first day and attained a maximum of 110,000/g after 60 days of curing. Numerous workers have suggested that food with a staphylococcal population of 500,000 organisms/g may contain emetic levels of toxin. The staphylococcal population that occurred in the cheese from vat 2 probably did not present any hazard, but obviously staphylococci can grow well in vats of milk lacking proper acid development.

Figure 4A contains data from the analysis of cheese manufactured from milk inoculated with 480,000 cells of S. aureus/ml (vat 3) which produce type A enterotoxin. The acidity of the whey-at-milling (Table 3) was 0.63% suggesting good lactic starter development. The pH range of the cheese during ripening was normal, varying from 4.9 to 5.4 (Figure 4A).

The apparent decrease in total population of the cheese from vat 3 (Figure 4A) during the first 20 days of ripening probably reflects the effect of freezing the samples as mentioned before. The staphylococcal population was 8,000/g on the first day of curing and increased until a maximum population of 130,000 organisms/g was reached after 60 days of ripening. The S. aureus population then decreased gradually until termination of ripening.

Data in Figure 4B represent the results of analysis of samples of Cheddar cheese manufactured from milk inoculated with 480,000 cells of type A toxin producing S. aureus/ml of milk (vat 4). Penicillin was also added to the milk at a concentration of 0.54 U/ml. The percent acidity of the whey-at-milling was 0.125 and indicates that this was a dead vat during manufacture. The pH of this cheese during curing was higher and ranged from 5.9 to 6.4.

The total population in the cheese from vat 4 (Figure 4B) reached a peak of 120 million organisms/g after 30 days. The staphylococcal population was 5,000 organisms/g on the first day after manufacture and increased to a maximum of 110 million organisms/g after 30 days of curing. The total and staphylococcal populations then decreased slightly during the remainder of the ripening period. The high total

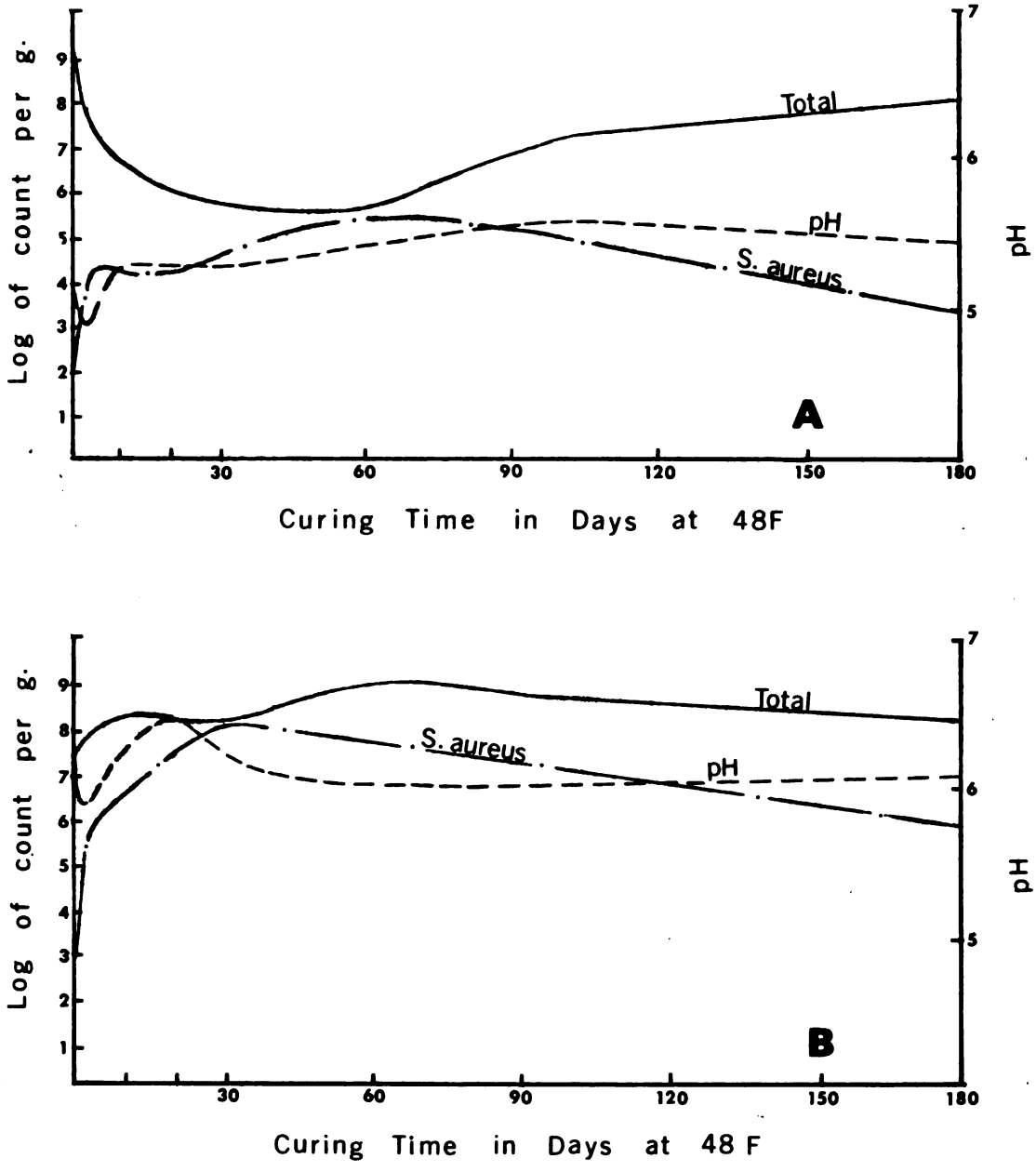

Figure 4: Total and S. aureus populations and pH of Cheddar cheese manufactured from pasteurized milk (A) inoculated with 480,000 type A enterotoxin producing S. aureus cells/ml or (B) inoculated as above and containing 0.54 Units of penicillin/ml (cured at 48 F).

population probably results from the high staphylococcal count, since the low acidity of the whey-at-milling and the high pH of this cheese indicates poor lactic acid starter development. Comparing the two cheeses from vats 3 and 4 (Figure 4A and 4B) one is also able to see very dramatically, the influence of the presence of penicillin in reducing lactic culture activity, causing poor acid development and permitting staphylococci to grow well in the milk and cheese.

Data in Figure 5A indicate the results from the analysis of samples of Cheddar cheese manufactured from milk inoculated with 450,000 cells of S. aureus/ml which produce type B toxin (vat 5). The percent acidity of the whey-at-milling was 0.51 (Table 3) which is indicative of good lactic starter growth. The pH of the cheese during ripening was normal and varied from 5.2 to 5.6.

The total and staphylococcal population in the cheese from vat 5 (Figure 5A) seem to follow the same trend throughout curing. The curd contained over 1 million staphylococci/g when pressed, reached a peak of approximately 18 million/g after 60 days and gradually decreased to about 2,500 organisms/g after 180 days. The high population of S. aureus was unexpected since normal lactic starter development occurred in vat 5 during manufacture.

The data in Figure 5B are from samples of cheese made from milk in vat 6 which contained 450,000 cells of type B producing S. aureus and 0.49 Units of penicillin per milliliter of milk. The percent acidity of the whey-at-milling was 0.125 and indicates that this was a dead vat. The cheese from vat 6 (Figure 5B) had a pH range during

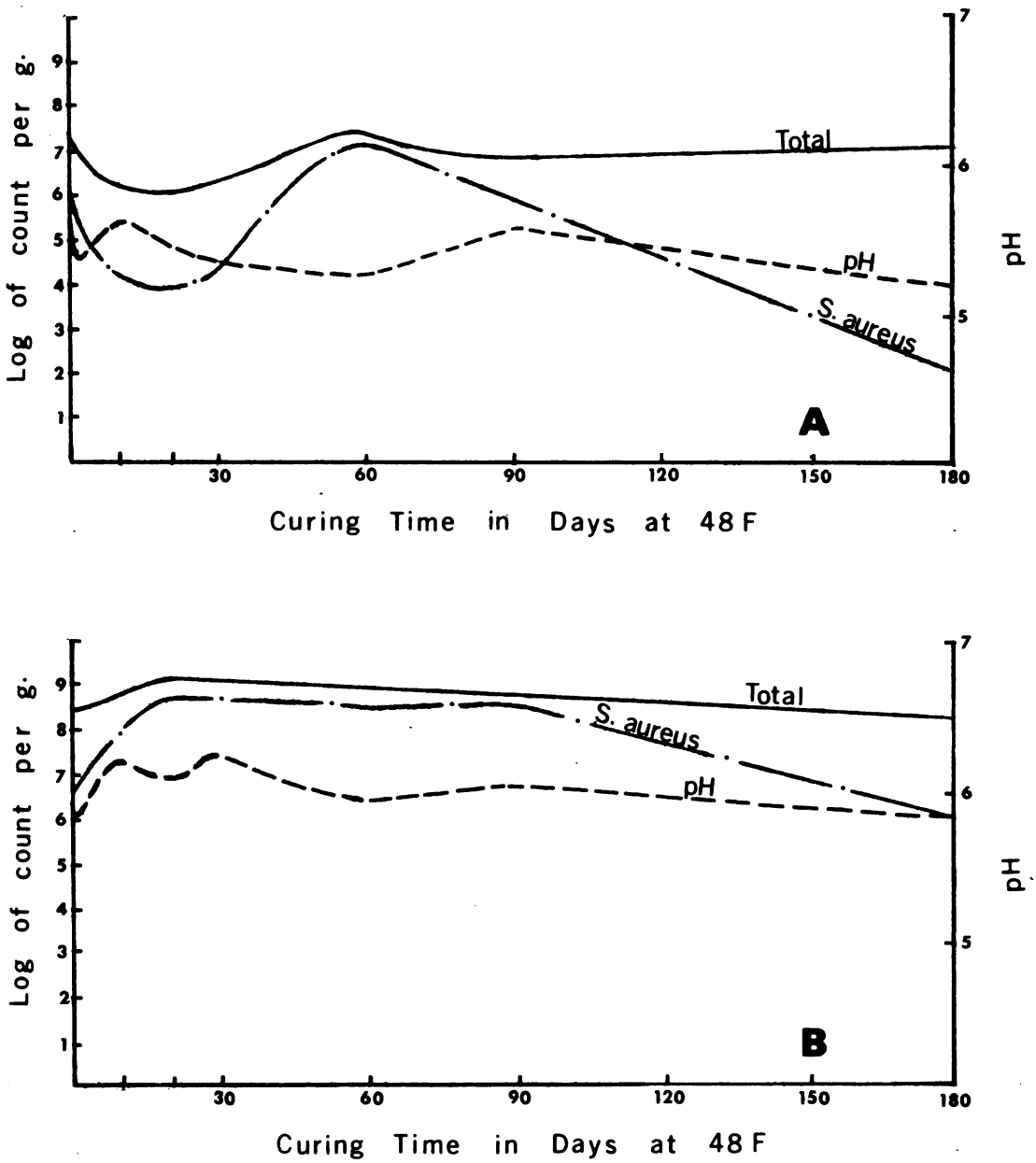

Figure 5: Total and S. aureus populations and pH of Cheddar cheese manufactured from pasteurized milk (A) inoculated with 450,000 type B enterotoxin producing S. aureus cells/ml or (B) inoculated as above and containing 0.49 Units of penicillin/ml (cured at 48 F).

curing which was higher than normal, varying from 5.9 to 6.4. The total population reached a maximum of about 1 billion organisms/g 20 days after manufacture and was relatively constant for the remainder of the curing period. The staphylococcal population in vat 6 was nearly maximum 10 days after manufacture, stationary until 90 days, and then decreased slowly during the remainder of the ripening period. It is evident that the staphylococci contribute substantially to the total population throughout curing. The cheeses from vats 5 and 6 (Figure 5A and 5B) could possibly be hazardous since their respective staphylococcal populations at the time the curd was pressed were 1.1 million and 1.9 million organisms/g.

Effect of penicillin upon pH, lactic acid bacteria and staphylococcal populations in colby cheese. Colby is a lower acid cheese than Cheddar because the curd is washed after the whey is drained, thus removing some of the lactic acid. The initial pH of the cheese is usually about 5.3 or 5.4 and increases to 5.7 or 5.8 during ripening.

Figures 6, 7, and 8 show the total and staphylococcal populations and pH of samples of colby cheese during ripening at 48 F for 180 days. Samples of the curd at pressing represent zero time.

Data in Figure 6A show the results of analysis of samples of colby cheese manufactured in vat 7 which contained milk that lacked the addition of penicillin or staphylococci. The acidity of the whey-at-dipping is an indication of acid development during colby cheese manufacture and normally should be 0.13 to 0.15%. A summary of the acidity of the whey-at-dipping for the colby cheese made in this investigation is shown in Table 4. The percent acidity in the colby

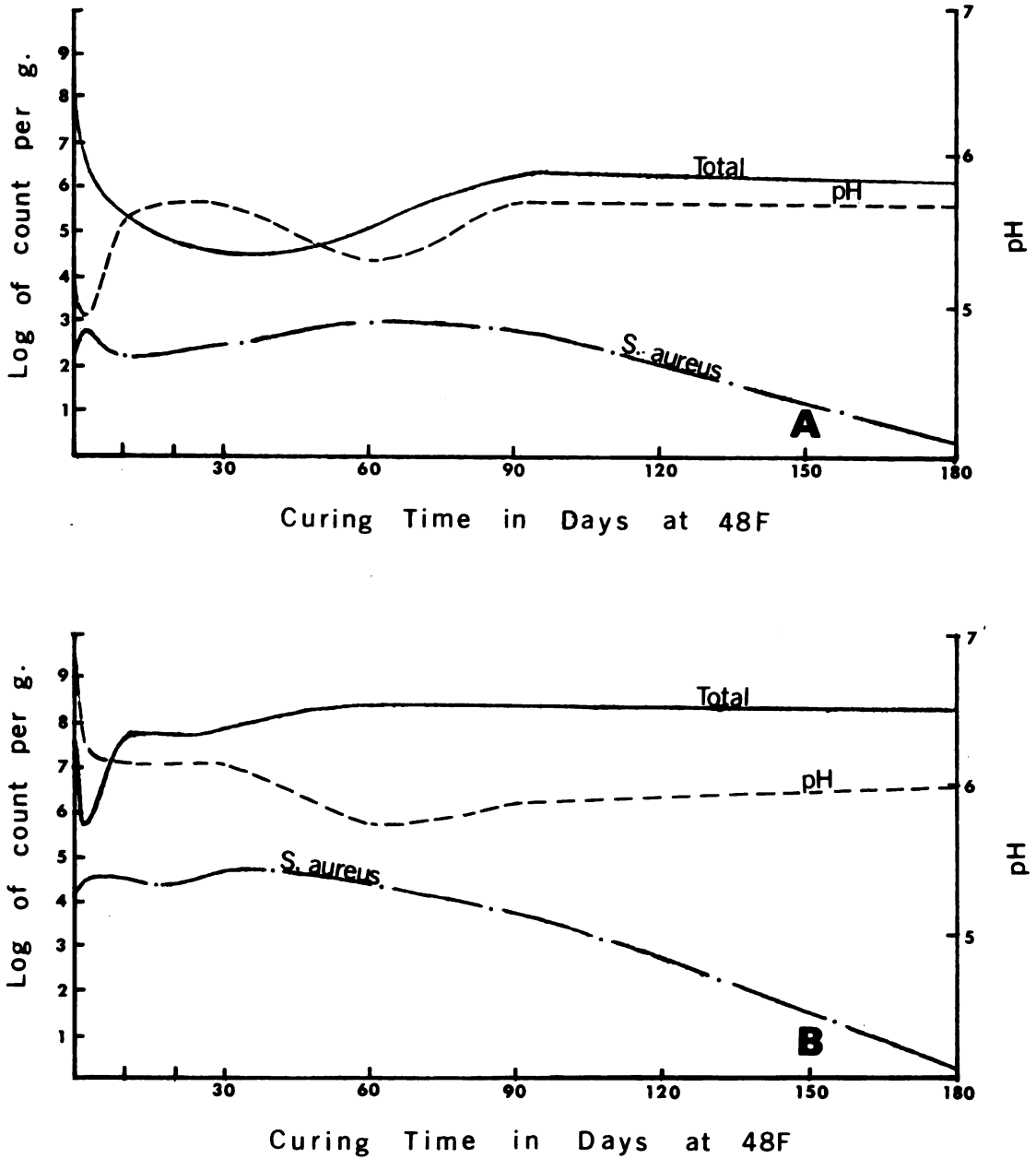

Figure 6: Total and S. aureus populations and pH of colby cheese manufactured from pasteurized milk containing (A) no penicillin or (B) 0.49 Units of penicillin/ml (cured at 48 F).

Table 4: Percent acidity of the whet-at-dipping during the manufacture of colby cheese.

Vat number	Acidity (% lactic acid)
7	0.130
8	0.120
9	0.130
10	0.120
11	0.135
12	0.120

cheese from vat 7 was 0.13 which is within the normal range. Data presented in Figure 6A (vat 7) show that the pH of the control colby cheese during ripening varied from 5.0 to 5.7. The apparent initial decrease in total population of this cheese is attributed to loss of viability of organisms during freezing. A total population of approximately 1 million organisms/g prevailed during the latter half of the curing period. The cheese from vat 7 also contained approximately 800 S. aureus per gram when pressed, probably introduced during the manufacturing process from adjacent vats. The staphylococcal population increased to a maximum of 1000 organisms/g at 60 days and decreased to less than 10 organisms/g at 180 days of ripening.

Figure 6B shows data from the analysis of samples of colby cheese manufactured from milk in vat 8 that contained 0.49 Units of penicillin per milliliter of milk. The acidity of the whey-at-dipping in vat 8 was 0.12% (Table 4) which indicates that this was a "slow" vat. The pH of this cheese during curing as shown in Figure 6B was higher than normal, varying from 5.7 to 5.9. The high pH during curing and abnormal dipping acidity applied to all colby cheeses in this investigation which were manufactured from milk that contained penicillin. However, the inhibition of the lactic acid starter bacteria may be less in colby than in Cheddar because some penicillin may be washed out of the colby during manufacture.

The cheese made from milk in vat 8 (Figure 6B) contained a low population of staphylococci, probably caused by contamination as previously mentioned. The staphylococci reached a maximum of 50,000

organisms/g in the cheese after 30 days of curing and gradually decreased to 3 organisms/g after 180 days.

Figure 7A contains data from the analysis of samples of cheese manufactured from milk inoculated with 440,000 cells of S. aureus/ml, which produce type A toxin (vat 9). Table 4 shows the acidity of the whey-at-dipping was 0.13% suggesting good lactic starter development. Figure 7A shows the pH range of the cheese during ripening was normal at 5.2 to 5.6. The total population attained in the cheese from vat 9 was maximum after 90 days of curing with 300 million organisms/g. The staphylococci attained a maximum of 300,000 organisms/g after 20 days of ripening. Both the S. aureus and total populations were fairly constant throughout the remainder of the curing period.

Data in Figure 7B represent the results of analysis of samples of colby cheese manufactured from milk in vat 10 which was inoculated with 440,000 cells of type A toxin producing S. aureus/ml and contained penicillin at a concentration of 0.49 U/ml. Table 4 shows that the acidity of the whey-at-dipping was 0.12% and indicates that vat 10 was a slow vat. During ripening, the pH of this cheese varied from 5.1 to 5.9. Figure 7B (vat 10) reveals that the staphylococcal population attained a maximum of 6.5 million organisms/g 20 days after manufacture, decreased to about 56,000/g after 90 days and increased slightly until the termination of the investigation. The two cheeses made from vats 9 and 10 (Figure 7A and 7B) contained sufficient numbers of staphylococci during ripening to make them potentially hazardous after only 1 day of curing.

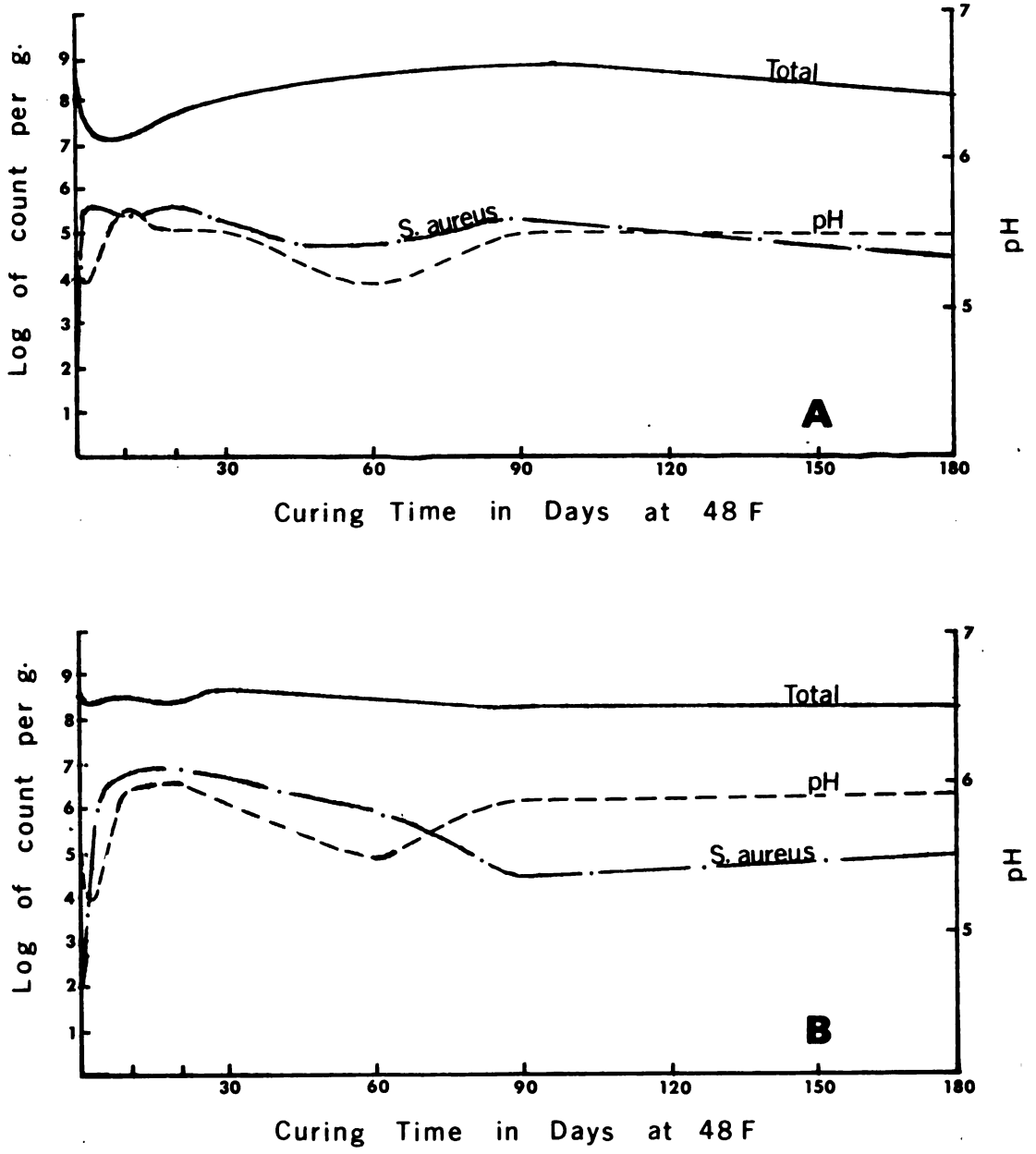

Figure 7: Total and S. aureus populations and pH of colby cheese manufactured from pasteurized milk (A) inoculated with 440,000 type A enterotoxin producing S. aureus cells/ml or (B) inoculated as above and containing 0.49 Units of penicillin/ml (cured at 48 F).

Data in Figure 8A show the results obtained from samples of colby cheese made from milk in vat 11 inoculated with 450,000 cells of S. aureus/ml, which produce type B toxin. Table 4 indicates that the percent acidity of the whey-at-dipping for the cheese made from vat 11 was 0.135% and suggests the lactic acid bacteria developed normally during manufacture. The pH of the cheese during ripening was normal and ranged from 5.3 to 5.8. The total population was relatively uniform throughout the ripening period except for a slight decrease during the first 30 days. The staphylococci inoculated into the milk during manufacture failed to grow to any significant population in the cheese and reached a maximum of only 2,400 organisms/g 30 days after the beginning of ripening.

Figure 8B shows data obtained by analysis of samples of colby cheese manufactured from milk in vat 12 inoculated with 450,000 cells of S. aureus/ml, producing type B enterotoxin. The milk also contained 0.49 U/ml of penicillin during manufacture. The dipping-acidity of the whey from vat 12 (Table 4) was 0.12% and indicates that this was a slow vat. Figure 8B indicates that the pH of this cheese was high and varied from 5.8 to 6.5 during curing.

Figure 8B (vat 12) shows that the staphylococci attained a maximum population of 43 million organisms/g 90 days after manufacture and decreased steadily until 1,600 organisms/g remained after 180 days of ripening. The total population in the cheese gradually increased throughout the entire storage period and reflects the presence of a high staphylococcal population along with the lactic acid bacteria. Of the

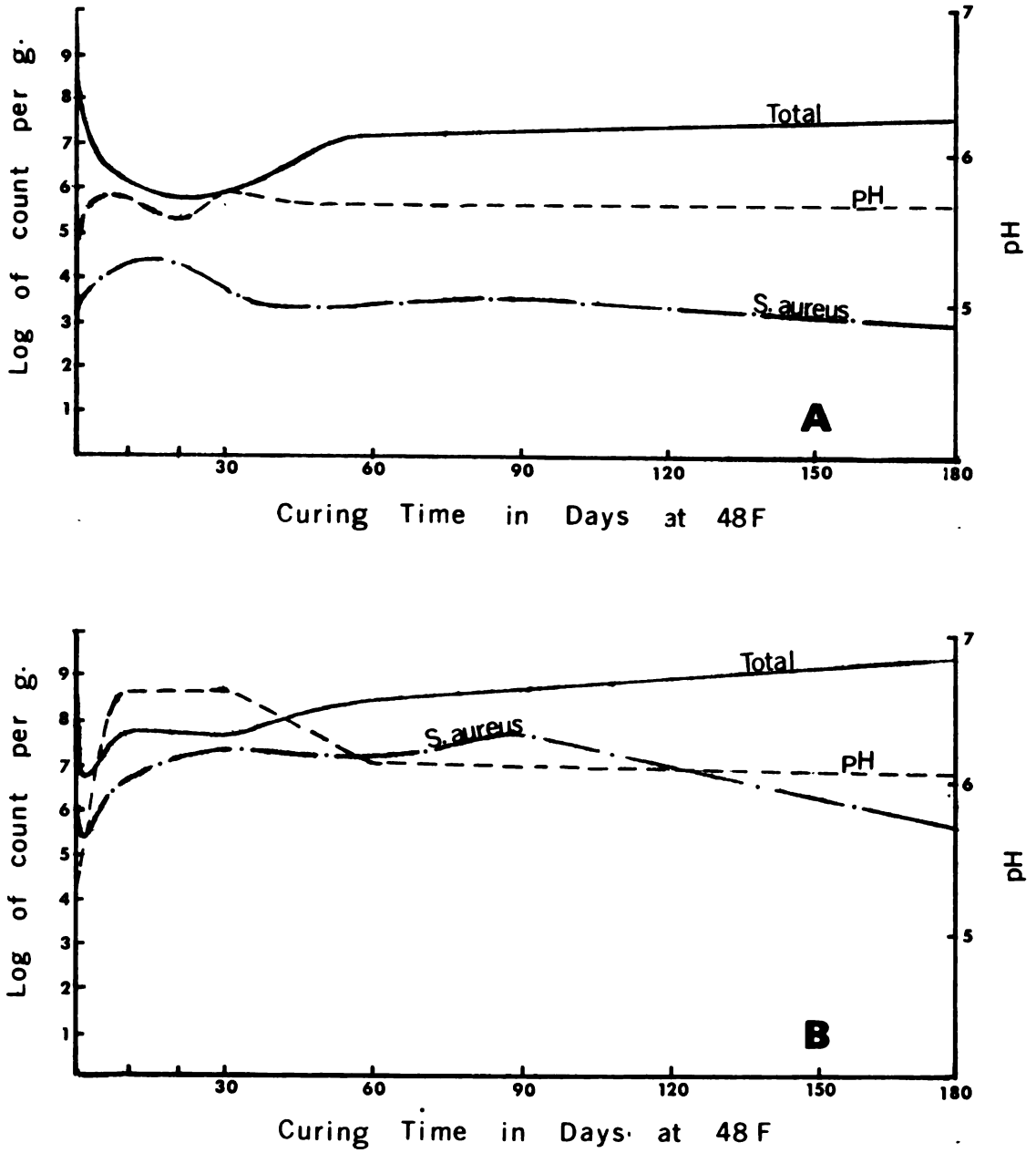

Figure 8: Total and S. aureus populations and pH of colby cheese manufactured from pasteurized milk (A) inoculated with 450,000 type B enterotoxin producing S. aureus cells/ml or (B) inoculated as above and containing 0.49 Units of penicillin/ml (cured at 48 F).

two vats of milk inoculated with the type B toxin producing staphylococci, only the cheese manufactured from vat 12, a typical slow vat as indicated by its low dipping acidity during manufacture, contained staphylococci in a sufficiently high number to suggest a hazard.

Production of enterotoxin in Cheddar and colby cheese manufactured from milk inoculated with toxigenic strains of S. aureus. One hundred gram samples of Cheddar and colby cheese manufactured in the Michigan State University dairy plant were removed from storage at -30 C and analyzed for enterotoxin by the procedure already discussed. Cheese manufactured from non-inoculated milk was examined after curing at 48 F for 180 days. Cheese manufactured from inoculated milk was examined after curing at 48 F for 60, 90, and 180 days; and if toxin was present then analyses were performed on additional samples taken earlier in the ripening period to determine the age of the cheese when toxin first appeared. Tables 5 and 6 present data obtained by performing slide gel-diffusion titrations on the cheese extracts.

Table 5 shows the summary of the assays for toxin as conducted on samples of Cheddar cheese made for this research. There was no toxin found in either of the non-inoculated control Cheddar cheeses during curing. The cheese from vat 1 was made from milk that did not contain penicillin. The cheese from vat 2 was made from milk that contained 0.54 Units of penicillin per milliliter of milk. Both of these cheeses contained low S. aureus populations during ripening which did not reach a concentration high enough to suggest a hazard (Figure 3A and 3B).

The Cheddar cheese made from vat 3 was manufactured from milk inoculated with a strain of S. aureus which produces type A entero-

Table 5: Enterotoxin produced during curing at 48 F of Cheddar cheese manufactured from pasteurized milk inoculated with toxigenic strains of S. aureus and containing penicillin as indicated.

Vat number	Age of cheese (days)	Penicillin added (U/ml milk)	S. aureus added (organisms/ml milk)	µg toxin/100 g cheese
1	180	none	none	none
2	180	0.54	none	none
3	180	none	480,000 type A	none
4	90	0.54	480,000 type A	4
4	60	0.54	480,000 type A	3
4	30	0.54	480,000 type A	4
4	20	0.54	480,000 type A	none
5	180	none	450,000 type B	none
6	180	0.49	450,000 type B	none
6	90	0.49	450,000 type B	none
6	60	0.49	450,000 type B	none

toxin. The staphylococci attained a maximum population of 130,000 cells/g after 60 days of curing (Figure 4A) and the cheese did not contain toxin when analyzed after 180 days at 48 F.

Figure 4B (vat 4) indicates that the S. aureus population of the cheese was approximately 1 million organisms/g after 10 days of curing and reached a maximum of 110 million organisms/g after 30 days. The cheese sample obtained after 30 days of ripening contained 4 µg of type A toxin/100 g of cheese. The cheese sample obtained after 20 days of curing gave no indication of toxin, which means that toxin was formed in the cheese from vat 4 between 20 and 30 days of ripening at 48 F. Table 5 indicates that samples of the cheese from vat 4 after 30 and 90 days of ripening contained 4 µg of toxin/100 g cheese. The cheese sample obtained after 60 days of curing contained only 3 µg of toxin/100 g cheese. The fluctuation in toxin content probably reflects variation in recovery since the toxin does not disappear after production. Evidence will be presented later that associates toxin production with an actively growing S. aureus population and not a stationary or declining population. One would not have expected an increase in the amount of toxin contained in the cheese from vat 4 after 30 days of curing since the S. aureus population began to decline after this time.

Casman and Bennett (1965) have reported type A enterotoxin in food poisoning incidents from coconut cream pie which contained 200 million staphylococci/g and banana cream pie which contained 72 million staphylococci/g. With such populations as indices, one would certainly

have expected toxin to be present in the cheese from vat 4 which contained an S. aureus population of 110 million organisms/g in one sample during ripening.

Toxin was not detected in the Cheddar cheese manufactured from the remaining vats of milk inoculated with a strain of S. aureus that produces type B toxin. The milk in vat 6 contained 0.49 Units of penicillin per milliliter of milk but the milk in vat 5 lacked the addition of any antibiotic. During curing, the cheese made from vat 5 (Figure 5A) contained a maximum of 18 million staphylococci/g after 60 days. Lactic acid bacteria developed normally during cheese manufacture in vat 5 and the pH of the cheese varied from 5.2 to 5.6 during curing. The cheese made from vat 6 (Figure 5B) contained a maximum of 48 million staphylococci/g after 90 days of ripening. Lactic acid bacteria developed poorly in vat 6 during manufacture and the pH of the cheese during ripening varied from 5.8 to 6.3.

The population of S. aureus required to produce measurable amounts of type B toxin in food has not been reported. No conclusions can be made regarding the absence of toxin and the maximum population of staphylococci in the cheese produced from vats 5 and 6.

Table 6 shows the summary of the assays for toxin conducted on samples of colby cheese made for this research. No toxin was detected in either of the non-inoculated colby cheeses during curing. The cheese from vat 8 (Figure 6B) was manufactured from milk that contained 0.49 Units of penicillin per milliliter of milk. The cheese from vat 7 (Figure 6A) was made from milk that did not contain penicillin. Both of these cheeses contained low S. aureus populations throughout ripening.

Table 6: Enterotoxin produced during curing at 48 F of colby cheese manufactured from pasteurized milk inoculated with toxigenic strains of S. aureus and containing penicillin as indicated.

Vat number	Age of cheese (days)	Penicillin added (U/ml milk)	S. aureus added (orfanisms/ml milk)	µg toxin/100 g cheese
7	180	none	none	none
8	180	0.49	none	none
9	180	none	440,000 type A	none
10	180	0.49	440,000 type A	2
10	90	0.49	440,000 type A	2
10	60	0.49	440,000 type A	1
10	30	0.49	440,000 type A	2
10	20	0.49	440,000 type A	1
10	10	0.49	440,000 type A	1
10	1	0.49	440,000 type A	none
11	180	none	450,000 type B	none
11	90	none	450,000 type B	none
12	180	0.49	450,000 type B	none
12	90	0.49	450,000 type B	none

The colby cheese produced from vat 9 (Figure 7A) was made from milk inoculated with a strain of S. aureus that produces type A enterotoxin. No toxin was detected in this cheese after 180 days of curing although the staphylococcal population was 850,000 organisms/g on the first day of ripening.

The colby cheese produced from vat 10 (Figure 7B) during manufacture contained 1 µg of type A enterotoxin/100 g of cheese after 10 days of curing. The maximum amount of toxin detected in this cheese was 2 µg/100 g of cheese and occurred after 30 days of ripening. The measurable toxin content fluctuated between 1 and 2 µg/100 g of cheese throughout the remainder of the curing period. This fluctuation is attributed to the lack of precision inherent in the extraction procedure because the toxin is stable.

Figure 7B shows an S. aureus population in the cheese from vat 10 of 3 million organisms/g after one day of curing. This cheese was potentially hazardous after only one day yet no toxin was detected. The number of organisms and the amount of toxin necessary to induce illness in humans is not known. It is possible that emetic quantities of toxin are below the sensitivity of the extraction method used in this investigation. Toxin was not detected in the cheese from vat 10 (Figure 7B) until after 10 days of curing when the staphylococcal population was approximately 1.4 million cells/g. The maximum staphylococcal population in the cheese from vat 10 was 6.5 million organisms/g after 20 days of curing. The staphylococcal population in the cheese was declining after 30 days of curing when the maximum concentration of toxin was indicated. Production of toxin by a declining population

is unlikely, as will be shown later. Inconsistencies in the extraction procedure probably explain the reason maximum toxin concentration in the cheese was not indicated at 20 days.

Casman and Bennett (1965) have reported type A enterotoxin in a food poisoning incident involving macaroni salad which contained one million staphylococci/g. If such a population can produce measurable amounts of toxin, one would have expected toxin in the cheese from vat 10 which contained 6.5 million S. aureus organisms/g during ripening. However, the staphylococcal population occurring in the cheese from vat 10 is several times less than the 110 million organisms/g in the toxic Cheddar cheese from vat 4 (Figure 4B).

Data in Table 6 indicate that no toxin was detected in either of the colby cheeses manufactured from milk inoculated with a strain of S. aureus that produces type B toxin (vats 11 and 12). The milk in vat 12 contained 0.49 Units of penicillin per milliliter of milk but the milk in vat 11 lacked the addition of any antibiotic. During ripening, the cheese made from vat 11 (Figure 8A) exhibited a maximum staphylococcal population of approximately 2,500 organisms/g after 20 days. The development of the lactic acid bacteria in vat 11 was normal during cheese manufacture and the pH of the cheese varied from 5.3 to 5.8, which is normal for colby cheese. The cheese manufactured from vat 12 (Figure 8B) exhibited an S. aureus population exceeding one million organisms/g after one day and a maximum of 33 million/g after 90 days of ripening. Lactic acid starter development in vat 12 during manufacture was above normal with the pH of the cheese varying from 5.8 to 6.5 during curing.

The results of this pilot plant experiment support the suggestion of Casman (1963), Brandly (1965), Harmon (1967) and the observation by Zehren and Zehren (1968b) that staphylococci can grow well and reach hazardous populations in foods receiving treatments to lower the natural bacterial population. Zehren and Zehren (1968a) examined a large quantity of cheese for type A enterotoxin and found that toxic cheese resulted only from those vats of milk failing to show proper lactic starter growth and acid development, which is partially supported by the results of this investigation. Two cheeses manufactured from milk receiving penicillin to artificially inhibit the lactic bacteria and containing a type A toxigenic strain of S. aureus, contained toxin during ripening. However, two cheeses also manufactured from milk treated with penicillin and receiving a type B toxigenic strain of S. aureus, failed to indicate the presence of toxin even though extremely high populations of the organisms occurred during ripening. The particular type B toxin producing strain of S. aureus used in this research produces very high amounts of toxin in broth cultures but has never been associated with a food poisoning incident.

Other workers have investigated the growth and persistence of toxigenic strains of S. aureus inoculated into milk that was manufactured into cheese (Takahaski and Johns, 1959; Walker et al., 1960). These workers found that staphylococci concentrated in the curd during cheese manufacture and that the curd at pressing and cheese after one day of curing often contained potentially hazardous numbers of staphylococci. No pattern of staphylococcal growth was evidenced by the work

reported herein, however. Potentially hazardous numbers were present for the first time after 1, 10, 20 and 30 days of ripening for both Cheddar and colby cheeses manufactured from milk inoculated with staphylococci.

Production of enterotoxin in Brain Heart Infusion (BHI) broth at different pH values. Non-buffered BHI broth was suggested by Casman (1967) as the best medium to use for production of enterotoxin resulting from the growth of toxigenic strains of S. aureus. This medium served as the control for observing growth and toxin production in a series of experiments to determine if pH influenced toxin formation.

Cheddar cheese is a highly buffered food material, normally having a pH of 5.2 to 5.6. Cheddar cheese produced from dead vats of milk has a pH of 6.0 to 6.8 as reported previously herein. To evaluate the effect of normal and low acid conditions as they can exist in Cheddar cheese, BHI broth was prepared from 0.2 M stock buffer solutions of pH 5.4 and 6.0. The BHI prepared from the pH 5.4 buffer corresponded to the pH of normal Cheddar cheese, while the BHI prepared from pH 6.0 buffer corresponded to the pH of low acid Cheddar cheese.

Both type A and type B toxin producing strains of S. aureus failed to grow after 14 days in BHI at pH 5.4. This was unexpected because both strains grew to some extent in Cheddar cheese that had normal acid development during manufacture. However, in making cheese the organism may become well established before the pH decreases enough to be inhibitory. The strain of S. aureus producing type A toxin was inoculated into the milk in vat 3 and reached a maximum population in the cheese of 130,000 organisms/g after 60 days of curing. The pH of the

cheese from vat 3 varied from 4.9 to 5.4 during that 60 day period. The strain of S. aureus producing type B toxin was inoculated into the milk in vat 5 and reached a maximum population in the cheese of 18 million organisms/g after 60 days of curing. The pH of the cheese from vat 5 varied from 5.2 to 5.6 during the first 60 days of ripening. Casman and Bennett (1963) have also reported that semisolid BHI agar with pH 5.3 is a suitable medium for the production of types A and B enterotoxin from several different strains of S. aureus. Casman and Bennett did not use a buffered BHI, but simply adjusted the medium to pH 5.3 with HCl before sterilization. Both of the toxigenic strains used in the research described herein grew well in non-buffered BHI at pH 5.4. During growth, both strains of S. aureus raised the pH of the medium to 8.6 after 48 hours. The supernatant from the culture of the toxigenic strains of S. aureus in non-buffered BHI at pH 5.4 was not examined for toxin.

Data in Tables 7, 8, and 9 show the toxin concentration, optical density at 620 mμ, viable count, and pH of the supernatant from the extended growth of the type A toxin producing S. aureus in BHI broth at various pH values.

The data in Table 7 indicate that toxin was first detected in the BHI broth at pH 7.4 after 5.0 hours of growth when the O.D. was 0.59 and the staphylococcal population was 270 million cells/ml. The maximum toxin concentration attained was 8 μg/ml after 12.0 hours of growth when the population was 4.3 billion organisms/ml. The pH of the supernatant (Table 7) decreased from 7.4 to 6.5 between 5.0 and 6.0

Table 7: Time, optical density at 620 mμ, viable count as determined on S-110 medium, pH of the supernatant, and enterotoxin concentration when S. aureus producing type A enterotoxin was grown in 3.7% BHI broth at pH 7.4 (nonbuffered) and incubated at 37 C. N_o = 51,000/ml broth.

Time (hr)	O.D. (620mμ)	Viable count (000 omitted)	pH	μg toxin/ml
0.0	0.00	51	7.40	NT[a]
1.5	0.01	1,200	7.40	NT
3.0	0.02	14,000	7.40	NT
4.5	0.29	98,000	7.40	--[b]
5.0	0.59	270,000	7.40	.25[c]
5.5	0.90	590,000	6.90	1.00
6.0	1.30	1,280,000	6.50	2.00
6.5	1.40	1,700,000	6.60	4.00
7.0	1.50	2,300,000	6.70	5.00
7.5	1.50	2,900,000	6.85	NT
12.5	1.60	4,300,000	7.50	8.00
24.0	1.60	5,500,000	8.40	8.00
36.0	1.60	3,500,000	8.75	8.00
48.0	1.60	2,100,000	8.90	8.00

a) Not tested
b) 100-fold concentrated sample of supernatant
c) 50-fold concentrated sample of supernatant

hours and then increased throughout the remainder of incubation to reach a maximum of 8.9 after 48.0 hours. Additional toxin was not produced after 12.0 hours of growth which is near the time the population reached the stationary phase. Toxin production was not observed when the staphylococcal population was decreasing.

Data in Table 8 show that in the BHI broth at pH 6.0, toxin first appeared after 24.5 hours when the O.D. was 0.58 and the population was approximately 260 million cells/ml. Estimates of the S. aureus populations occurring in samples of BHI broth at pH 6.0 and 8.0 (Tables 8 and 9) were obtained by comparing the sample O.D. to the standard population versus optical density curve in Figure 9. Figure 9 was obtained by plotting the log of population versus optical density of culture samples taken during the growth of the type A toxigenic strain of S. aureus in non-buffered BHI broth at pH 7.4. Presumably, the population occurring in the non-buffered BHI broth and the BHI broth at pH 6.0 and 8.0 were the same when the optical densities were the same.

The S. aureus population in the Cheddar cheese made from vat 4 (Figure 4B) when toxin was first detected was 110 million cells/g. The staphylococcal population in the cheese from vat 4 compares reasonably well to the staphylococcal population of 260 million organisms/ml when toxin was first detected in the BHI broth at pH 6.0. The toxin concentration in the BHI broth at this time was 0.50 µg/ml. When the S. aureus population was 110 million organisms/g in the cheese from vat 4, approximately 0.15 µg of toxin/g of cheese was present.

Table 8: Time, optical density at 620 mμ, estimated viable count, pH of the supernatant and enterotoxin concentration when S. aureus producing type A enterotoxin was grown in 3.7% BHI broth, buffered at pH 6.0 with 0.2 M sodium phosphate and incubated at 37 C. N_o = 8,000/ml broth

Time (hr)	O.D. (620 mμ)	Estimated viable count (000 omitted)	pH	μg toxin/ml
0.0	0.00		6.00	NT[a]
6.0	0.00		6.00	NT
13.0	0.00		6.00	NT
23.5	0.38	110,000	6.00	NT[b]
24.0	0.51	210,000	6.00	--[b]
24.5	0.58	260,000	6.00	0.50[b]
25.0	0.64	325,000	6.00	1.00
28.0	0.85	550,000	6.00	3.00
30.0	0.93	600,000	6.00	5.00
32.0	0.93	600,000	6.00	6.00
48.0	1.50	2,300,000	6.50	8.00
72.0	1.50	2,300,000	6.70	8.00

a) Not tested
b) Supernatant concentrated 50-fold

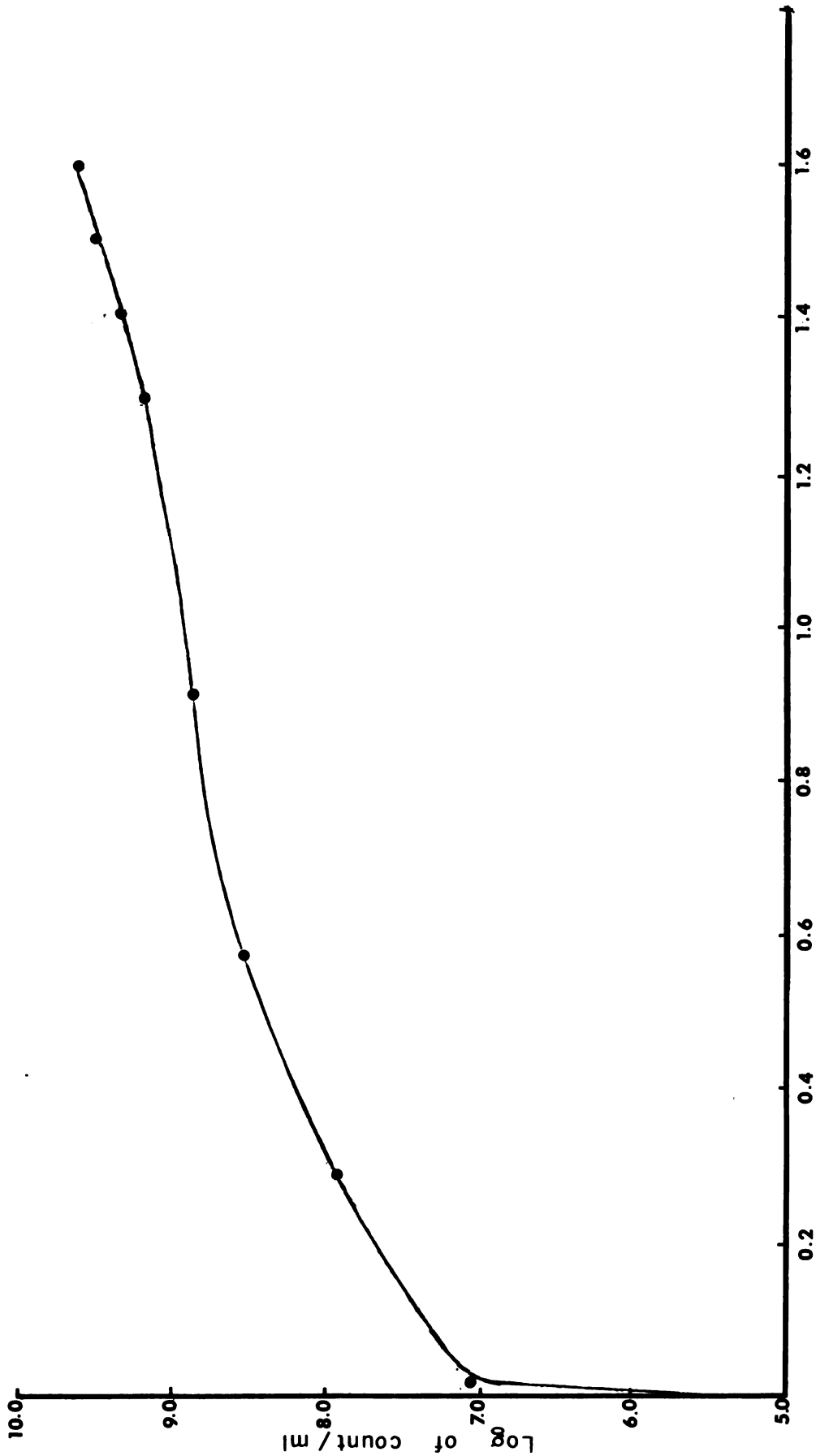

Figure 9: Relationship between optical density and population when cells of S. aureus producing type A enterotoxin are grown in non-buffered 3.7% BHI broth at pH 7.4 and incubated at 37 C.

This amount of toxin is the result of correcting the amount detected in the cheese, 0.04 µg/g, for the percent recovery known for the extraction procedure used in this investigation. Lower concentrations of toxin in cheese as compared to broth cultures of S. aureus, even with nearly equal populations, was expected since aeration in the cheese is lower than in the broth. Increased aeration has been correlated with increased toxin production by type A toxin producing strains of S. aureus (Kato et al., 1966).

The toxic colby cheese from vat 10 (Figure 7B) had a maximum S. aureus population of 6.5 million organisms/g during curing. Toxin was never detected in any of the BHI broths containing less than 260 million organisms/m of the type A producing strain of S. aureus. Unless there is some unknown difference between toxin development in cheese and in broth, two things are evident about the toxic colby cheese from vat 10: (1) the S. aureus population in the cheese during manufacture and ripening was higher than indicated, possibly because of enumeration errors or (2) false-positives were obtained in the test for toxin. The former is more tenable than the latter because positive tests for toxin were obtained in 6 different samples.

Data in Table 9 represent results of analysis when the type A toxigenic strain of S. aureus was grown in BHI broth at pH 8.0. Toxin was not detected until after 12.5 hours of growth and the population was approximately 530 million organisms/ml. This is approximately two times the population present when toxin was first detected in the BHI at pH 7.4 and 6.0. The maximum toxin concentration observed in the BHI broth at pH 8.0 was 6 µg/ml as compared to a maximum of 8 µg/ml

Table 9: Time, optical density at 620 mμ, estimated viable count, pH of the supernatant and enterotoxin concentration when S. aureus producing type A enterotoxin was grown in 3.7% BHI broth, buffered at pH 8.0 with 0.2 M tris and incubated at 37 C. N_o = 8,000/ml broth

Time (hr)	O.D. (620 mμ)	Estimated viable count (000 omitted)	pH	μg toxin/ml
0.0	0.00		8.00	NT[a]
8.0	0.00		8.00	NT
9.0	0.07		7.95	NT
10.0	0.22	87,000	7.85	NT
11.0	0.38	110,000	7.80	NT
11.5	0.52	218,000	7.70	NT
12.0	0.67	330,000	7.70	-- [b]
12.5	0.83	530,000	7.70	0.25[c]
13.0	1.00	615,000	7.70	1.00
13.5	1.20	815,000	7.70	3.00
23.0	1.30	1,000,000	7.70	5.00
24.0	1.40	1,700,000	7.80	6.00
48.0	1.45	2,000,000	8.20	6.00
72.0	1.50	2,300,000	8.50	6.00

a) Not tested
b) Supernatant concentrated 100-fold
c) Supernatant concentrated 50-fold

observed in the control BHI broth at pH 7.4 and the BHI broth at pH 6.0. Growth of the type A producing strain of S. aureus in BHI at pH 8.0 was not significantly inhibited but toxin production was less than when growth occurred in BHI broth at pH 6.0 and 7.4. Similar observations of reduced toxin production but little inhibition of growth for type B toxin producing strains of S. aureus have been reported. Genigeorgis (1966) observed that strain S-6 of S. aureus grew normally in BHI broth with 10% NaCl added, but failed to produce any toxin.

Tables 10, 11, and 12 contain data showing the toxin concentration, optical density at 620 mμ, viable counts and pH of the supernatant from the extended growth of the type B toxin producing strain of S. aureus in BHI broth with various pH values. Estimates of S. aureus populations occurring in samples of BHI broth at pH 6.0 and 8.0 (Tables 11 and 12) were obtained by comparing the sample O.D. to the standard population verses optical density curve in Figure 10.

The data in Table 10 show the result of growing the type B toxin producing strain of S. aureus in non-buffered BHI broth at pH 7.4. The first indication of toxin occurred after 6.0 hours when the optical density was 0.36 and the population was 47 million organisms/ml which is lower than the population present in the BHI broth at pH 7.4 when type A toxin was first detected. The type B strain produced a maximum of 382 μg of toxin/ml of supernatant in the BHI at pH 7.4 at which time the population of S. aureus was 6.6 billion cells/ml. When the type A toxin producing strain of S. aureus was grown in the BHI broth at pH 7.4, the maximum toxin concentration occurred when the population

Table 10: Time, optical density at 620 mμ, viable count as determined on S-110 medium, pH of the supernatant and enterotoxin concentration when S. aureus producing type B enterotoxin was grown in 3.7% BHI broth at pH 7.4 (nonbuffered) and incubated at 37 C. N_o = 31,000/ml broth

Time (hr)	O.D. (620 mμ)	Viable count (000 omitted)	pH	μg toxin/ml
0.0	0.00	200	7.40	NT[a]
3.0	0.00	460	7.40	NT[b]
5.0	0.18	27,000	7.20	--
6.0	0.36	47,000	7.16	1.00
6.5	0.46	61,000	7.00	NT
7.0	0.78	280,000	6.85	NT
7.5	1.10	400,000	6.50	NT
8.0	1.30	720,000	6.58	NT
8.5	1.35	2,400,000	6.60	24.00
10.5	1.40	NT	6.60	64.00
12.0	1.50	6,000,000	7.20	112.00
24.0	1.60	6,600,000	8.35	382.00
36.0	1.60	NT	8.75	NT
48.0	1.60	1,700,000	8.90	382.00
72.0	1.60	NT	8.90	382.00

a) Not tested
b) Supernatant concentrated 50-fold

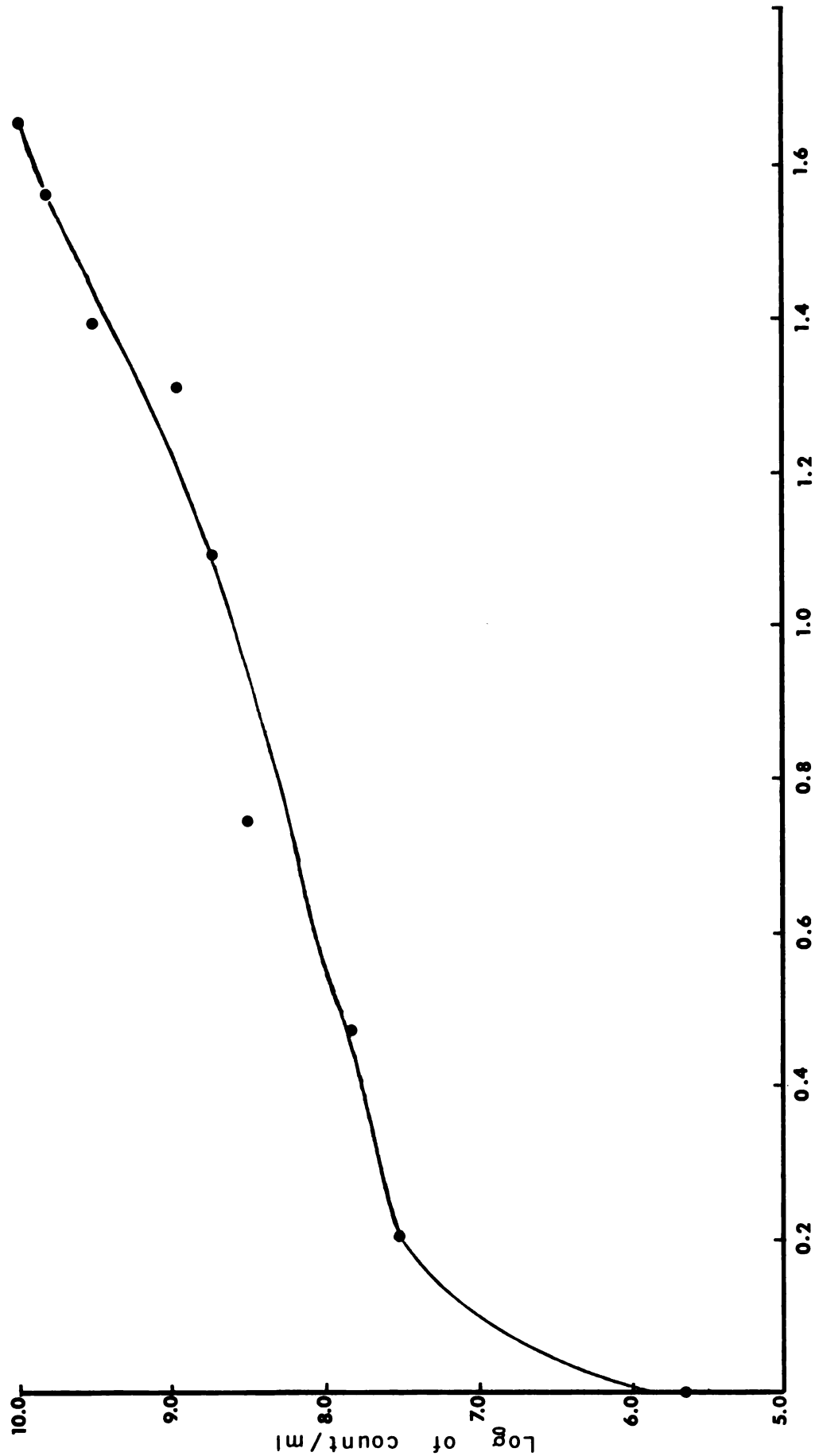

Figure 10: Relationship between optical density and population when cells of S. aureus producing type B enterotoxin are grown in non-buffered 3.7% BHI broth at pH 7.4 and incubated at 37 C.

was 4.3 billion cells/ml. The relationship between maximum population and maximum toxin production by toxigenic strains of S. aureus is not clear. Genigeorgis (1966), working with strain S-6 and McLean et al. (1968) working with strain 243, both of which produce type B enterotoxin, reported the first indication of toxin occurred as the population entered the stationary phase of growth. Because of this observation, Genigeorgis suggested that toxin production was the result of the accumulation of inhibitory materials in the growth medium. The observations reported herein, suggest that toxin production is associated with active growth of the organisms and toxin production ceases as the population stops active division.

Table 11 contain data which show that the growth of the type B toxin producing strain of S. aureus caused the pH of the BHI broth buffered at pH 6.0 to decrease after 9.0 hours until a minimum of 5.75 was reached at 12.0 hours. The pH then increased until a maximum of 8.9 was attained after 48.0 hours of growth.

Type B toxin was not detected in cheese manufactured from milk having poor acid development and a high population of a strain of S. aureus producing type B toxin during ripening. Maximum staphylococcal populations of 20, 38, and 480 million organisms/g of cheese were observed for three separate cheeses during ripening. When the type B toxin producing strain of S. aureus was grown in BHI broth at pH 6.0, toxin was detected at a population of 180 million organisms/ml. Theoretically then, the Cheddar cheese from vat 6 which contained 480 million staphylococci/g at one time during curing should have been

Table 11: Time, optical density at 620 mμ, estimated viable count, pH of the supernatant, and enterotoxin concentration when S. aureus producing type B enterotoxin was grown in 3.7% BHI broth, buffered at pH 6.0 with 0.2 M sodium phosphate and incubated at 37 C. N_o = 270,000/ml broth

Time (hr)	O.D. (620 mμ)	Estimated viable count (000 omitted)	pH	μg toxin/ml
0.0	0.00		6.00	NT[a]
6.0	0.09		6.00	NT
7.0	0.15	26,000	6.00	NT
8.0	0.34	45,000	6.00	NT
9.0	0.54	200,000	6.00	--[b]
10.0	0.72	250,000	5.90	4.00
11.0	0.93	340,000	5.80	NT
12.0	1.10	400,000	5.75	32.00
18.0	1.40	3,000,000	6.00	64.00
24.0	1.50	6,000,000	6.50	382.00
48.0	1.80	6,600,000	6.90	NT
72.0	1.80	6,600,000	7.00	382.00

a) Not tested
b) Supernatant concentrated 100-fold

toxic. No explanation as to why toxin was not detected in the cheese from vat 6 is offered, but apparently environmental factors are involved.

Growth and toxin production by the type B toxin producing strain of S. aureus in BHI broth at pH 8.0 is indicated by data in Table 12. Toxin was first detected after 9.5 hours of growth when the population was approximately 350 million cells/ml which is higher than when toxin was first detected in the BHI broth at pH 7.4 and 6.0. At the time when toxin was first detected in the BHI at pH 7.4, the staphylococcal population was 47 million organisms/ml. The staphylococcal population in the BHI at pH 6.0 was 180 million/ml when toxin was first detected. The maximum toxin concentration observed in the BHI broth at pH 8.0 was 32 µg/ml after 24 hours of growth which is considerably lower than the 382 µg/ml produced in the control BHI and BHI at pH 6.0. The maximum population occurring in the BHI broth at pH 8.0 was approximately 5 billion organisms/ml, which corresponds to a maximum population of 6 billion organisms/ml in the control BHI at pH 7.4. Apparently the BHI broth at pH 8.0 is inhibitory to toxin production by the strain of S. aureus producing type B toxin but is only slightly inhibitory to cell growth. Cellular growth but reduced toxin production were also observed when the type A toxin producing strain of S. aureus was grown in BHI broth at pH 8.0. No explanation of why this occurred can be made, but the phenomenon merits investigation.

Table 12: Time, optical density at 620 mμ, estimated viable count, pH of the supernatant, and enterotoxin concentration when S. aureus producing type B enterotoxin was grown in 3.7% BHI broth, buffered at pH 8.0 with 0.2 M tris and incubated at 37 C. N_o = 31,000/ml broth

Time (hr)	O.D. (620 mμ)	Estimated viable count (000 omitted)	pH	μg toxin/ml
0.0	0.00		8.00	NT[a]
7.5	0.14	23,000	7.80	NT
8.0	0.26	36,000	7.80	NT
8.5	0.42	59,000	7.70	NT[b]
9.0	0.72	250,000	7.70	--
9.5	0.96	350,000	7.65	0.01
10.0	1.30	2,500,000	7.50	1.00
12.0	1.40	3,000,000	7.80	8.00
24.0	1.45	5,000,000	7.80	32.00
48.0	1.45	5,000,000	7.80	32.00
72.0	1.50	6,100,000	8.70	32.00

a) Not tested
b) Supernatant concentrated 100-fold

SUMMARY AND CONCLUSIONS

Milk treated with 0.49 to 0.54 Units of penicillin per milliliter of milk and inoculated with toxigenic strains of Staphylococcus aureus, yielded cheese which contained high populations of staphylococci during ripening. One of the Cheddar and one of the colby cheeses manufactured from milk treated with penicillin and inoculated with a strain of S. aureus producing type A enterotoxin was found to contain enterotoxin during ripening at 48 F. The toxic Cheddar cheese had a maximum S. aureus population of 110 million organisms/g of cheese after 30 days of curing. Toxin was formed in this Cheddar cheese between 20 and 30 days of ripening. The maximum amount of toxin detected in the cheese was 4 µg/100 g of cheese after 30 days of curing. The toxic colby cheese had a maximum staphylococcal population of 6.5 million organisms/g of cheese after 20 days of curing. Toxin was formed in the colby cheese between 1 and 10 days of ripening. The maximum amount of toxin detected in the cheese was 2 µg/100 g of cheese.

No toxin was detected in the cheese manufactured from milk inoculated with the strain of S. aureus producing type B enterotoxin. However, one of the Cheddar cheeses manufactured from milk containing penicillin and inoculated with the S. aureus producing type B toxin, contained 480 million staphylococci per gram of cheese after 60 days of curing.

Growth of the types A and B enterotoxin producing strains of S. aureus was inhibited in buffered BHI broth at pH 5.4. Growth of the strains in non-buffered BHI broth at pH 5.4 appeared to be uninhibited.

The type A producing strain of S. aureus produced maxima of 8, 8 and 6 µg of toxin/ml of supernatant in aerated BHI broth with respective pH values of 6.0, 7.4 and 8.0. These maximum toxin concentrations were present after 12, 48, and 32 hours of growth at 37 C in BHI broth with the same respective pH values. None of the BHI broths inoculated with the type A producing strain of S. aureus contained toxin before at least 270 million organisms/ml were present in the culture. This population relates reasonably well to the maximum of 110 million S. aureus organisms/g that occurred during ripening of the toxic Cheddar cheese. The toxic colby cheese contained only 6.5 million staphylococci/g when the maximum population occurred.

The type B toxin producing strain of S. aureus produced maxima of 382, 382, and 32 µg of toxin/ml of supernatant in aerated BHI broth with respective pH values of 6.0, 7.4 and 8.0. The toxin concentration was at a maximum after 24 hours of growth at 37 C in BHI broth with these same respective pH values. Toxin was first indicated in the BHI broth at pH 7.4 when the S. aureus population of 40 million organisms/ml was present in the culture. No explanation is offered as to why toxin was not detected in the Cheddar cheese made from milk inoculated with the type B enterotoxin producing strain of S. aureus and which attained 480 million staphylococci/g during ripening.

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